Remediation of soils polluted by metal(loid)s based on waste valorization and bioremediation by symbiotic and saprobic microorganisms

Mario Paniagua López

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Restauración de suelos contaminados por metales pesados a través de la revalorización de residuos y la biorremediación con microorganismos simbióticos y saprobios

PhD Thesis - Tesis Doctoral

Programa de Doctorado en Ciencias de la Tierra (UGR)

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DOCTORAL THESIS

Remediation of soils polluted by metal(loid)s based on waste valorization and bioremediation by symbiotic and saprobic microorganisms.

Directores: Dr. Manuel Sierra Aragón
Dr. Inmaculada García Romera

Memoria de Tesis Doctoral presentada por **D. Mario Paniagua López** para optar al grado de Doctor con mención internacional por la Universidad de Granada.

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Summary

Soil pollution by potentially toxic elements (PTEs) (e.g., Pb, As, Zn, Cu, Cd, and Sb) is a significant environmental problem worldwide, mainly associated with anthropogenic sources and activities, including the mining industry. These elements, while naturally occurring in the environment, can reach toxic concentrations and persist for long periods, causing severe damage to ecosystems and posing serious risks to human health.

In scenarios where extensive and severe soil pollution occurs, active soil remediation actions are needed to mitigate environmental damages and protect humans and other living organisms health. The Guadiamar Green Corridor (GGC) (Seville, SW Spain) represents an exemplary scenario of a natural ecosystem severely damaged over the long term by anthropogenic activities, and more specifically by PTEs pollution. This area was severely affected by the Aznalcóllar mining spill occurred in 1998, which resulted in high levels of persistent soil pollution despite initial cleanup efforts. Still nowadays, 25 years after the accident, residual polluted areas remain evident along the GGC by the existence of bare highly acidic soil patches with elevated PTEs concentrations. These residual polluted soils represent a risk not only to the environment but also to human health, requiring continuous monitoring and the development of comprehensive protocols and effective and feasible remediation strategies.

This thesis addresses the need to develop sustainable and effective strategies for remediation PTE-polluted soils, using the GGC as a case study. The research is focused on the valorization of wastes and by-products from anthropogenic activities and the application of symbiotic microorganisms as viable strategies for remediation of soils polluted by PTEs. The aim of this thesis is to evaluate the effectiveness and feasibility of a set of cost-effective and environmentally friendly soil remediation techniques and strategies for the ecological remediation of PTE-polluted soils. For this purpose, various soil remediation treatments, including inorganic liming amendments (gypsum mining spoil and marble sludge), organic amendments (vermicompost and biotransformed dry olive residue (DOR) by saprobic fungi), and physical techniques such as landfarming and biopiles, were applied in situ to polluted soils in the GGC. Furthermore, a soil bioremediation approach based on arbuscular mycorrhizal fungi (AMFs) was implemented under greenhouse conditions to evaluate the potential of this bioremediation strategy to enhance the effectiveness of the physicochemical treatments.

The thesis evaluates the impact of the treatments applied on the main soil properties and pollution levels over time, as assessed in chapters 2, 3, and 4. Liming treatments, based on gypsum mining spoil and marble sludge, were highly effective in neutralizing the strong acidity of the polluted soils, particularly marble-based treatments, which led to complete pH neutralization. Water-soluble and EDTA-extractable fractions of the PTEs were measured to assess the changes in soil mobility and bioavailability of PTEs following the application of the treatments to the polluted soil (PS). Liming treatments were also the most effective in reducing both fractions of these elements. This was associated with a significant increase in soil pH, resulting in the effective immobilization of highly mobile elements such as Cu, Zn, and Cd. However, excessively high pH levels could limit the immobilization of other PTEs such as As, thus increasing its bioavailability. Resolubilization of As and Pb could also occur in the presence of organic matter by competing mechanisms for sorption sites in the soil. Therefore, the doses of liming and organic amendments should be accurately estimated to effectively control the mobility of PTEs in polluted soils.

In chapter 2, an ecotoxicological assessment of soil pollution in the treated soils was carried out using a variety of bioassays selected as indicators of PTEs stress in polluted soils, to evaluate the effectiveness of the remediation treatments applied to the PS. A set of ecotoxicological tests were performed using both the solid and liquid phase of the soil, involving target organisms from different taxonomic groups and trophic levels, including microorganisms, plants, and invertebrates. The results showed that marble-based treatments were the most effective in reducing soil toxicity, primarily due to their strong pH neutralization, which reduced PTEs solubility and minimized toxicity risks. Moreover, bioassays using the liquid phase showed higher sensitivity to toxicity compared to those using the solid phase, thus providing a better estimation of soil toxicity.

The remediation treatments evaluated aimed to facilitate and accelerate natural ecological succession in areas of the GGC where it was hindered by the extremely limiting conditions. The success of these treatments in triggering succession and facilitating the recovery of biological communities in the highly degraded soil was assessed by analyzing the status of these communities at various levels. In chapter 3, the vegetation status and spontaneous recolonization of the reference and treated soils by native plants were measured. In general, the treatments were effective in promoting spontaneous vegetation growth by improving soil properties and reducing PTEs availability. Among them, those based on vermicompost showed the greatest vegetation cover and species richness, approaching the conditions of the adjacent naturally recovered soils (RS). These treatments significantly increased soil organic carbon content and improved water retention capacity, essential for facilitating plant recolonization and growth in polluted soils. In the studied soils, two native plant species, Lamarckia aurea and Spergularia rubra, acted as pioneer species colonizing the soil, exhibiting remarkable ability to accumulate Pb and As in their roots. They can be considered key species in the area, as they not only serve as pioneers in recolonizing the degraded soils but also facilitate the subsequent recolonization by other species less tolerant to high levels of pollution. Thus, the success of the remediation strategy can be promoted by the early recolonization of the soil by these highly pollution-tolerant species, which enhance further evolution of the soil physical, biological, and chemical conditions.

The presence of pioneer plant species in treated soils can enhance microbial activity by providing soil microbial communities with suitable habitat, along with essential organic carbon and nutrient contents, which are crucial for key soil processes and overall ecosystem functioning at the local scale. For this reason, when evaluating the rate of ecological succession driven by remediation treatments, it is important to take into account not only aboveground processes, such as vegetation response, but also those occurring belowground. In this regard, the soil microbiological status of both reference and treated soils was evaluated in **chapter 4**. The results showed that PS had low total abundances, community structure, and diversity of microbial communities, confirming that microbial biomass and taxonomic diversity of soil communities were significantly affected by the PTEs concentrations. On the other hand, the soil treatments evaluated for their microbiological status, specifically marble sludge and biopile, showed effectiveness in restoring soil quality. The abundance and structure of microbial populations under both treatments were restored to levels proximate to those found in RS.

The application of the remediation treatments to PTE-polluted soils in the GGC led to significant improvements in soil quality, reducing the mobility and toxicity of PTEs, and allowing the establishment of vegetation. However, despite these improvements, the conditions and quality of the treated soils remained less favorable compared to the RS, with a persistent potential risk of PTEs remobilization that requires to be monitored over time. This highlights that physicochemical techniques for the remediation of polluted soils alone may not be enough to permanently alleviate pollution risks. Consequently, soil bioremediation processes could be implemented in parallel to promote the remedial effects of other techniques. In this sense, **Chapter 5** explores the addition of a biological element, AMFs inoculation, to the soil remediation treatment that showed the highest overall effectiveness in the field-based in-situ remediation approach (marble sludge), combined with organic amendments. Biotransformed DOR showed high effectiveness in improving soil physicochemical and biological status when combined with marble sludge, promoting plant growth and survival, and reducing PTEs toxicity and plant uptake. Thus, DOR biotransformed by saprobic fungi can represent an efficient organic amendment for remediating PTE-polluted soils. Furthermore, the combined application of marble sludge and DOR along with AMF inoculation, further enhanced PTEs immobilization in polluted soils by stimulating the phytostabilization process induced by AMFs. This bioremediation approach improved plant protection and significantly increased the overall effectiveness of the remediation process, showing potential as a sustainable bioremediation strategy for restoring soil functions and reducing toxicity in areas polluted by PTEs.

In conclusion, this thesis provides valuable insights into the remediation of PTE-polluted soils. Overall, the results demonstrated that combining physicochemical treatments with a bioremediation approach, incorporating AMF inoculation and organic amendments, significantly enhanced the effectiveness of soil remediation processes. This approach not only improves soil health and reduces PTEs mobility, but also supports the re-establishment of vegetation and microbial communities in the degraded soils. These findings contribute to the development of more effective and sustainable soil remediation strategies that can be applied to similar polluted sites worldwide. Moreover, the results emphasize the importance of a holistic approach to soil remediation that considers not only the soil physicochemical status but also its biological health and capacity for ecological recovery.

Resumen

La contaminación del suelo por elementos potencialmente tóxicos (PTEs) (ej., Pb, As, Zn, Cu, Cd y Sb) representa un serio problema medioambiental a nivel mundial, asociado principalmente a fuentes y actividades antropogénicas, entre las que se incluye la industria minera. Estos elementos, a pesar de encontrarse de forma natural en el medio ambiente, pueden alcanzar concentraciones tóxicas y persistir en el suelo durante largos periodos de tiempo, causando graves daños a los ecosistemas y planteando serios riesgos para la salud humana.

En escenarios en los que se produce una contaminación severa del suelo, la implementación de medidas activas de remediación del suelo se hace necesaria para mitigar los daños ambientales y proteger la salud de los seres humanos y otros organismos vivos. El Corredor Verde del Guadiamar (GGC) (Sevilla, SO España) representa un escenario ejemplificador de un ecosistema natural severamente dañado a largo plazo por actividades antropogénicas, y más concretamente por contaminación por PTEs. Esta zona se vio gravemente afectada por el vertido minero de Aznalcóllar ocurrido en 1998, que dio lugar a altos niveles de contaminación persistente del suelo a pesar de los esfuerzos iniciales de limpieza. Aún hoy en día, 25 años después del accidente, persisten suelos afectados por contaminación residual a lo largo del GGC, que se hacen evidentes por la aparición de zonas delimitadas de suelo desnudo que presentan una fuerte acidez y elevadas concentraciones de PTEs. Estos suelos residualmente contaminados representan un riesgo no sólo para el medio ambiente sino también para la salud humana, requiriendo una vigilancia continua así como el desarrollo de protocolos integrales y estrategias de remediación eficaces y viables para su recuperación.

Esta tesis aborda la necesidad de desarrollar estrategias sostenibles y eficaces para la remediación de suelos contaminados por PTEs, utilizando el GGC como caso de estudio. La investigación se centra en la valorización de residuos y subproductos de actividades antropogénicas y la aplicación de microorganismos simbióticos como estrategias viables para la remediación de suelos contaminados por PTEs. El objetivo de esta tesis es evaluar la eficacia y viabilidad de una serie de técnicas y estrategias de remediación de suelos económicas y sostenibles para la remediación ecológica de suelos contaminados por PTEs. Para ello, se aplicaron in situ a los suelos contaminados del GGC diversos tratamientos de remediación del suelo, para los que se seleccionaron enmiendas inorgánicas encalantes (residuos de yeso y lodos del corte y pulido del mármol), enmiendas orgánicas (vermicompost y alperujo (DOR) biotransformado por hongos saprobios), así como técnicas físicas como el laboreo del suelo y la aplicación de biopilas. Además, se aplicó un enfoque de biorremediación del suelo basado en hongos micorrícicos arbusculares (AMFs) bajo condiciones de invernadero con el fin de evaluar el potencial de esta estrategia de biorremediación para mejorar la eficacia de los tratamientos fisicoquímicos.

En esta tesis se evalúa el impacto de los tratamientos aplicados sobre las principales propiedades del suelo y los niveles de contaminación a lo largo del tiempo (**capítulos 2**, **3** y **4**). Los tratamientos encalantes, consistentes en residuos de yeso y lodos de mármol, mostraron una alta eficacia en la neutralización de la fuerte acidez de los suelos contaminados, especialmente los tratamientos a base de mármol, que condujeron a una neutralización completa del pH del suelo. Además, se midieron las fracciones solubles en agua y extraíbles con EDTA de los PTEs para evaluar los cambios en su movilidad en el suelo y su biodisponibilidad tras la aplicación de los tratamientos al suelo contaminado (PS). Los tratamientos encalantes fueron asimismo los más eficaces en la reducción de las fracciones solubles y biodisponibles de los PTEs. Esto se asoció con el aumento significativo del pH del suelo, lo que resultó en la inmovilización efectiva de los elementos altamente móviles como el Cu, Zn y Cd. Sin embargo, niveles de pH excesivamente elevados pueden limitar la inmovilización de otros PTEs como el As, provocando así un aumento en su biodisponibilidad. La resolubilización del As y Pb también podría producirse en presencia de materia orgánica, debido a mecanismos de competencia por los sitios de adsorción en el suelo. Por tanto, las dosis de aplicación de enmiendas encalantes y orgánicas deben estimarse cautelosamente con el fin de controlar eficazmente la movilidad de los PTEs en suelos contaminados.

En el **capítulo 2** de esta tesis se lleva a cabo una evaluación ecotoxicológica de la contaminación del suelo en los suelos tratados mediante una serie de bioensayos seleccionados como indicadores del estrés producido por los PTEs, con el fin de evaluar la eficacia de los tratamientos de remediación aplicados al PS. Para ello, se realizó un conjunto de pruebas ecotoxicológicas utilizando tanto la fase sólida como la líquida del suelo, y en las que se utilizaron organismos indicadores de diferentes grupos taxonómicos y niveles tróficos, incluyendo microorganismos, plantas e invertebrados. Los resultados mostraron que los tratamientos basados en mármol fueron los más eficaces para reducir la toxicidad del suelo, principalmente debido a su fuerte neutralización del pH, que a su vez redujo la solubilidad de los PTEs minimizando los riesgos toxicológicos. Además, los bioensayos que utilizaron la fase líquida mostraron una mayor sensibilidad a la toxicidad en comparación con los que utilizaron la fase sólida, proporcionando así una mejor estimación de la toxicidad del suelo.

Los tratamientos de remediación evaluados pretenden facilitar y acelerar la sucesión ecológica natural en zonas del GGC donde ésta se ve dificultada por las condiciones extremadamente limitantes del suelo. El éxito de estos tratamientos a la hora de desencadenar la sucesión y facilitar la recuperación de las comunidades biológicas en el suelo altamente degradado se evalúa analizando el estado de estas comunidades a varios niveles. En el **capítulo 3**, se evalúa el estado de la vegetación y la recolonización espontánea por plantas autóctonas de los suelos tanto de referencia como tratados. En general, los tratamientos resultaron eficaces para promover el crecimiento espontáneo de la vegetación al mejorar las propiedades del suelo y reducir la disponibilidad de los PTEs. Entre ellos, los tratamientos basados en vermicompost mostraron la mayor cobertura vegetal y riqueza de especies, acercándose a las condiciones de los suelos adyacentes recuperados naturalmente (RS). Estos tratamientos aumentaron significativamente el contenido de carbono orgánico del suelo y mejoraron la capacidad de retención de agua, factores esenciales para facilitar la recolonización y el crecimiento de las plantas en los suelos contaminados. En los suelos estudiados, dos especies nativas de plantas, *Lamarckia aurea* y *Spergularia rubra*, actuaron como especies pioneras colonizadoras del suelo, mostrando una notable capacidad para acumular Pb y As en sus raíces. Estas pueden considerarse especies clave en la zona de estudio, dado que no sólo actúan como especies pioneras en la recolonización de los suelos degradados, sino que también facilitan la recolonización posterior por parte de otras especies menos tolerantes a los altos niveles de contaminación. Por tanto, el éxito de la estrategia de remediación puede verse favorecido por la pronta recolonización del suelo por estas especies altamente tolerantes a la contaminación, cuya presencia potencia la evolución y mejora de las condiciones físicas, biológicas y químicas del suelo.

La presencia de especies de plantas pioneras en los suelos tratados puede mejorar la actividad microbiana al proporcionar a las comunidades microbianas del suelo un hábitat adecuado, así como contenidos esenciales de materia orgánica y nutrientes, cruciales para los procesos clave del suelo y el funcionamiento general del ecosistema a escala local. Por esta razón, a la hora de evaluar la tasa de sucesión ecológica impulsada por los tratamientos de remediación, es importante tener en cuenta no sólo los procesos que ocurren sobre el nivel del suelo, tales como la respuesta de la vegetación, sino también aquellos que tienen lugar bajo la superficie. A este respecto, en el **capítulo 4** se evalúa el estado microbiológico tanto en los suelos de referencia como en los tratados. Los resultados mostraron que el PS muestra una baja abundancia total, estructura y diversidad de las comunidades microbianas, lo que confirma que la biomasa microbiana y la diversidad taxonómica de las comunidades del suelo se ven afectadas significativamente por las concentraciones de PTEs. Por otra parte, los tratamientos seleccionados cuyo estado microbiológico del suelo fue evaluado, en concreto los basados en mármol y la biopila, fueron eficaces en la restauración de la calidad del suelo. Bajo ambos tratamientos, la abundancia y estructura de las poblaciones microbianas se restablecieron a niveles próximos a los encontrados en el RS.

La aplicación de los tratamientos de remediación a los suelos del GGC contaminados por PTEs conducen a mejoras significativas en la calidad del suelo, reduciendo la movilidad y toxicidad de los PTEs, y permitiendo el establecimiento de la vegetación. Sin embargo, a pesar de estas mejoras, las condiciones y la calidad de los suelos tratados siguen siendo menos favorables en comparación con el RS, existiendo un riesgo potencial persistente de removilización de los PTEs que requiere ser monitorizado a lo largo del tiempo. Esto pone de manifiesto que las técnicas fisicoquímicas para la remediación de suelos contaminados pueden no ser suficientes por sí solas para aliviar de forma permanente los riesgos de contaminación. En consecuencia, procesos basados en la biorremediación del suelo podrían aplicarse en paralelo a estas técnicas para potenciar sus efectos remediadores. En este sentido, el **Capítulo 5** explora la adición de un elemento biológico, consistente en la inoculación de AMFs, combinado con enmiendas orgánicas, al tratamiento de remediación que muestra una mayor eficacia global en la fase experimental de remediación in situ abordada en los capítulos previos (basado en la adición de lodo de mármol). La enmienda orgánica consistente en DOR biotransformado por hongos saprobios, en combinación con el mármol, destaca por su gran eficacia en la mejora del estado fisicoquímico y biológico del suelo, promoviendo la supervivencia y el crecimiento de las plantas, así como reduciendo la toxicidad de los PTEs y su acumulación en las plantas. Por ello, el DOR biotransformado por hongos saprobios puede representar una enmienda orgánica eficiente para ser aplicada en la remediación de suelos contaminados por PTEs. Además, la aplicación combinada de lodo de mármol y DOR junto con la inoculación de AMFs, aporta una eficacia aún mayor en la inmovilización de los PTEs en suelos contaminados, mediante la estimulación del proceso de fitoestabilización inducido por los AMFs. Este enfoque de biorremediación mejora la protección de las plantas y aumenta significativamente la eficacia global del proceso de remediación, mostrando así un elevado potencial como estrategia de biorremediación sostenible para restaurar las funciones del suelo y reducir la toxicidad en áreas contaminadas por PTEs.

En consecuencia, esta tesis aporta valiosos conocimientos en relación con la remediación de suelos contaminados por PTEs. En general, los resultados demuestran que la combinación de tratamientos fisicoquímicos con un enfoque de biorremediación, incorporando la inoculación de AMFs y enmiendas orgánicas, mejora significativamente la eficacia de los procesos de remediación del suelo. Este enfoque no sólo mejora la salud del suelo y reduce la movilidad de los PTEs, sino que también favorece el restablecimiento de la vegetación y las comunidades microbianas en los suelos degradados. Estos hallazgos contribuyen al desarrollo de estrategias de remediación del suelo más eficaces y sostenibles que puedan ser aplicadas en zonas contaminadas similares en cualquier parte del mundo. Además, los resultados subrayan la importancia de un enfoque holístico de la rehabilitación del suelo que tenga en cuenta no sólo su estado fisicoquímico, sino también su salud biológica y su capacidad de recuperación ecológica.

Chapter 1

General introduction

1.1. Soil pollution

Soil, as a fundamental element of ecosystems, plays a crucial role in providing essential environmental functions and ecosystem services. Among these, soil productivity is one of the most essential services for the human well-being and its development (Kabata-Pendias 2010; Adhikari and Hartemink 2016). Nevertheless, soil faces several soil degradation processes that may threaten its quality, both natural and human-induced. Of the anthropogenic causes, soil pollution represents one of the major threats and concerns worldwide, since it poses a risk to ecological systems, human health, and food production. Soil pollution refers to the presence in the soil of a chemical or substance out of place and/or present at a higher than normal concentration that has adverse effects on any non-targeted organism (FAO and ITPS 2015). In Europe alone, it is estimated that there are approximately 2.8 million potentially polluted sites, many of which may need remediation or risk-reduction measures (FAO and UNEP 2021).

Soil has a great capacity to respond to any type of pollution. Its high regulatory potential, associated with its physical, chemical, and biological properties, makes soil play a key role in protecting other ecosystem elements such as water, air and living organisms (Adhikari and Hartemink 2016). However, the resilience and protective capacity of soil is finite, and intense or continuous degradation processes over time can exceed its buffering capacity and prevent the performance of one or more of these functions, leading to serious damage to the ecosystem and humans (Lal 1997; Seybold et al. 1999).

Heavy metals (e.g. Pb, Zn, Cu, Cd) are elements whose specific density is equal to or greater than 5 g ml^{-1}, or whose atomic number exceeds 20 but do not include elements in groups I and II of the periodic table of elements (Adriano, 2001). Other elements such as arsenic (As) and Antimony (Sb) are metalloids belonging to Group 15 of the periodic table (Wilson et al. 2010). They all occur naturally in the environment at trace levels, although the toxicity of these elements is not defined by classification based on density, atomic weight, atomic number, or other properties. Rather, toxicity may be defined by the concept of Potentially Toxic Elements (PTEs) (Meier et al. 2012), which includes connotations to their intrinsic potential toxicity and bioaccumulation. To refer to all of these elements as a homogeneous group, hereinafter the terms PTEs and metal(loid)s are used interchangeably.

Among all chemical pollutants, PTEs, including heavy metals and metalloids, can accumulate and persist tightly bound in soil (Pilon-Smits 2005; Igalavithana et al. 2022), affecting ecosystem functions and representing a significant potential toxicological risk to organisms (Song et al. 2009), which can lead to cumulative impacts. Soil pollution by PTEs has become an increasingly pressing issue especially in the last decades, being the main causes associated with anthropogenic sources and activities, such as mining, and resulting in the accumulation of pollutants in soils that may reach levels of concern (Hu et al. 2013; Cachada et al. 2018). Actually, of the total polluted soil, more than 80% derives from PTEs soil pollution, while organic contaminants represent the rest (Zhang et al. 2024).

Although certain PTEs can act as essential elements and are involved in biological functions, such as Cu and Zn, they can turn potentially toxic when specific thresholds are exceeded (Gall et al. 2015). Other elements such as Pb, As, Cd, and Sb are non-essential elements, being among the most hazardous PTEs since very low concentrations of them can exert toxic effects (Rahman and Singh 2019), causing serious problems of contamination in the food chain, with the consequent health risk (Simon 2014) (Figure 1.1).

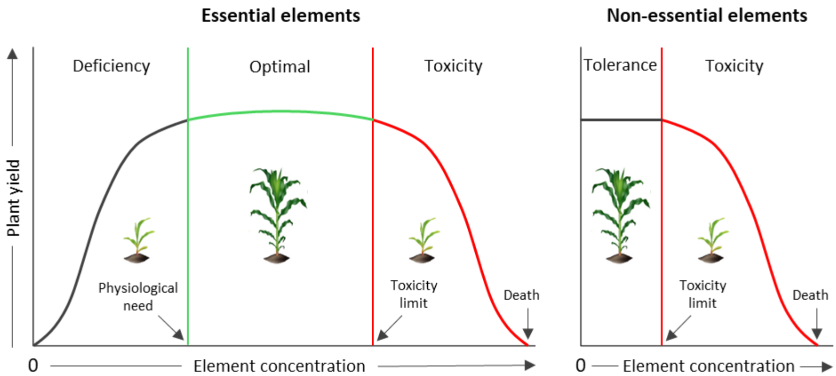

Figure 1.1. Typical dose-response curves for essential (left) and non-essential (right) trace elements (adapted from Alloway 1995).

1.2. Soil remediation

In scenarios where extensive and severe soil pollution happen, active soil remediation actions are needed, and the correct management of these sites is essential to reduce the damages to the environment and to the health of humans and other living organisms. Remediation is considered as the cleanup of a polluted area to safe levels through actions that remove or isolate pollutants from the environment, alleviating their negative impacts on the ecosystem and promoting its recovery (Finger et al. 2017).

A wide range of in-situ and ex-situ remediation techniques have been developed to contain, clean up, or restore PTEs polluted soils, including physical, chemical, and biological remediation. Strategies based on conventional clean-up technologies, such as ex-situ or physical remediation methods, are costly, environmentally destructive, and only applicable to small-scale polluted sites where rapid and complete decontamination is required (Saifullah et al. 2009). Chemical remediation techniques can facilitate the reduction in the mobility, bioavailability, and toxicity of PTEs in soil environments (Zhang et al. 2024). However, these techniques do not remove them from the polluted soil, and long-term monitoring is needed to prevent undesired PTEs mobilization or leaching over time under changing environmental conditions (Bolan et al. 2014; Palansooriya et al. 2020). Biological methods are generally time-consuming and effective only for low to moderate levels of PTEs. Thus, it is generally accepted that the used techniques should not only be significantly effective in reducing the amount, bioavailability, and toxicity of the PTEs, but also cause minimal disturbance to the natural environment or local ecological systems (Gong et al. 2018).

1.2.1. Assisted natural remediation of PTEs polluted soils

When PTEs are present in the soil, natural attenuation processes may occur, although they may not be sufficient in mitigating the risks or negative effects from the pollutants in the ecosystem, especially when their levels are high. In these cases, accelerating these processes that occur naturally in soils with human interference (i.e., assisted natural remediation) might be a viable option as a cleanup tool, and which may be based on the use of soil amendments that enhance key biogeochemical processes in soils that effectively immobilizes PTEs (Adriano et al. 2004; Pérez-de-Mora et al. 2006a).

In the remediation of soils polluted with PTEs, in-situ chemical stabilization through the addition of soil amendments is more widely employed than other remediation technologies to reduce the mobility and bioavailability of these elements and minimize their uptake by plants (Zhang et al. 2013). The addition of amendments to the soil, both inorganic and organic, represents an economical and environmentally efficient solution that can be feasibly applied even at large scale, and which has been widely implemented to assist natural remediation processes of soils polluted with PTEs (Park et al. 2011, Liu et al. 2018).

1.2.2. Waste valorization for soil remediation

The revalorization of low-cost mining and agro-food industry wastes through their application as amendments in degraded soils aligns with zero-waste and circular economy strategies (Greyson 2007; Pietzsch et al. 2017; Tayebi-Khorami et al. 2019). In this sense, numerous case studies have explored the use of materials at the end-of-life-cycle from different sectors to remediate residually polluted soils by metal mining activity, and corroborate the viability of these organic and/or inorganic soil amendments under field conditions (Fernández-Caliani and Barba-Brioso 2010; González et al. 2012). More precisely, liming or calcium and organic matter-rich amendments are among the most effective ones, through the correction of soil acidic pH, the enhancement of soil physicochemical properties, the increase of nutrient availability and the immobilization of certain PTEs (Bernal et al. 2007; Pérez-de-Mora et al. 2007a), which prevent from the potential spread of pollutants into the ecosystem.

Among the liming materials derived from human wastes or by-products, gypsum mining spoil or marble sludge are examples that may be used as soil amendments for their valorization. Gypsum mining spoil is the part discarded during gypsum processing since its purity does not reach the standards to be used in the construction industry. Being gypsum among the largest mined natural minerals (Escavy et al. 2012), great quantities of residue are produced. Therefore, gypsum mining spoil is a low-cost waste material mainly formed of gypsum ($CaSO_4 \cdot 2H_2O$), calcium carbonate ($CaCO_3$), and clay minerals, with the capacity to control soil pH, improve soil texture, reduce soil crusting, provide essential nutrients, and decrease PTEs toxicity (Amezketa et al. 2005; Gadepalle et al. 2007; García-Robles et al. 2022). Marble sludge is another waste material very rich in $CaCO_3$ that derives from marble stone cutting and polishing. Considering that marble is the largest produced natural stone in the world, large amounts of this low-cost waste material are produced annually (Alyousef et al. 2019), potentially producing negative effects on the ecosystem if incorrectly discarded (El-Sayed et al. 2018), so that it must be sustainably managed. This amendment has been previously tested by several authors with promising results for increasing pH and reducing PTEs toxicity in soils affected by mining activities (Pérez-Sirvent et al. 2007; del Moral et al. 2010; González et al. 2017).

Moreover, organic wastes, such as compost or those produced by the agri-food industry, can be used as organic matter-rich soil amendments due to their high content in organic matter (OM), enhancing soil pH, improving microbial structure, and reducing PTEs bioavailability in polluted soils, thus recovering their quality and functionality (García-Sánchez et al. 2015, 2019). For instance, vermicompost is an organic material that derives from processing different organic wastes with earthworms to optimize the composting process, enhancing the remediation potential of the final product (Huang et al. 2016). Its use as soil amendment could enhance the overall soil conditions by improving its physical structure, water holding capacity, cation exchange capacity, content and availability of nutrients (i.e. N, K, P, Ca and Mg) and, thus, by reducing PTEs toxicity, which may also promote the recovery of vegetation in polluted soils (Pérez-Esteban et al. 2012).

1.3. Biotransformation of dry olive residue (DOR) and its use in soil remediation

Dry olive mill residue (DOR) is a solid waste arising from the olive oil two-phase extraction system (Sampedro et al. 2007), which can be transformed for its use as an organic amendment for remediating PTE-polluted soils. Annually, large volumes of this residue are produced by the olive oil industry, representing a great environmental problem. Therefore, the management and treatment of this waste is a major concern for this industry and considered a priority, especially in Mediterranean countries, which are the major world producers of olive oil and where the olive sector has a great importance at social and economic level (Tortosa et al. 2012). In order to minimize its associated environmental impacts, producers have been obliged to treat or even reduce substantially their wastes (Justino et al. 2012).

Various alternative solutions have been proposed for the use and revalorization of DOR, including its combustion for energy production, which poses negative implications due to polyaromatic hydrocarbons and CO_2 release in the process, or its composting, considered an appropriate low-cost technology for organic waste recycling and organic fertilizer production (Sampedro et al. 2007; Tortosa et al. 2012; Siles et al. 2014a). DOR has also been proposed as a soil organic amendment for its high content in organic matter and mineral nutrients (Siles et al. 2015). However, the direct application of DOR to the soil could negatively impact on soil microorganisms and plant growth due to its phytotoxicity, primarily from its high levels of phenolic compounds and other substances like fatty acids (García-Sánchez et al. 2012; Siles et al. 2014a). For this reason, transformation of DOR prior to its application into soil is required.

The inoculation with saprobe fungi able to both stabilize the waste and to degrade phytotoxic compounds such as phenols represents a rapid and effective technique to reduce the phytotoxic effects of DOR and facilitate its use as an organic amendment (Sampedro et al. 2005, 2009; Aranda et al. 2006). The ability of these fungi to degrade a wide variety of pollutants like phenolic compounds may be attributed to the release of extracellular enzymes such as laccases and peroxidases (García-Sánchez et al. 2012). The application of DOR remediated by these fungi (mycoremediated DOR) has a beneficial impact on plant growth, increases fungal biomass, and decreases the bioavailability of PTEs (Hovorka et al. 2016; Reina et al. 2017).

1.4. Bioremediation

Physicochemical techniques for the soil remediation of polluted sites are in some cases not enough to permanently alleviate pollution risks. Soil bioremediation processes may be implemented in parallel to promote the remediating effects of other techniques applied. Bioremediation techniques have emerged as an economical and environmentally sustainable alternative for the management of polluted soils (Sales da Silva et al. 2020). These techniques use the capacity of microorganisms or microbial processes to degrade and transform environmental pollutants into harmless or less toxic forms (Garbisu and Alkorta 2003), as well as to reduce their toxicity risk through volatilization, binding, or immobilization in the soil (Verma and Kuila 2019), thereby offering a practical and viable solution for the restoration of degraded ecosystems.

The bioremediation processes may occur by a wide variety of mechanisms, including biosorption, bioaccumulation, biotransformation, biomineralization, biodegradation, bioleaching, and other microbe-metal interactions (H. Zhang et al. 2020; Yaashikaa et al. 2022). However, these processes are gradual, so natural bioremediation strategies usually require a relatively long time for remediation, which limits their application on large scales. Therefore, several methods have been proposed to increase the rate of biodegradation and to promote the microorganism's viability (Dehnavi and Ebrahimipour 2022). In this regard, bioremediation may be approached by two different procedures: bioaugmentation, by the external addition of microorganisms into the polluted environment to increase their populations (e.g. plant inoculation with beneficial microorganisms), and biostimulation, based on the addition of nutrients and carbon sources to promote the growth and activity of microorganisms and thus increase degradation and other bioremediating processes (Pino Rodríguez et al. 2012).

1.5. Arbuscular mycorrhizal fungi (AMFs) for soil bioremediation

Stressful environments, such as polluted soils, can cause a negative impact on plant growth and development. However, the stress-induced negative effects can be alleviated or minimized by naturally occurring microorganisms, including bacteria and fungi (Nadeem et al. 2014). Mycorrhiza is a symbiotic association between plant roots and fungi, with arbuscular mycorrhizae being the most abundant type involved in such association and commonly present in soil. These fungi represent a critical component of the ecosystem due to their major contribution to plant nutrition and resistance to several environmental stresses (Nadeem et al. 2014; Bhantana et al. 2021). Arbuscular mycorrhizal fungi (AMFs) also play a significant role in ecosystem functioning by participating in nutrient cycling and carbon sequestration (Garg et al. 2017).

The arbuscular mycorrhizal association is a widespread terrestrial symbiosis, involving more than 80% of vascular plants in almost all ecosystems that are able to establish these mutualistic associations with AMFs (Kim et al. 2017). Furthermore, AMFs are capable of inhabiting extremely harsh environments, including PTE-polluted soils (Miransari 2011). In this regard, the application of AMFs to soils polluted by PTEs represents a strategy for the biological remediation of these scenarios, since their presence can promote the efficiency of assisted natural remediation (Raklami et al. 2021). AMFs can provide plants protection against PTEs by their immobilization, extraction, and concentration in their tissues (Arriagada et al. 2009; Meier et al. 2012; Leung et al. 2013), and they also improve plant nutrition and tolerance to excess of certain PTEs (Janoušková et al. 2005; Cabral et al. 2015).

The mechanisms developed by AMFs to alleviate PTEs stress are very varied, and can be divided into direct and indirect mechanisms (Figure 1.2). Direct influence of AMFs includes immobilization strategies such as accumulation of PTEs in fungal structures and production of chelating agents and glomalin, stabilizing these elements in the mycorrhizosphere. For promoting PTEs tolerance in host plants, exclusion mechanisms mediated by AMFs were identified, in which fungal structures such as intra- and extra-radical mycelium and arbuscules, are involved by accumulating large part of these elements, thereby decreasing the uptake and the toxicity in the plant (González-Chávez et al. 2011; Riaz et al. 2021). In particular, the toxicity of PTEs for plants and the environment may be mitigated by the high metal sorption capacity of mycorrhizal mycelia and their ability to transfer and sequester excess element concentrations into vacuoles and root cell walls. This process also involves retaining metal ions in chemically inactive form complexes (Colpaert et al. 2011; Shi et al. 2019). Moreover, AMFs indirectly influence PTEs tolerance of plants by enhancing plant growth, increasing nutrient and water uptake, improving the antioxidative defense system in the plant, and reducing translocation of PTEs to the aerial part by enhanced extraction by the roots (Janeeshma and Puthur 2020). In consequence, AMFs may represent an important tool for the recovery of PTE-polluted sites (Leung et al. 2006; Cabral et al. 2015).

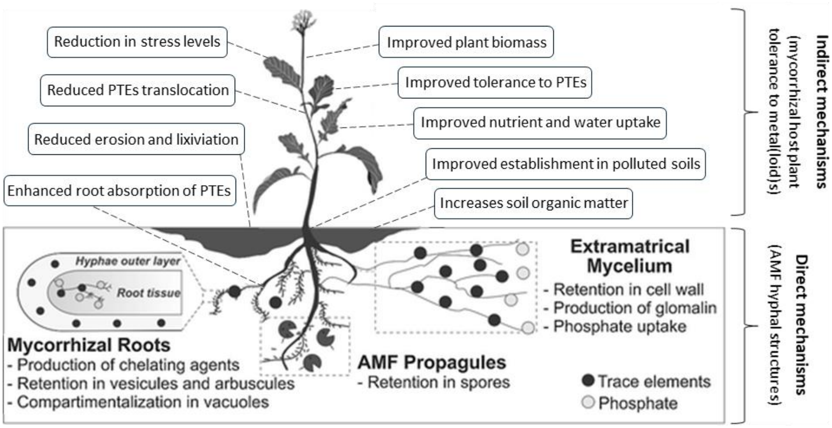

Figure 1.2. Mechanisms developed by AMFs to alleviate PTEs stress and induce tolerance in mycorrhizal plants (adapted from Cabral et al. 2015).

1.6. Ecotoxicological risk assessment of soils polluted by PTEs

1.6.1. Toxicity bioassays

In order to ensure the effectiveness of remediation techniques in reducing toxicity risks in the ecosystem, it is not sufficient to determine the forms of PTEs present. Evaluating the potential toxic effects that PTEs in soils can cause on living organisms is essential for a correct risk assessment (Song et al. 2006). In this sense, ecotoxicological risk assessment is used as a diagnostic tool to assess the potential toxic effects of pollutants on the environment and living organisms, using a combination of chemical and biological techniques (Abbasi et al. 2022). Therefore, to reliably address the negative impacts of PTEs in a polluted area, it is first required to evaluate exposure levels by measuring their concentrations. Then, the potential hazard that these concentrations pose to target organisms is assessed by biological tests (Moermond et al. 2017), altogether allowing a final evaluation of the existing ecotoxicological risk.

Commonly, the ecotoxicological assessment includes the performance of bioassays, obtaining direct responses of indicator organisms exposed to the pollutants. The application of bioassays in real scenarios of soil pollution can reflect the response to both simple and complex mixture of pollutants, being of great relevance for taking into account the interactions between pollutants (antagonistic, additive, and synergistic effects) and their bioavailability (Gong et al. 2020), These factors are major determinants of the actual toxicity of pollutants on soil organisms. However, the effects of these pollutants on living organisms may greatly differ due to different uptake pathways, thus requiring the use of a set of organisms for a more realistic ecotoxicity assessment (Romero-Freire et al. 2018). Therefore, multiple toxicity tests with representative species from different taxonomic groups and trophic levels should be conducted to accurately evaluate toxic effects. For terrestrial ecosystems, target soil organisms for toxicity evaluation include microorganisms, plants, and invertebrates (Song et al. 2006).

Performing ecotoxicity tests using both liquid (e.g. soil extract, pore water, leachate) and solid phase is also crucial for a more reliable biological approach of soil toxicity, since organisms can interact differently with soil particles and liquid soil components (Pastor-Jáuregui et al. 2022). In this regard, bioassays using soil microorganisms can be carried out with both fractions. The bioluminescent bacteria *Vibrio fischeri* is used as an indicator organism in toxicity bioassays using the soil-water fraction (Martín-Peinado et al. 2010), while soil heterotrophic basal respiration rate using the soil-solid fraction is also employed as biological indicator of microbial stress caused by soil pollution (Niemeyer et al. 2012). Moreover, plant-based bioassays to assess soil phytotoxicity, measuring parameters such as germination and growth rates, are performed using both liquid and solid soil fractions. The liquid-phase bioassay with lettuce (*Lactuca sativa* L.) has been recommended by many international organizations for determining the ecological effects of toxic substances and for standard toxicity tests (Lyu et al. 2018). Similarly, a bioassay using barley (*Hordeum vulgare* L.) is applied directly to the solid phase to evaluate toxic effects in soil (Schwertfeger and Hendershot 2013). The solid fraction is also commonly used in bioassays based on soil invertebrates such as *Eisenia* spp. earthworms, widely used as indicators of soil health and toxicity in standard tests (OECD 1984), and in studies to evaluate the toxic effects of single or mixture of pollutants (Natal-da-Luz et al. 2011). Nonetheless, not only terrestrial organisms, but also non-terrestrial ones, like freshwater algae (e.g. *Raphidocelis subcapitata*) and crustaceans (e.g. *Daphnia magna*), can be used for conducting soil toxicity bioassays with the liquid phase. All these represent examples of the wide variety of living organisms that can be employed as indicators to assess soil toxicity. Moreover, all these bioassays, using different groups of organisms and soil fractions, are considered cost-effective tools for monitoring soil pollution for being simple, fast, reliable, and inexpensive, often not requiring expensive equipment.

In addition to toxicity bioassays, to achieve a comprehensive perspective of the ecosystem's health status and a complete evaluation of the effectiveness of the remediation process, it is important to monitor the in situ soil conditions both aboveground and belowground, due to their direct linkage with ecosystem processes and their high sensitivity (Pérez-Vázquez et al. 2024).

1.6.2. In situ evaluation of vegetation development

To assess soil phytotoxicity of PTE-polluted soils, a wide variety of biological tests using model plants can be performed as an approximation of pollution levels for these organisms. However, when evaluating the remediation processes of polluted soils, it is crucial to perform an in situ evaluation of vegetation status, as this is a key component of the ecosystem. This approach provides a more indicative assessment of the extent of soil pollution and the changes induced by remediation treatments. PTEs toxicity in plants can limit processes such as seed germination, root development, and growth, or disrupt the uptake of essential nutrients, potentially leading to plant death (Kabata-Pendias 2010). However, PTEs can also accumulate in plants, able to adapt to varying levels of environmental pollution. Thus, the presence of certain plant communities or species can be indicative of the pollution levels present in the soil.

The evaluation of passive restoration, which involves the natural recolonization of a polluted area by native vegetation as the conditions improve, is crucial when assessing the effectiveness of remediation measures (Álvarez-Rogel et al. 2021). These native plants may act as "nurse plants", promoting the growth of species with lower tolerance to stress conditions (Navarro-Cano et al. 2018). In this regard, the development of vegetation cover is relevant when considering the effectiveness of a specific treatment applied to polluted soil, since it provides physical protection and may reduce potential re-movement of pollutant particles and migration to groundwater (Pérez-de-Mora et al. 2006a). Additionally, the species richness under the influence of a particular treatment can be measured to evaluate the PTE-pollution levels. Decreases in species richness, along with reductions in vegetation cover, are indicators of increased pollution levels (Bayouli et al. 2021), as fewer species are able to adapt and survive under certain limiting conditions. Nevertheless, certain plant species exhibit exceptional capacity to tolerate high soil PTEs concentrations by a wide variety of strategies both physiological and behavioral, allowing them to develop in limiting soil conditions (Baker et al. 2010). Consequently, these species may contribute to improve the physicochemical and biological conditions of polluted soils by handling PTEs concentrations, enhancing nutrient availability, and increasing soil organic carbon (Bolan et al. 2011). Finally, they can also be used as indicator species, since their presence or absence can be used to detect changes and evolution in the soil conditions (Hernández and Pastor 2008).

1.6.3. In situ evaluation of soil microbiological status

The study of soil microbiota including abundance, diversity, and metabolisms can provide valuable information about soil quality and indicate improvements in physicochemical and biological parameters (Giacometti et al. 2013).

Microbial biomass can be used as a biological indicator that provides information on soil quality. The response of soil microbiota to pollution or to the application of amendments can be used to assess changes in the soil, since microorganisms respond faster to these changes than shifts in soil physicochemical properties (Nannipieri et al. 2017). Exposure to PTEs can reduce microbial biomass due to cell death caused by the alteration of essential functions or an unaffordable increase in vital energy cost (Oijagbe et al. 2019). The decrease in microbial activity due to the presence of soil pollutants can also be used as an indicator of stress levels of microbial communities in polluted soils (Azarbad et al. 2013). The response of microbiological communities to soil pollution and remediation treatments can be assessed through the soil heterotrophic respiration rate, which serves as a measure of soil microbiological activity. Furthermore, soil enzymatic activities, which play a key role in soil biochemical processes and control the decomposition rate of organic matter and nutrient cycling, are highly sensitive to PTE-pollution stress (Hu et al. 2014). For this reason, they can also serve as bioindicator of changes in soil quality and biological activity after the application of remediation treatments (Silva-Castro et al. 2022).

In recent years, molecular biological methods such as real-time polymerase chain reaction (PCR) or massive sequencing have gained prominence due to their capacity to provide more detailed information (Hermansson et al. 2004). Real-time PCR has proven to be an effective method for quantifying genes, with high sensitivity that enables the characterization of microbial communities with high specificity and accuracy (Zheng et al. 2020). This technique is particularly useful to detect changes in the microbial community structure after the application of remediation treatments.

1.7. Study site and experimental design

1.7.1. Aznalcóllar mining accident

On April 25, 1998, one of the most important metal mining accidents worldwide took place in Aznalcóllar (Seville, SW Spain) (Nikolic et al. 2011). The breakage of the tailings dam of this metal mine located on the Iberian Pyrite Belt produced a spill of 36 x 10^5 m^3 of pyritic sludge and 9 x 10^5 m^3 of acidic water into the Agrio and Guadiamar river basins (Simón et al. 2001). The sludge was composed mainly of pyrite (68-78%, w/w), and presented high concentrations in Pb, As, Cu, Zn, Cd, Sb, and Tl, which were considered the main pollutants in the soils affected by the spill (Simón et al. 1999). The accident was caused by the excessive volume of waste deposited in the tailings dam, which had been expanded several times to increase its storage capacity, reaching between 21 and 27 m in height at the time of the accident. In total, the spill of the sludge affected an area of over 43 km^2, while the acidic water even reached the vicinity of the Doñana National Park, 58 km downstream from the tailings dam (Grimalt et al. 1999; Ayala-Carcedo 2004).

Immediately after the accident, one of the most important soil-remediation plans in Europe was executed to restore the affected area (Domínguez et al. 2008). First, efforts were focused mainly on the removal of the tailings and the upper part of the highly polluted soils by heavy machinery. Following this, remediation measures primarily involved the extensive application of different amendments to neutralize pollution and reduce the toxicity risk in the surrounding environment. These amendments were based on liming, organic matter, and iron-rich clayey materials, followed by tilling of the upper 20 cm of the soils (Martín-Peinado et al. 2015). These actions significantly reduced the concentrations of soil pollutants, and the affected area was reconverted by the Regional Government of Andalusia into a natural protected area, the Guadiamar Green Corridor (GGC) (CMA 2003), in order to preserve it and facilitate its complete restoration.

1.7.2. Research background in the area and site description

The Aznalcóllar mining spill affected area, currently the GGC, has been scenario of continuous research focused on testing a wide range of materials and remediation strategies to identify effective approaches to remediate and restore the soil quality and ecosystem's health, which were highly degraded after the mining spill. Firstly, Aguilar et al. (2004a, 2004b) and Simón et al. (2008a) evaluated the effectiveness of the remediation actions applied to the area straight after the accident in the reduction of soil pollution levels. These actions involved the successive application of amendments based on sugar-refinery scum, iron-rich clayey materials, and organic matter (compost from various sources and manure), combined with tilling of the soil. They concluded that the most feasible remediation measure was the chemical immobilization of the pollutants, combining liming with organic matter and amendment materials such as soils rich in iron oxides and clay. Other organic and liming amendments were also subsequently tested in the same area as pollutant-stabilization materials, including biosolid and municipal waste composts, cow manure, leaf litter, coal, sugar beet lime, and leonardite (Clemente et al. 2003; Burgos et al. 2005; Madejón et al. 2006, 2009), showing effectiveness in reducing PTEs solubility, facilitating the vegetation establishment, and thus enhancing phytostabilization processes. The potential of these amendments for remediating PTEs-polluted soils was also evaluated by Pérez-de-Mora et al. (2007b) with disparate results among the materials tested. Moreover, the potential of phytoremediation practices has also been tested in the area using both local wild and cultivated species (de Haro et al. 2000; del Río et al. 2002;

Clemente et al. 2005; Vázquez et al. 2006), which evidenced a limited capacity for phytoextraction remediation approaches in these heavily polluted soils. These are some examples of relevant soil remediation studies conducted in the area following the mining accident. However, the GGC scenario represents a very important and widely used natural laboratory for evaluating the effectiveness of a broad range of soil remediation approaches and contributes to a deeper understanding of the evolution of ecosystems after a major soil pollution episode, as well as the long-term fate of pollutants in soils under varying conditions (Madejón et al. 2018).

Currently, the area is considered as recovered. However, although the remediation measures were effective and gradually decreased the concentration of pollutants in the affected soils, even 25 years after the accident, high levels of residual pollution may be still found within the area affected by the spill, especially in the upper part (the first 18 km downstream from the source of the pollution), where it was estimated that around 7% of the affected soils remained unrecovered 15 years after the accident (Martín-Peinado et al. 2015). The persistence of pollution in these soils is mainly related to the presence of residual tailings in the area, caused by a deficient and non-uniform cleanup of the tailings right after the accident (Simón et al. 2005a; Martín-Peinado et al. 2015). This fact becomes evident by the presence of bare soil patches of different sizes, easily identifiable by the complete absence of vegetation, which are heterogeneously distributed along the GGC. In these unvegetated soil patches the soils are mainly characterized by a strong acidic pH, low organic matter and carbonate contents, and high salinity and total concentration of PTEs (Martín-Peinado et al. 2015).

These residual polluted soils pose a high risk to the surrounding areas linked to the potential spreading of pollution by wind and water erosion or leaching (Bopp et al. 2016), as they are not stabilized by vegetation. Therefore, they represent a risk not only to the environment but also to human health from exposure to these elements (Timofeev et al. 2018; Rinklebe et al. 2019). In this regard, Pastor-Jáuregui et al. (2022) assessed the human toxicity risk of the soils in the GGC since this area is currently intended for recreational use, and therefore exposure to PTEs that may be present in soils by local population is constant. Although they found that the GGC soils do not represent a potential risk to human health for this use, their results also revealed a specific potential risk for children to arsenic exposure, requiring continuous monitoring of the evolution of toxicity in the area. In any case, these unvegetated patches pose a high risk of pollution dispersion that must be monitored and addressed, making the design and development of effective and feasible remediation strategies for these soils highly relevant for the environment and the society.

1.7.3. Experimental set up

1.7.3.1. Field experimental plots based on soil amendment treatments

An in-situ evaluation of the effectiveness of different soil treatments, using inorganic and organic amendments, for the remediation of PTE-polluted soils was carried out in the soils of the GGC affected by the Aznalcóllar mining spill.

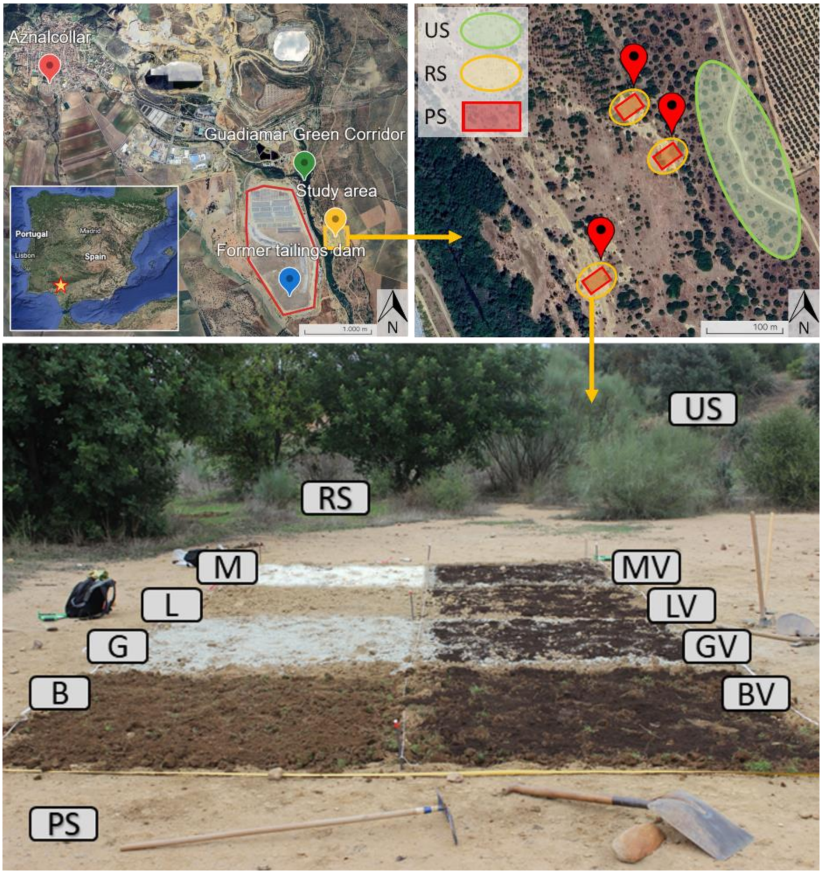

Figure 1.3. Study area and field experimental plots location and design. US: Unaffected reference soil; RS: Recovered reference soil; PS: Untreated polluted reference soil; B: Biopile (50% w/w mixture of PS with adjacent RS; BV: Biopile + vermicompost; G: Gypsum mining spoil; GV: Gypsum mining spoil + vermicompost; L: Landfarming; LV: Landfarming + vermicompost;7. M: Marble sludge; MV: Marble sludge + vermicompost.

This evaluation involved chemical, physiological, and microbiological assessment of soils and plants. For this purpose, three unvegetated residually polluted soil patches within the GGC and located in the area closest to the former mine tailings dam were selected (Figure 1.3). Within each of the three selected patches, an experimental plot of 32 m^2 was set up and divided into eight subplots of 4 m^2 with a different treatment applied in each of them (Table 1.1).

Table 1.1. Description of the studied soils and treatments used for soil remediation in this thesis, and their corresponding acronyms.

Acronym	Treatment	Description
US	UNAFFECTED SOIL	Natural soil adjacent to the affected area
RS	RECOVERED SOIL	Soil naturally revegetated within the affected area
PS	POLLUTED SOIL*	Unvegetated polluted soil within the affected area
B	BIOPILES	50% w/w mixture of RS and PS
BV	BIOPILES + VERMICOMPOST	50% w/w mixture of RS and PS + vermicompost
G	GYPSUM	Gypsum mining spoil
GV	GYPSUM + VERMICOMPOST	Gypsum mining spoil + vermicompost
L	LANDFARMING	Soil crust breaking through tillage
LV	LANDFARMING + VERMICOMPOST	Soil crust breaking through tillage + vermicompost
M	MARBLE SLUDGE	Marble cutting and polishing residue
MV	MARBLE SLUDGE + VERMICOMPOST	Marble cutting and polishing residue + vermicompost

*Equivalent to control/contaminated soil (CT) in chapter 4.

The selected amendments were chosen for being calcium and organic matter-rich amendments, and in the case of the inorganic amendments, for representing low-cost waste materials generated in very large amounts that need to be sustainably managed (Rayed et al. 2019; García-Robles et al. 2022). The doses of gypsum mining spoil and marble sludge (5 kg m^{-2}) were selected according to doses previously applied in the area for calcium-rich amendments (Madejón et al. 2018), and the ratio for the BS treatment was successfully tested in a previous ex-situ experiment (García-Carmona et al. 2017). In all cases, the dose of vermicompost was 5 kg m^{-2} (equivalent to 50 t ha^{-1}) because the organic amendments had been previously applied in the area at a rate of 15-25 t ha^{-1} (Cabrera et al. 2005, 2008) and resulted insufficient to promote vegetation growth.

In addition to the treatments, and based on the different degrees of pollution present in the study area, three reference soils were established and considered as control soils for different soil conditions. Thus, one subplot within the unvegetated soil patch was designated as untreated polluted control (PS), one vegetated subplot adjacent to the unvegetated soil patch was selected as recovered control (RS), and soil unaffected by the spill near the experimental area was used as a reference for the natural unaffected soil (US) (Table 1.1).

1.7.3.2. Bioremediation treatments

A soil bioremediation approach based on AMFs was implemented under greenhouse conditions as a pilot study. This study aimed at evaluating the potential of bioremediation treatments to improve the effectiveness of physicochemical remediation treatments applied to PTE-polluted soil of the GGC, with future perspectives of transferring this approach to the field using the knowledge gathered in this assessment.

Residual PTE-polluted soil from the area affected by the Aznalcóllar mining spill, proximate to the location of the field experimental plots, was sampled for use in the greenhouse experiment. The sampled soil was amended with inorganic and organic materials, some of which had been selected previously in in-situ treatments, along with additional ones. A marble-based amendment was selected as inorganic material according to previous experiments. Meanwhile, two different organic materials were used, vermicompost and DOR previously biotransformed by two different saprobic fungi (Coriolopsis rigida (EEZ-92) and Coprinellus radians (EEZ-84)), thus leading to a total of three different organic amendments applied to the polluted soil.

The AMFs used in this assessment were added to the polluted soil in combination with the amendment treatments. Two different AMF species were selected for this experiment, *Rhizophagus irregularis*, formerly known as *Glomus intraradices*, and *Rhizoglomus custos* MUCL47214. Both species are known for their ability to support plant survival and development in mining soils (Silva-Castro et al. 2022). The model fungus *R. irregularis* was used as the control, while *R. custos* was selected for being an indigenous species isolated from a proximate mining area with naturally high concentrations of PTEs. The AMFs were applied to the treatments by inoculating pre-germinated wheat plants (*Triticum aestivum* L.) selected as model plant, which were grown individually in pots containing the mixture of polluted soil with the correspondent amendments. After 45 days, soil and plant samples were individually collected for subsequent analysis (Figure 1.4).

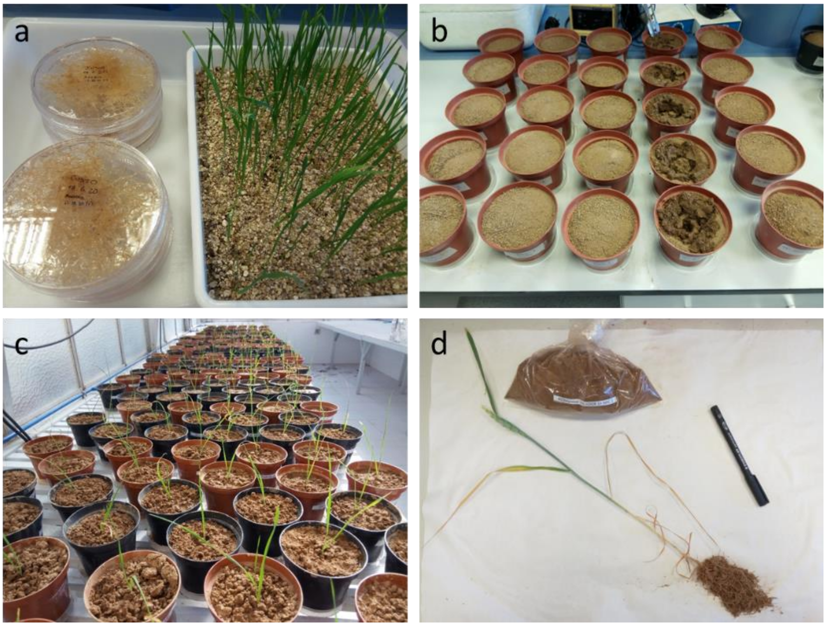

Figure 1.4. Experimental set up of the AMF-based bioremediation treatments. a) mycorrhizal fungi and pre-germinated wheat plants prepared to be added to the soil; b) PTE-polluted soil pots with the addition of the AMF inoculum; c) greenhouse pot model; d) soil samples and plants collected after 45 days.

1.8. Aim and outline of this thesis

The focus of this thesis is based on the premise that the utilization of residues from anthropogenic activities and the application of symbiotic microorganisms can be viable strategies for the remediation of soils polluted by PTEs. The use of stabilized residues as organic and inorganic amendments improves the chemical, physical and biological properties of soils. Similarly, the use of AMFs can help plants tolerate the stress of polluted soils through nutritional, physiological and structural improvements as well as their ability to immobilize, extract and concentrate PTEs.

The main goal of this thesis is, therefore, to evaluate the effectiveness and feasibility of a set of soil remediation techniques and strategies for the ecological remediation of areas polluted by PTEs in the Guadiamar Green Corridor. For this purpose, these remediation techniques based on the valorization of wastes and by-products derived from human activities combined with the use of beneficial soil microorganisms, such as AMFs, were applied both in-situ and under greenhouse conditions.

In **chapter 2**, an ecotoxicological assessment of soil pollution in the treated soils was carried out through the application of a variety of bioassays selected as indicators of PTEs stress in polluted soils. In **chapter 3**, the vegetation status and spontaneous recolonization in the reference and treated soils were measured. The soil microbiological status of the reference and treated soils was evaluated in **chapter 4**. The addition of a biological element based on AMFs inoculation to the soil remediation treatments tested in the field was explored in **chapter 5** as a potential strategy for enhancing the effectiveness of the remediation process. Finally, in the concluding chapter of this PhD thesis (**chapter 6**), the results obtained in previous chapters are summarized, discussed, and integrated.

Chapter 2

Ecotoxicological assessment of polluted soils one year after the application of different soil remediation techniques

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Abstract

The present work evaluates the influence of eight different soil remediation techniques, based on the use of residual materials (gypsum, marble, vermicompost), on the reduction of metal(loid)s toxicity (Cu, Zn, As, Pb and Cd) in a polluted natural area. Selected remediation treatments were applied in field exposed to real conditions and they were evaluated one year after the application. More specifically, five ecotoxicological tests were carried out using different organisms on either the solid or the aqueous (leachate) fraction of the amended soils. Likewise, the main soil properties and the total, water-soluble and bioavailable metal fractions were determined to evaluate their influence on soil toxicity. According to the toxicity bioassays performed, the response of organisms to the treatments differed depending on whether the solid or the aqueous fraction was used. Our results highlight that the use of a single bioassay may not be sufficient as an indicator of toxicity pathways to select soil remediation methods, so that the joint determination of metal availability and ecotoxicological response will be determinant for the correct establishment of any remediation technique carried out under natural conditions. Our results indicated that, of the different treatments used, the best technique for the remediation of metal(loid)s toxicity was the addition of marble sludge with vermicompost.

Keywords: soil pollution; circular economy; remediation; bioavailability; ecotoxicology; bioassay; *Hordeum vulgare*; *Lactuca sativa*; *Raphidocelis subcapitata*; *Daphnia magna*

2.1. Introduction

Soil pollution by potentially toxic elements (PTEs) is one of the most pressing public concerns, since it could directly affect not only the environment, but also public health. It denotes a major degradation of the ecosystem (Burgos et al. 2008a), and a significant potential toxicological risk to organisms (Song et al. 2009). Over 10 million sites worldwide have been reported with soil pollution by several anthropogenic sources and activities (industry, mining, smelters, agriculture, etc.) (He et al. 2015), and, therefore, the correct management of these sites is essential to avoid damages to human health or the environment. Of the usual sources of soil pollution, the current pace and development of the mining industry is a major concern worldwide, mainly due to the pollution by metal(loid)s (e.g. Cr, Hg, Ni, Cu, Zn, Cd, As, Pb). They can accumulate and persist in soils, affecting ecosystem functions, compromising soil quality and the balance of ecosystem communities (Liu et al. 2017; Palma et al. 2019).

The Aznalcóllar disaster (Seville), considered one of the largest mining disasters in Europe (Nikolic et al. 2011), is a notable example of this type of pollution and the damage that can be caused by PTEs. In this mining accident a total of 45 km^2 of mainly agricultural soils were affected by the discharge of 45 x 105 m^3 of acidic waters and toxic tailings after the rupture of the Aznalcóllar pyrite mine sludge pond in 1998 (Simón et al. 2001). Although the environmental impact was greatly minimized, thanks to the high buffering capacity of the soils (Aguilar et al. 2007; Simón et al. 2008b), one of the most ambitious soil remediation programs in Europe was carried out. However, more than 20 years after the accident, residual pollution is still found in the area (Pastor-Jáuregui et al. 2020a), posing a potential toxic risk to living organisms (Romero-Freire et al. 2016a; García-Carmona et al. 2017). In the first 18 km downstream of the tailings pond, unvegetated soil patches remained, with high concentrations of PTEs and unfavorable physicochemical properties (i.e. acidic pH, low organic matter content, etc.) (Sierra-Aragón et al. 2019). These areas represent approximately 7% of the total area affected and can act as a source of diffuse pollution that must be addressed (Martín-Peinado et al. 2015). According to García-Carmona et al. (2017), these unvegetated soil patches pose an environmental risk, assessed by risk quotients (USEPA 2007) for the total concentration of As, Cu and Pb, and for Cu and Zn when considering water-soluble concentrations.

Recently, the development of a large number of safer, cleaner, less expensive, and more environmentally friendly soil remediation techniques is being studied (Kulshreshtha et al. 2014). In the case of residual pollution, the usual techniques aim to reduce the toxicity of pollutants in the soil by controlling their mobility and bioavailability, since the toxicity risk strongly depends on them, rather than of their total concentration (Quevauviller et al. 1998; Romero-Freire et al. 2015a, 2015b). Thus, the remediation of soils affected by residual pollution must consider the main soil properties related to the reduction of mobility, availability, and toxicity such as pH, organic matter, calcium carbonate content, texture, cation exchange capacity and iron oxy(hydr)oxides (García-Carmona et al. 2017; Romero-Freire et al. 2014, 2015b; Kabata-Pendias 2010). One of the most widely applied remediation technologies is the addition of amendments to polluted soils. Especially low-cost ones, such as waste from several human activities, to tackle both the negative impacts of PTEs on the environment and to connect with the circular economy strategy (Stahel 2016; Lèbre et al. 2017; Tayebi-Khorami et al. 2019). In this sense, some research has explored the use of materials at the end-of-life cycle from different sectors to remediate residually polluted soils by mining activity. For example, marble waste sludge amendment can significantly reduce soil acidity and PTEs mobility in polluted mines by shifting the water-soluble forms of PTEs to fractions associated with carbonates, metaloxides, organic matter, and insoluble secondary oxidation minerals and residual phases (Fernández-Caliani et al. 2022). Likewise, it was also demonstrated that the application of organic amendments on the non-vegetated soil patches in Aznalcóllar area has resulted in significant changes in the main soil properties, as well as in reductions in the soluble and exchangeable forms of several PTEs (Sierra-Aragón et al. 2019). However, caution should be exercised as in some cases the addition of amendments to residually polluted soils has been reported to be ineffective in remediating some PTEs (e.g., As) in relation to changes in environmental conditions (reducing or oxidizing conditions) (Beiyuan et al. 2017; Aguilar-Garrido et al. 2022). Nevertheless, further in-situ techniques are available and in use. For example, landfarming (periodic tilling of the polluted soils to remove surface crusts and aerate) and biopiles (mixing polluted soils with recovered soils) were applied together with composting in García-Carmona et al. (2017) in bare patches of Aznalcóllar area with the aim of reducing the mobility and bioavailability of soil pollutants.

In order to ensure that remediation techniques are effective, the determination of PTEs forms present is not enough, and the potential effect that the studied PTEs can cause in living organisms is essential. Commonly, the assessment of the potential harmful effect of the PTEs in soils is done by obtaining direct responses of living organisms exposed to them. However, the effects of the pollutants on organisms will greatly differ due to different uptake pathways, thus requiring the use of a set of organisms for a more realistic ecotoxicity assessment (Romero-Freire et al. 2018). Therefore, multiple toxicity tests must be conducted with representative species of different taxonomic groups to evaluate toxic effects.

The aim of the present work is to assess if the use of different remediation techniques, based on the use of residual materials (circular economy), could be helpful in reducing the metal(loid)s toxicity in a polluted natural area. To perform a complete toxicity assessment, selected amendments were applied in field exposed to real conditions and they were evaluated one year after the application. More specifically, five ecotoxicological tests, on either the solid or the aqueous (leachate) fraction of the amended soils, were performed. The main soil properties were also measured and the total, water-soluble and bioavailable metal(loid)s content (Cu, Zn, As, Pb and Cd) were determined to evaluate which properties and metal(loid)s fraction most influence soil toxicity and to select the best remediation technique.

2.2. Materials and methods

2.2.1. Study area and remediation treatments

The study site is located in the area affected by the Aznalcóllar mine spill (Seville, SW Spain), in the sector nearby to the mine and highly affected by the pyritic tailings spill in 1998. The high pollution soil levels were evidenced by the presence of soil patches of varied surface with no vegetation growth and heterogeneously dispersed among re-vegetated areas (Martín-Peinado et al. 2015). Within the affected area, in an unvegetated soil patch near the mine, different remediation treatments were tested (Figure 2.1).

Specifically, an experimental plot of 32 m^2 consisting of 8 subplots (4 m^2 each) with eight different treatments was established (Table 2.1). In all cases, the dose of amendment applied, both inorganic (gypsum and marble) and organic (vermicompost), was 5 kg m^{-2} (equivalent to 50 t ha^{-1}). In addition, one vegetated subplot adjacent to the unvegetated soil patch to the experimental plot was selected as recovered control (RS), and one subplot within the unvegetated soil patch as unrecovered control (PS).

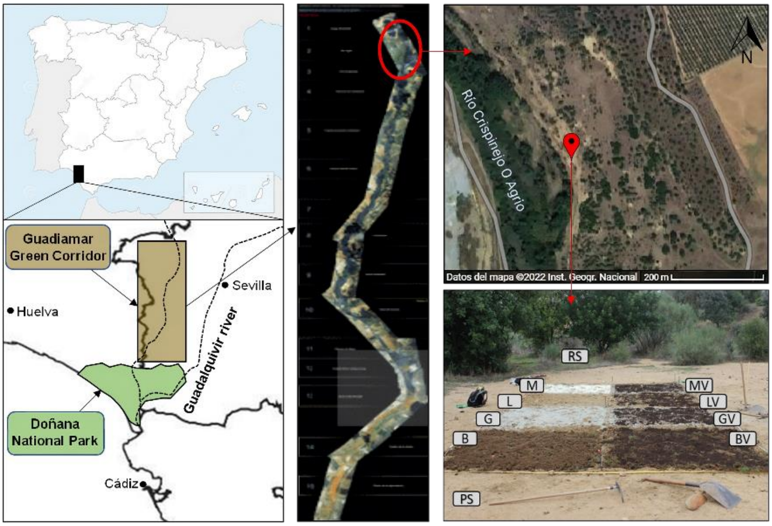

Figure 2.1. Study area, plot localization in the unvegetated soil nearby to the mine and treatments used for the remediation.

2.2.2. Soil properties

After one year of exposure to the different remediation techniques used, soil samples, in each studied subplot, were taken by mixing 200 g of topsoil (0–10 cm) from each corner and midpoint of a square 1 m per side (composite samples; n=3). Samples were air dried at room temperature in laboratory and passed through a 2-mm sieve. This fraction was used to characterize the main

Table 2.2. Description of the treatments used for soil remediation, and the corresponding descriptor acronym.

Acronym	Treatment	Description
RS	RECOVERED SOIL	Soil naturally revegetated within the affected area
PS	POLLUTED SOIL	Unvegetated polluted soil within the affected area
B	50% BIOPILES	50% w/w mixture of RS and PS
BV	BIOPILES + VERMICOMPOST	50% w/w mixture of RS and PS + vermicompost
G	GYPSUM	Gypsum mining spoil
GV	GYPSUM + VERMICOMPOST	Gypsum mining spoil + vermicompost
L	LANDFARMING	Soil crust breaking through tillage
LV	LANDFARMING + VERMICOMPOST	Soil crust breaking through tillage + vermicompost
M	MARBLE SLUDGE	Marble cutting and polishing residue
MV	MARBLE SLUDGE + VERMICOMPOST	Marble cutting and polishing residue + vermicompost

soil properties and for the toxicity bioassays, and when determinations required, soil samples were also finely ground. Soil pH was determined in a soil:water ratio 1:2.5 with a 914 pH/Conductometer Metrohm (Herisau, Switzerland); soil:water extract (1:5) was prepared to determine the electrical conductivity (EC, dS m^{-1}) using a Eutech CON700 conductivity-meter (Oakton Instruments, Vernon-Hills, IL, USA); calcium carbonate content (%$CaCO_3$) by volumetric gases (Barahona 1984); organic carbon (%OC) was determined according to Tyurin (1951); total nitrogen (%N) was analyzed by dry combustion using an elemental analyzer TruSpec CN LECO® (St. Joseph, MI, USA); soil texture was determined by the Robinson pipette method (USDA 1972); base content and cation exchange capacity (CEC) were determined according to the methodology of (USDA 1972); and the water field capacity (%WFC) and available water content (%AWC) were determined from Richards methodology (Richards 1947).

2.2.3. Soil metal content determination

To determine total metal content (Xx_T), soil samples were acid digested (HCl:HNO_3, 3:1); water-soluble content (Xx_W) was obtained from soil:water extract (1:5) (Blakemore et al. 1987), and bioavailable content (Xx_B) was obtained by extraction with 0.05 M EDTA (pH 7) as described by (Quevauviller 1998). All samples were analyzed by ICP-MS in a PerkinElmer NexION® 300D spectrometer (Waltham, MA, USA). The ICP-MS operating conditions included three replicates in each measurement. For calibration, two sets of multi-element standards containing all the analytes of interest were prepared using rhodium as the internal standard. All standards were prepared from ICP single-element (Zn, Cu, Pb, As and Cd) standard solutions (Merck, Darmstadt, Germany) after dilution with 10% HNO_3. The accuracy of the method was confirmed by analyzing Certified Reference Material CRM025-050 (Sandy Loam Soil), six replicates. For Zn, Cu, Pb, As and Cd the average recoveries were 98.4±12% of the CRM.

2.2.4. Ecotoxicological approach

2.2.4.1. Ecotoxicological tests in soil-solid fraction

Root elongation toxicity test with *Hordeum vulgare* L. (barley, monocotyledonous, Poaceae) evaluates the toxic effect directly in soil fraction (Schwertfeger and Hendershot 2013). Soil moisture content was fixed at WFC and soils were incubated in cylindrical pots (8 cm in diameter and 11 cm in height). Barley seeds were pregerminated at 20 °C in darkness 48 h in petri dishes. Six seeds with a radical length lower than 2 mm were planted in each pot, approximately 10 mm beneath the surface of the soil. Plants were placed under controlled growing conditions (16 h/8 h light/darkness, 20±2 °C). After 5 days of growth, each soil was gently washed out of the pots. Plant roots were carefully washed preventing root damage and the result was expressed as the measured longest root per seedling (cm).

Heterotrophic soil respiration (Rs) was measured by determining the CO_2 flux from studied soils with a Microbiological Analyser μ-Trac 4200 SY-LAB model (SY-LAB Geräte GmbH, Neupurkersdorf, Austria) (ISO 2002). Soil moisture content was fixed at WFC and soils were incubated at a constant temperature of 30 °C with addition of glucose (3 mg per gram of soil). CO_2 production was determined by absorption during 6 h in vessels with two electrodes containing a solution of potassium hydroxide (KOH 0.2%) and hermetically sealed. The results obtained indicate the average of induced soil respiration and were expressed as the μg of CO_2 respired for day and gram of soil (μg CO_2 day^{-1} g^{-1} soil).

2.2.4.2. Ecotoxicological tests in soil-water fraction

Root elongation toxicity test with *Lactuca sativa* L. (lettuce, dicotyledonous, Asteraceae) was done according to a modification of US EPA recommendations (USEPA 1996). In Petri dishes 15 seeds of *L. sativa* were incubated in 5 ml of soil:water extract (1:5) from the studied soils. The dishes were placed in an incubator at 25 ± 1 °C and the length of the developing roots was measured after 120 h. The endpoint calculated was the % of root elongation reduction compared to a control performed only with distilled water.

The algal growth inhibition test with *Raphidocelis subcapitata* (freshwater algae) was carried out based on (ISO 2012). Exponentially growing algae (10^4 cell mL^{-1}) were exposed to soil:water extract from the studied soils over a period of 72 h under defined conditions, as described elsewhere (Russo et al. 2018). Growth was quantified from measurements of the algal biomass density (cell counts) by spectrometry (670 nm) as a function of time. The specific growth rate of *R. subcapitata* was calculated from the logarithmic increase in cell density in the intervals from 0 to 72 h. The results were expressed as the mean of the % inhibition of the algae growth of the sample compared with a negative control (ISO media).

Daphnia magna (Crustacea) immobilization test was carried out according to OECD 202 guideline (OECD 2004). Daphnid neonates, up to 24 h old, were transferred to 6-well polycarbonate test plates with soil:water extract and incubated for 48 h under control conditions (darkness and 20±2 °C) without feeding for the duration of the experiment. After 48 h of exposure, daphnids were observed under magnification and those that were not able to swim within 15 s under gentle agitation were recorded as immobilized. The calculated endpoint was the % of immobilized daphnids compared to the control performed with distilled water.

2.2.5. Statistical analysis

Data distributions were established by calculating the mean values and the standard deviations by cumulative frequency–distribution curves. The differences between the individual means of the study samples were compared using ANOVA and Duncan's post hoc test ($p<0.05$) using SPSS v.21.0 (SPSS Inc. Chicago, USA). In order to study the relation between soil properties, metal content and ecotoxicological approach in the studied soils, a non-metric multidimensional scaling (NMDS) analysis was carried out using the "vegan" package of RStudio software (RStudio Inc.(R), 250 Northern Ave, Boston).

2.3. Results and discussion

2.3.1. Improvement of soil properties after remediation

The use of the different techniques selected for the remediation of polluted soil showed efficient results in the increase of soil pH (Table 2.2). The acidic solution from the oxidation of the pyrite-tailings infiltrates the soil and the H^+ are neutralized by exchangeable bases, by weathering of silicate mineral or, more intensely, by carbonates (Cravotta and Trahan 1999). According to our results, the use of marble (M) was the best treatment to reach pH near neutrality. This amendment was the one that contributed the highest $CaCO_3$ values, this being the main factor affecting the increase in pH. According to Williams et al. (1982) the potential acidity of 1 g of pyritic sulfur is neutralized by approximately 3 g of $CaCO_3$ and, for this reason, liming is one of the most widespread remediation actions to control soil acidity issues (Simón et al. 2005b). For all remediation amendments the electric conductivity (EC), as a measurement of salinity, decreased by half, but without reaching the values present in the recovered soil (RS). The use of vermicompost, as part of the amendments, was directly related to the higher increase in OC and N content

Table 2.3. Main soil properties for the different remediation techniques (see table 2.1) one year after field application. EC: electrical conductivity; $CaCO_3$: calcium carbonate content; OC: organic carbon; N: total nitrogen; C/N: carbon-to-nitrogen ratio; CEC: cation exchange capacity; AWC: available water content.

Sample	pH	EC	$CaCO_3$	OC	N	C/N	Silt	Clay	CEC	AWC
		dS m^{-1}	%	%	%		%	%	$cmol_c\ kg^{-1}$	%
RS	6.7 *cd*	1.0 *a*	2.30 *a*	1.28 *e*	0.11 *d*	11.4 *c*	28.1	15.3	11.4	15.1
PS	3.3 *a*	4.1 *c*	1.25 *a*	0.36 *a*	0.07 *a*	5.6 *a*	26.6	15.5	7.0	8.8
B	4.6 *b*	2.2 *b*	0.93 *a*	0.71 *bc*	0.09 *bc*	7.8 *b*	27.8	21.8	8.9	12.3
BV	5.1 *b*	2.2 *b*	1.07 *a*	0.90 *cd*	0.10 *cd*	8.6 *b*	32.7	23.0	10.5	12.4
G	6.0 *c*	2.3 *b*	1.25 *a*	0.61 *ab*	0.08 *ab*	8.0 *b*	27.4	22.2	8.6	11.7
GV	6.0 *c*	2.3 *b*	1.12 *a*	1.00 *d*	0.11 *cd*	9.0 *b*	27.3	19.6	9.4	11.9
L	5.0 *b*	2.2 *b*	1.05 *a*	0.49 *ab*	0.06 *a*	7.9 *b*	24.9	14.8	9.9	12.2
LV	4.3 *b*	2.2 *b*	0.99 *a*	0.72 *bc*	0.10 *cd*	7.5 *ab*	28.2	22.2	13.6	11.2
M	7.0 *d*	2.2 *b*	8.35 *b*	0.59 *ab*	0.06 *a*	9.4 *bc*	30.9	16.7	8.5	12.7
MV	7.2 *d*	2.3 *b*	3.18 *a*	0.99 *d*	0.11 *d*	8.8 *b*	29.5	20.7	9.9	14.4

Lowercase letters indicate significant differences among treatments according to Duncan's post hoc test ($p<0.05$).

(BV, GV, LV and MV). The addition of compost increases the content of stable organic compounds in soils (Park et al. 2011), improves chemical soil fertility of polluted soil promoting favorable conditions for plants and microorganism (Nakamaru and Martín-Peinado 2017), and reduces the mobility of potential toxic elements (Bolan and Duraisamy 2003). Vermicompost increased OC content in soils, but this content did not reach the values observed in RS, where natural vegetation appears improving soil properties related to fertility (García-Carmona et al. 2019a). In general, the use of the selected amendments showed a slight increase in the CEC of soils, higher when vermicompost was used in the mixture. It was the landfarming + vermicompost (LV) and biopiles + vermicompost (BV) which showed CEC values close to those of the recovered soil (RS). This is consistent with García-Carmona et al. (2017), who reported a positive correlation between soil OC content and CEC in soils that remained polluted 18 years after the Aznalcóllar accident and that were treated at laboratory scale with landfarming, biopiles and composting. The use of the selected amendments modified soil texture as well, with the higher values of fine granulometries (silt and clay) in BV. All applied amendments improved soil water retention (AWC). This may be related to the increased OC content in the amended soils, which increases soil water retention and infiltration capacity (Maylavarapu and Zinati 2009; Whelan et al. 2013), improves soil structural stability (Owen et al. 2021) and reduces soil bulk density (River et al. 2022).

2.3.2. Availability and solubility of the studied elements

Table 2.3 shows the total (Xx_T), bioavailable (Xx_B), and water-soluble (Xx_W) content of Cu, Zn, As, Pb and Cd in recovered soil (RS), polluted soil (PS), and polluted soil after the application of the amendments of study and incubation in field during one year.

Metal(loid)s pollution is usually determined based on the total metal content in soils, and there are several legislations that specify total concentration values that are intervention values for polluted soils. According to the Andalusian government, where soil samples are located, the intervention values for Cu, Zn, As, Pb and Cd are 595, 10,000, 36, 275 and 25 mg kg^{-1} respectively (BOJA 2015). According to this regulation, only total As and Pb showed contents above the guideline values. But even nowadays there is not a clear consensus for determining the metal guideline values in soils. Not all countries have defined the corresponding guidelines, and when compared, the concentrations differ significantly from one place to another (See table S2.1 for more detail). In Spain, intervention values for some elements vary more than 10 times depending on the regional government. This is the case of Galicia guideline (northwest Spain), where for Cu, Zn and Cd guidelines values are 50, 300 and 2 mg kg^{-1}, more than 10 times lower than the Andalusian ones (DOG 2009). If we compare the total content of these 3 elements in our soils with the Galician legislation, we have also risk of pollution for Cu, Zn and Cd that, in principle, were out of risk in Andalusia. In the case of other countries, values differ even more, i.e. in China the risk screening value for Cd is 0.2 mg kg^{-1} (MEEPRC 2018) or in Canada the intervention value for As is 12 mg kg^{-1} (CCME 2007) (Table S2.1). Thus, the risk of pollution could be over or under-estimated when studies are based in total concentrations only, and dependent on the local regulations and guideline values. Furthermore, it is not expected that the use of soil amendments modifies the total metal content, as it was the case for the treatments used in this study, being their influence more related to the available metal forms. Indeed, the total concentration of a PTE may not be relevant in terms of toxicity and its behavior in the soil, and its ecotoxicity being more related to its mobility in soil (Giannakis et al. 2021). Therefore, it is also important to determine the available forms of PTEs before and after remediation, and regulations be based on them and not only in total concentrations, since they are more accurately related to their actual risk of toxicity.

Table 2.4. The total (Xx_T) soil concentration, the bioavailable concentration (Xx_B), and the water-soluble concentration (Xx_W) of the studied trace elements (Cu, Zn, As, Pb and Cd) for the recovered soil (RS), the polluted soil (PS), and the different treatments used (see table 2.1.) one year after field application. Values in mg kg^{-1} dry soil.

Sample	RS	PS	B	BV	G	GV	L	LV	M	MV
Cu_T	248 *e*	149 *abc*	185 *d*	170 *cd*	142 *ab*	155 *abc*	160 *bcd*	151 *abc*	130 *a*	136 *ab*
Zn_T	926 *a*	359 *b*	316 *b*	335 *b*	235 *b*	232 *b*	319 *b*	213 *b*	240 *b*	349 *b*
As_T	209 *a*	309 *bc*	330 *c*	398 *d*	292 *bc*	326 *bc*	306 *bc*	317 *bc*	284 *b*	289 *bc*
Pb_T	314 *a*	430 *b*	560 *c*	838 *d*	496 *bc*	560 *c*	547 *c*	548 *c*	492 *bc*	436 *b*
Cd_T	4.4 *bcd*	6.6 *e*	5.0 *cde*	6.1 *de*	4.0 *bc*	2.9 *ab*	4.3 *bcd*	6.1 *de*	2.3 *a*	5.6 *cde*
Cu_B	67.96 *d*	37.95 *c*	33.59 *bc*	33.90 *bc*	31.52 *bc*	32.37 *bc*	29.96 *bc*	29.54 *bc*	16.87 *a*	24.45 *ab*
Zn_B	105.90 *ab*	239.84 *b*	78.91 *a*	72.35 *a*	42.30 *a*	46.70 *a*	96.81 *ab*	57.08 *a*	18.94 *a*	56.87 *a*
As_B	1.58 *c*	0.88 *bc*	0.11 *ab*	0.24 *ab*	0.13 *ab*	0.88 *bc*	<0.01	0.47 *ab*	0.09 *ab*	0.30 *ab*
Pb_B	2.47 *b*	0.30 *ab*	2.19 *ab*	1.65 *ab*	0.13 *a*	0.61 *ab*	<0.01	0.19 *a*	0.07 *a*	0.21 *a*
Cd_B	1.34 *c*	0.74 *b*	0.52 *ab*	0.53 *ab*	0.40 *ab*	0.37 *a*	0.46 *ab*	0.30 *a*	0.26 *a*	0.43 *ab*
Cu_W	0.41 *a*	19.30 *b*	1.09 *a*	0.40 *a*	0.15 *a*	0.22 *a*	0.65 *a*	0.95 *a*	0.15 *a*	0.18 *a*
Zn_W	0.56 *a*	206.14 *b*	31.95 *a*	18.38 *a*	0.22 *a*	0.22 *a*	38.76 *a*	38.16 *a*	<0.01	<0.01
As_W	0.25 *b*	0.10 *ab*	0.06 *a*	0.06 *a*	0.05 *a*	0.05 *a*	0.05 *a*	0.06 *a*	0.07 *a*	0.06 *a*
Pb_W	0.21 *a*	0.10 *a*	0.10 *a*	0.08 *a*	0.06 *a*	0.04 *a*	0.05 *a*	0.05 *a*	0.07 *a*	0.02 *a*
Cd_W	<0.01	0.78 *b*	0.18 *a*	0.08 *a*	0.01 *a*	0.01 *a*	0.16 *a*	0.14 *a*	<0.01	<0.01

Lowercase letters indicate significant differences among treatments according to Duncan's post hoc test ($p<0.05$).

In the case of bioavailable content (Xx_B) obtained by extraction with EDTA, metal concentrations were even higher in the recovered soil (RS) than in the polluted one (PS). These results agree with those reported by Sierra-Aragón et al. (2019) and Paniagua-López et al. (2021), that reported an increase in EDTA-extractable Cu, As, Pb and Cd with increasing soil organic matter content, as organic matter can be considered as a key soil property for the extractability of metals by chelators such as EDTA (Fang et al. 2007; Xu et al. 2022). Anyway, the use of the selected amendments decreased for all studied elements their bioavailable content compared to the PS, being the application of marble sludge (M) the remediation technique that showed the highest decrease in bioavailability for all elements. Marble sludge influenced the most the increase in the $CaCO_3$ content of studied soils, which implies that metals could precipitate as insoluble carbonates (García-Carmona et al. 2017; Simón et al. 2005b) and be extracted by the EDTA (Santos et al. 2010).

Moreover, the determination of water-soluble forms is one of the easiest and cheapest techniques to study metal pollution. The water-soluble forms could also be a good indicator of metal pollution. In this regard, the water-soluble concentrations (Xx_W) observed were greater in the polluted soil compared with the recovered one in all cases, except for As and Pb. Regarding As, authors such as Martín-Peinado et al. (2015) and Pastor-Jáuregui et al. (2022) have reported increases in As solubility in recovered soils in the Guadiamar Green Corridor due to an increase in the organic matter content of the soils over time, 15 and 20 years after the pyritic sludge spill, respectively. If we compare our results with the reported from Rajendran et al. (2022), who showed the maximum pollutant level in mg L^{-1} for the different studied elements, we obtained that, in our case, after unit transformation, we have values higher than the maximum pollutant level for polluted soil (PS) in the case of Cu (0.25 mg L^{-1}), Zn (0.8 mg L^{-1}) and Cd (0.01 mg L^{-1}). To remark that, water-soluble content decreased with the amendments, while after the application of biopiles (B and BV) and landfarming (L and LV) techniques, and according to the related values, water-soluble concentrations in these cases were still higher than the maximum pollutant levels established for Zn and Cd, which implies that they are not feasible treatments for controlling metal pollution.

2.3.3. Effects of metal toxicity in different soil organisms after remediation

The determination of only the metal content, both total and in different available forms, could give some idea of the degree of remediation of a soil, but it is not enough to address a soil as not polluted. Therefore, the study of the behavior of living organisms in these soils by using toxicity bioassays is essential for a correct Ecotoxicological Risk Assessment (ERA) (Song et al. 2006). Exposure to high concentrations of different PTEs poses a serious threat to organisms (Ojuederie and Babalola 2017), as they can reduce microbial biomass (Shukurov et al. 2014), affect the taxonomic diversity of soil communities (Stefanowicz et al. 2008), and act on various soil microbial processes, thus disrupting nutrient cycling and the capability to perform key ecological functions (e.g., mineralization of organic compounds and synthesis of organic substances) (Giller et al. 1998; Moreno et al. 2009).

Romero-Freire et al. (2015a) observed high variability when different bioassays are compared for studying soil toxicity. Therefore, selecting different living organisms is crucial to obtain real approaches of remediation results. In the present study, residual toxicity after remediation was evaluated by a total of five different well-established ecotoxicological tests using different organisms and soil fractions. In soil-solid fraction toxicity was determined by barley and soil basal respiration, whereas in soil-water fraction with lettuce, daphnid and algae (Figure 2.2).

According to the tests performed in soil-solid fraction we observed a positive response for all treatments compared to the polluted soil (PS). In the toxicity test using barley, root elongation was close to that obtained in the recovered soil (RS) for all remediation techniques, with the highest root elongations in the case of addition of gypsum (G, GV) and marble (M, MV). In the case of soil respiration, it showed recoveries compared to the PS for all treatments. In general, for this bioassay the addition of vermicompost showed higher respiration in all cases except in marble with vermicompost (MV) where values were similar to the application of marble alone. B and L techniques alone, without their combination with vermicompost, showed the lowest increases in soil respiration compared to PS.

In the toxicity tests performed in soil-water fraction, results obtained from the different treatments are not compared to the recovered soil, but to the response obtained from the control samples performed with distilled water instead of with soil extract. In relation to these tests, for the lettuce root reduction, total inhibition of root growth was found for PS, while no toxic effect was found under all remediation treatments. However, G and M stood out as the treatments that led to the most similar response to RS, enhancing the root growth to a greater extent. In the case of the test done by the algae, we observed no toxic effects for GV, M and MV and in the case of other remediation techniques the algae inhibition was even higher than in the PS in some cases (B, BV, G, L, LV). The test studying daphnid mortality showed results similar to the algae, and only the GV, M and MV presented a decrease in the toxicity for this organism, whereas the other remediation techniques used showed high toxicity, even with 100% of mortality in BV, L and LV, similar to the PS.

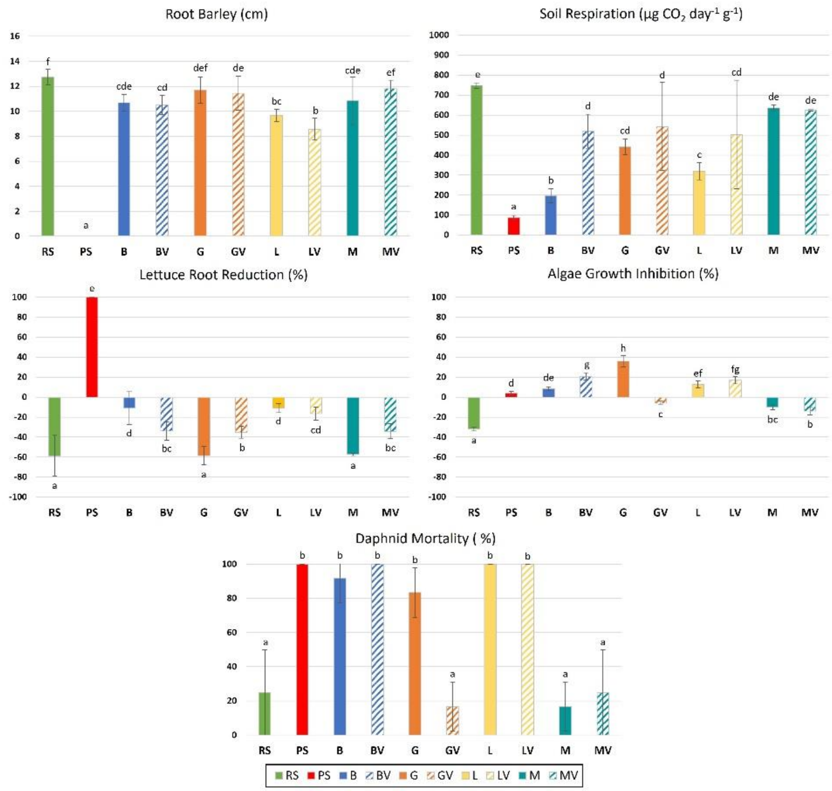

Figure 2.2. Ecotoxicological tests done in: a) soil-solid fraction: root barley elongation (cm) and soil induced respiration (µg CO2 day^{-1} g^{-1} soil) and, b) in soil-water fraction: lettuce root reduction (%), algae inhibition growth (%) and daphnid mortality (%). Block bars represent the recovered soil (RS), the control polluted soil (PS); and the different studied amendments (B: Biopiles, G: Gypsum, L: Landfarming, M: Marble sludge). Striped bars indicate the use of composite amendments, with the addition of vermicompost (BV, GV, LV and MV). Lowercase letters indicate significant differences among treatments according to Duncan's post hoc test (p<0.05).

To assess environmentally relevant soil toxicity, the use of a diverse set of exposure routes is recommended, thus, the set of bioassays selected should jointly take into account exposure to both the solid and the soluble phases of the soil (Rodriguez-Ruiz et al. 2014). In this study, according to the toxicity bioassays performed, the response of the organisms to the treatments differed depending on whether the solid fraction or the aqueous fraction was used. Lors et al. (2011) stated that for ecotoxicological assays, soil extracts (liquid fraction) could reflect the toxicity of the soil solid phase. However, metals can be present in the soil solution as free ion but also as complexes with organic or inorganic ligands. Therefore, partitioning of the metal ions between the solid phase and the solution depends on the composition of both phases (Smolders et al. 2009). An increase in organic carbon, for example, could lead to a decrease of metal availability (Sherene 2010), since organic carbon associated with clay minerals in the solid phase has a high capacity to bind metals and deplete the soil solution. However, the addition of vermicompost in our treatments did not reflect a better response for studied organisms, and only some improvements were obtained in the case of soil respiration (BV, GV, LV), for which the addition of vermicompost reflected a better response for this parameter. In this regard, some authors have stated that organic carbon content influence soil respiration (Balogh et al. 2011), while Romero-Freire et al. (2016b) verified that indeed soils rich in organic carbon have the highest CO_2 efflux emitted, using same technique as that applied in this bioassay. In this study, it appears that, in the solid fraction, the remediation techniques used recovered the polluted soil and present little toxic effect. However, the correlation between the total and soluble forms of a pollutant is not always direct, and in this case, with natural polluted soils we should take into account the toxicity of multi-component chemical mixtures. For example, the solubility and toxicity of As are usually more tightly controlled by the soil properties than by the total amounts present in the sample (Martín-Peinado et al. 2011, 2012). In this sense, bioassays using the liquid phase extract can more readily reflect the behavior of mobile phases, evaluating the short-term risk of dispersion, solubilization, and bioavailability of pollutants in the environment. In water fraction, according to the lettuce root reduction, all techniques were effective in the remediation, but if the results for algae or daphnid are observed, only GV, M and MV were effective techniques. According to soil properties, these three techniques were the remediation measures that showed a higher increase in pH. Organisms have their own sensitivity to pollutants and also show different responses under different exposure conditions (Matejczyk et al. 2011). It is generally considered that pH is one of the major factors controlling the toxicity of metals to aquatic organisms (de Schamphelaere et al. 2004), so the observed results could be masked not only by the metal but also by pH effect.

Our results support that the use of only one bioassay may be useful for early identifying pollution problems, but it will not be sufficient as an indicator of toxicity pathways in order to select remediation methods under natural conditions. A metal that causes toxicity in some organisms may not do so in others, and the complexity of natural polluted soils due to the presence of mixture of elements, as well as different soil properties and constituents, can mask the effects attributed to the different metals involved (Spurgeon and Hopkin 1995). Bioassays performed with samples taken from the polluted site after a period of application of the treatments allow assessing the real risk of pollution under natural conditions (Fernández et al. 2006).

2.3.4. Effect of soil properties and metal availability in organism toxicity

Figure 2.3 is the result obtained after applying and non-metric multidimensional scaling (NMDS) (stress=0.02) having in consideration soil properties, metal studied forms and the response to the bioassays performed. Results showed that the application of the selected amendment treatments, after one year, induced great distances from the polluted soil sample (PS), although they do not show similitude to the recovered soil (RS). The application of L (landfarming) and B (biopiles) were not useful remediation techniques for the recovery of the PS, since they follow similar distribution. According to metal(loid)s concentration, both water-soluble and bioavailable forms decreased compared to the PS. However, these two remediation techniques showed the lowest pH. Soil properties are known to be essential factors when comparing toxicity of polluted soils (Romero-Freire et al. 2014, 2015a). Usually, soil pH is an important path for decreasing solubility of several metal(loid)s, by precipitation, adsorption or co-precipitation processes (Barna et al. 2007; González et al. 2013). However, in real conditions, mixture of elements leads to synergistic or antagonistic effects (Romero-Freire et al. 2016a), and the toxic effects of some elements can be masked by others. In the pH range of the recovered soils samples (4.6-5), it is expected that some elements, as Zn, Cu or Pb, reach greater mobility (Alloway 1995). It is well-known that soil properties are the main factors affecting metal speciation and bioavailability (Sheppard and Evenden 1988), thus being directly related to the response of living organisms in metal polluted soils. The lowest distances from RS appear in the case of MV, followed by M, GV, G, and LV, which, according to the results observed previously, showed a good recovery of soil properties, low levels of metal(loid)s studied, and the best results from the ecotoxicological approach, mainly for treatments with marble sludge (M) and gypsum (G), although worse for the LV treatment (landfarming + vermicompost).

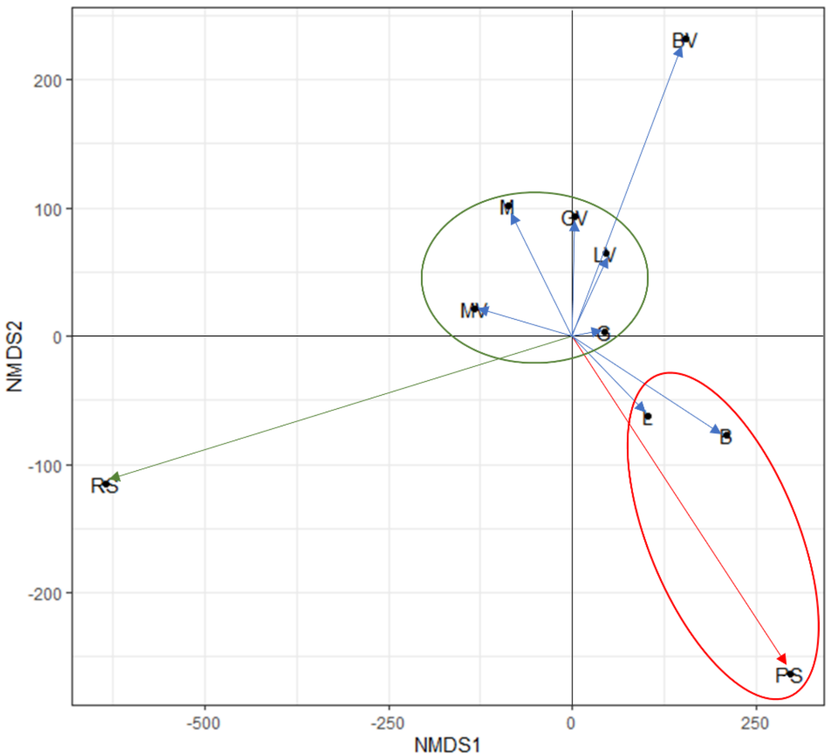

Figure 2.3. Non-metric multidimensional scaling (NMDS) for the different studied soils and treatments: the recovered soil (RS), the control polluted soil (PS), and the different studied amendments (B: Biopiles, G: Gypsum, L: Landfarming, M: Marble sludge; and the same with the addition of vermicompost (BV, GV, LV and MV)). The NMDS was done including the soil physicochemical properties; the total (Xx_T), bioavailable (Xx_B), and water-soluble (Xx_W) content of Cu, Zn, As, Pb and Cd; and the results for the five performed ecotoxicological tests.

From NMDS distances, and aiming at upgrading the treated polluted soil to similar conditions of the nearby recovered soil (RS), we could approximate that the best remediation technique used was the addition of marble sludge with vermicompost (MV). Previous studies using similar soil amendments in the same area have suggested the most appropriate doses of application for both marble and vermicompost (Madejón et al. 2018; García-Robles 2020), which were followed in this study (equivalent to 50 t ha^{-1}). Thus, according to the results obtained, performing future studies or remediation programs based on marble sludge amendments on a larger scale following the specified dose are encouraged and could be feasible. This is based on the elevated production of this residual material, which, in the near future, could even become a serious environmental problem to be addressed (Sánchez et al.

2010). In particular, MV technique increased the $CaCO_3$ and OC content and raised the pH to ranges of 7.2. In addition, MV was effective in reducing some water-soluble and bioavailable forms of Cu, Zn and Cd (Table 2.2). Soil carbonates, in addition to their direct effect in increasing soil pH, may affect metal solubility and availability through their surface interactions, providing specific adsorption or precipitation reactions (Martínez and Motto 2000; Simón et al. 2010). Metal toxicity is also influenced by metal binding state, being usually the complexation with organic ligands, from OC, a way to decrease toxicity (Rodriguez-Ruiz et al. 2014). Finally, and according to ecotoxicological responses, it must be highlighted that with the results showed for the 5 bioassays selected, MV showed toxic results close to those obtained for the recovered soil sample (RS) (Figure 2.2). Contrary to the findings of García-Carmona et al. (2017), who deduced that biopiles were an effective remediation action, mainly due to the increase in pH, our results suggest the opposite. This is explained by the fact that García-Carmona et al. (2017) performed these remediation techniques in laboratory with a short period of establishment. In contrast, our results demonstrated that the solubility, bioavailability, and toxicity of metal(loid)s may vary over time due to the influence of soil properties and, presumably, the reduction in soluble concentrations leads to a decrease in the risk of toxicity to the ecosystem (Romero-Freire et al. 2016a; Martín-Peinado et al. 2011; Lock and Janssen 2003). Consequently, when dealing with soil pollution by metal(loid)s from mining industry, and aiming to effectively assess soil toxicity risk and the success of soil remediation techniques, the determination of both metal availability and the toxicity for several organisms after an implementation period should be considered. In the case of this study, a temporal approach of one year was followed to observe the evolution of the soil toxicity after treatments implementation, which helped us to ensure the best remediation technique used. This approach could therefore be reliable for application in future studies aimed at a comprehensive assessment of toxicity in polluted soils by metal(loid)s.

2.4. Conclusions

Overall, the use of the different techniques selected for the remediation of a polluted natural area improved the main soil properties and led to significant changes in the mobility and availability of metal(loid)s one year after their application, thus being effective in reducing their toxicity. Of the different treatments used, the best technique for the remediation of soils affected by residual pollution and for reducing metal(loid)s toxicity was the addition of marble sludge with vermicompost. This technique was the one that most improved the main soil properties and was effective in reducing water-soluble and bioavailable forms of Cu, Zn and Cd. Also, and according to the ecotoxicological responses, this technique showed toxic results close to those obtained for the recovered soil.

The ecotoxicological approach performed to evaluate the responses of living organisms to metal(loid)s toxicity after remediation showed that their response to treatments differed depending on whether the solid or the aqueous fraction was used. According to the tests performed in soil-solid fraction, a positive response was found for all treatments compared to the polluted soil, so that the remediation techniques used seemed to effectively recover the polluted soil and present little toxic effect. However, the results obtained from the toxicity tests performed in soil-water fraction showed high toxicity for some of the remediation techniques used.

Our results highlight that the use of a single bioassay may not be sufficient as an indicator of toxicity pathways to select soil remediation methods, so that the joint determination of metal availability and ecotoxicological response of several organisms will be determinant for the correct establishment of any remediation technique carried out under natural conditions, where factors as multi-stress or climatic conditions are involved.

Supplementary Materials: Supplementary data to this article can be found online at https://www.mdpi.com/article/10.3390/toxics11040298/s1

Chapter 3

Vegetation establishment in soils polluted by heavy metal(loid)s after assisted natural remediation

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Abstract

Background and Aims This field-base study evaluates the long-term effectiveness of in-situ remediation measures applied to soils residually polluted by potentially toxic elements (PTEs) in an area affected by a mining spill in SW Spain.

Methods To evaluate the remediation treatments success, their influence on key soil properties and on the development of spontaneous vegetation in the treated soils was investigated. The treatments were based on human derived by-products valorization, and consisted of: biopiles, marble sludge and gypsum mining spoil addition, and their combination with an organic amendment (vermicompost).

Results Amendments application improved the soil properties and reduced PTEs availability. As a result, an enhancement in spontaneous development of vegetation cover and diversity of plant species in the treated soils was followed. *Spergularia rubra* and *Lamarckia aurea*, two primary plant species growing in the studied area and that exhibit strong association to soils with the highest levels of pollution, showed high Pb and As accumulation in shoots and in roots. Exceptionally, accumulation of these pollutants occurred in *L. aurea* roots, which can explain its high presence in soils with more limited vegetation development and in which no additional plant species can thrive.

Conclusions The occurrence of *S. rubra* and *L. aurea* in the amended soils may be indicative of improved soil conditions and reduced toxicity induced by the remediation measures implemented. They may also be considered key species in the area since their presence can promote the recolonization of the degraded soils by species less tolerant to their residual pollution.

Keywords: soil pollution; soil remediation; amendment; assisted natural remediation; *Lamarckia aurea*, bioaccumulation factor

3.1. Introduction

Soil, as a fundamental element of ecosystems, plays a crucial role in providing essential environmental functions and ecosystem services. Among these, soil productivity is one of the most essential services for the human well-being and its development (Kabata-Pendias 2010; Adhikari and Hartemink 2016). Nevertheless, soil faces several soil degradation processes that may threaten its quality, both natural and human-induced. Of the anthropogenic causes, soil pollution by potentially toxic elements (PTEs) represent one of the major threats and concerns worldwide (FAO and ITPS 2015), since it poses a risk to ecological systems, human health, and food production. Among all chemical pollutants, PTEs, including heavy metals and metalloids, can persist tightly bound in soil (Pilon-Smits 2005; Igalavithana et al. 2022). This can lead to cumulative impacts in organisms, ultimately making them a significant contributor to environmental pollution with implicit adverse effects to human health (Muyessar and Linsheng 2016). Although certain PTEs can act as essential elements and are involved in biological functions, such as Cu and Zn, they can turn potentially toxic when specific thresholds are exceeded (Gall et al. 2015). Other elements such as As and Pb are among the most hazardous PTEs, as very low concentrations of them can cause toxicity (Rahman and Singh 2019), causing serious problems of contamination in the food chain, with the consequent health risk (Simon 2014). Therefore, in order to preserve soil functions, degraded soil ecosystems polluted by PTEs would require assisted natural remediation (ANR) (Adriano et al. 2004; Raklami et al. 2021).

With the aim of reducing the PTEs harmful effects in polluted soils, a wide range of techniques for soil remediation, either in-situ or ex-situ, and involving the use of both organic and inorganic amendments, have been developed and tested to assist natural remediation processes (Adriano et al. 2004; Park et al. 2011; Liu et al. 2018). The addition of amendments to the soil is considered as an economical and environmentally efficient solution that addresses pollutant toxicity and enhances critical biogeochemical mechanisms (Rodríguez-Jordá et al. 2012; Nirola et al. 2016). Moreover, the revalorization of mining and agro-food industry wastes through their application as amendments in degraded soils aligns with zero-waste strategies (Greyson 2007; Pietzsch et al. 2017). Numerous case studies corroborate the viability of organic and/or inorganic soil amendments under field conditions (Fernández-Caliani and Barba-Brioso 2010; González et al. 2012). More precisely, liming or calcium and organic matter-rich amendments are among the most effective ones, through the correction of soil acidic pH, the enhancement of soil physicochemical properties, the increase of nutrient availability and the immobilization of certain PTEs (Bernal et al. 2007; Pérez-de-Mora et al. 2007a), which prevent from the potential spread of pollutants into the ecosystem. Consequently, a promotion in re-colonization and re-establishment in barren polluted soils by the vegetation from surrounding areas in the remediated soils may be achieved in turn (Fernández-Caliani and Barba-Brioso 2010; Xiong et al. 2015) through the enhancement of plant germination and growth (Clemente et al. 2015; Madejón et al. 2018; Sierra-Aragón et al. 2019).

Industrial activities, such as mining, represent one of the most significant potential sources of soil pollution by PTEs (Dermont et al. 2008; Liu et al. 2018), and several remediation treatments have been implemented in mining polluted areas. One of the greatest metal mining accidents worldwide took place in 1998 in Spain, after the collapse of the tailings dam of the Aznalcóllar pyrite mine, which resulted in the spill of acidic waters and tailings containing high concentrations of PTEs (Grimalt et al. 1999; Simón et al. 1999; Sanz-Ramos et al. 2022). Following, extensive cleanup and rehabilitation measures were implemented in the affected area to guarantee the safety of the local inhabitants and promote the full recovery of the area in the long term. However, several decades after the accident, numerous patches of residual polluted soils where vegetation cannot even germinate still remain in the area, and represent an environmental risk for their high PTEs concentrations (García-Carmona et al. 2017). Since the residual polluted patches are deeply integrated into a large, recovered area (the Protected Landscape of Guadiamar Green Corridor), the mixture of adjacent recovered soil with the remaining polluted soil represents a plausible, economic, and minimally invasive technique. This treatment has been tested in previous experiments in the area with successful results directly tied to the improvement of soil properties and reduced PTEs solubility (García-Carmona et al. 2017; Lorente-Casalini et al. 2021).

When evaluating the restoration processes of degraded or polluted areas, the presence of key components of ecosystems such as vegetation plays a crucial role as indicators of the extent of soil pollution. Trace elements, and particularly heavy metals, can be accumulated by plants, which are able to adapt to varying levels of environmental pollution (Kabata-Pendias 2010). Furthermore, passive restoration, which involves the natural recolonization of a polluted area by native vegetation and that occurs in parallel to the conditions improvement, is crucial when assessing the effectiveness of remediation measures (Álvarez-Rogel et al. 2021), as these native plants may act as “nurse plants”, promoting the growth of species with lower tolerance to stress conditions (Navarro-Cano et al. 2018). Additionally, the development of vegetation cover is relevant when considering the effectiveness of a specific treatment applied to a polluted soil, since it provides an extra physical protection and may reduce possible re-movement of pollutant particles and migration to groundwater (Pérez-de-Mora et al. 2006a). However, PTEs toxicity in plants can limit processes such as seed germination, root development, and growth, or disrupt the uptake of essential nutrients leading to plant death (Kabata-Pendias 2010). Nonetheless, a wide variety of strategies, both physiological and behavioral, allow numerous plant species to tolerate remarkably high PTEs concentrations (Baker 2010; Viehweger 2014), such as metallophytes species inhabiting metalliferous deposits (Baker 2010). Also, certain plant species not exclusive to metal-rich environments, known as pseudometallophytes (Baker 2010), also exhibit exceptional capacity to adapt to unfavorable soil conditions caused by pollution. Under moderate levels of soil metal toxicity, they can show higher biomass production and have competitive advantages over other species (Poschenrieder et al. 2001). Consequently, these species may contribute to improve the physicochemical and biological conditions of the polluted soils by handling the PTEs concentrations, enhancing nutrient availability, and increasing soil organic carbon (Arienzo et al. 2004; Bolan et al. 2011).

The aim of this study is to evaluate the long-term effects of in-situ remediation measures, based on the addition of inorganic and organic amendments (six different treatments), which were applied to soils residually polluted by PTEs. The influence of the treatments will be determined by analyzing: i) the changes induced in the main soil properties; ii) the mobility and bioavailability of PTEs after remediation and ageing; iii) the natural settling of PTEs tolerant plants, as an indicator of the success of the remediation actions used; and iv) the bioaccumulation and translocation factors of PTEs in these tolerant plant species, for a better understanding of the mechanisms that allow them to survive to the great concentrations present in the studied soils.

3.2. Materials and methods

3.2.1. Study site and experimental design

The study site was located in the area nearby the tailings dam of the Aznalcóllar mine (Seville, SW Spain), which was most severely affected by the Aznalcóllar mining spill. This area is characterized by the presence of heterogeneously distributed bare soil patches of varying sizes, where high levels of pollution are still detected (Figure 3.1a and 3.1b). For this study, three of these unvegetated residually polluted soil patches were selected (Figure 3.1c). Within each of these patches, an experimental plot of 24 m^2 was set up, and divided into six subplots of 4 m^2 with a different treatment applied in each of them (Figure 3.1d). The six treatments selected were the following: 1. B: Biopile (50% w/w mixture of polluted soil -PS- with adjacent recovered soil -RS-); 2. BV: Biopile + vermicompost; 3. G: Gypsum mining spoil (5 kg m^{-2}); 4. GV: Gypsum mining spoil + vermicompost; 5. M: Marble sludge (5 kg m^{-2}); 6. MV: Marble sludge + vermicompost. The doses applied of every amendment was 5 kg m^{-2} for each subplot. The selected amendments were chosen for being calcium and organic matter-rich amendments, and in the case of the inorganic amendments, for representing low-cost waste materials generated in very

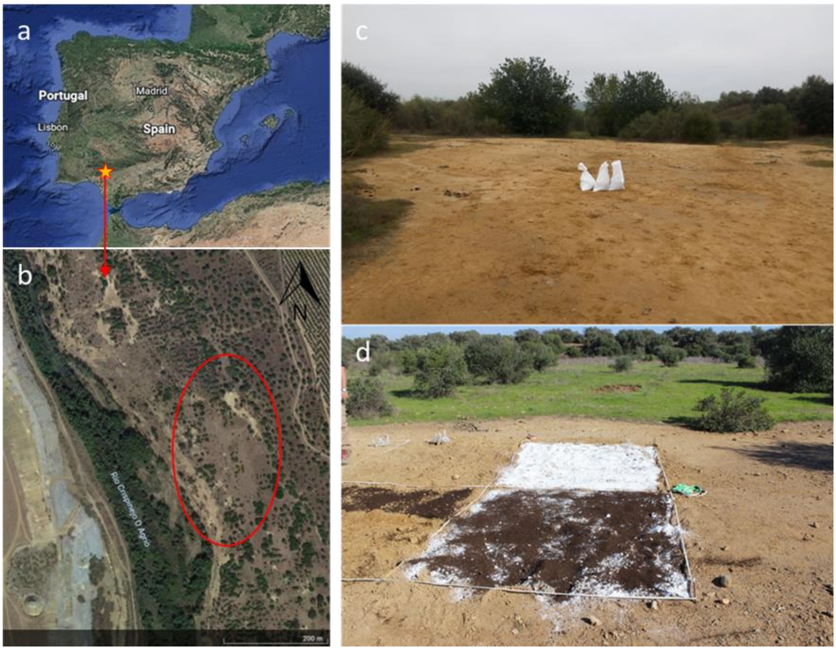

Figure 3.1. Left: Study area location (a) and aerial image of selected unvegetated patches (b). Right: Detailed image of bare soils (c) and treatments application (d).

large amounts that need to be sustainably managed (Rayed et al. 2019; García-Robles et al. 2022). The doses of gypsum mining spoil and marble sludge were selected according to the doses previously applied in the area for calcium-rich amendments (Madejón et al. 2018), and the ratio for the B treatment was successfully tested in a previous ex-situ experiment (García-Carmona et al. 2017). In all cases, the dose of vermicompost was 5 kg m^{-2} (equivalent to 50 t ha^{-1}) because the organic amendments had been previously applied in the area at a rate of 15-25 t ha^{-1} (Cabrera et al. 2005, 2008) and resulted insufficient to promote vegetation growth. The characterization of the amendments (biopile, vermicompost, marble sludge and gypsum mining spoil) is shown in Table S3.1.

3.2.2. Soil and vegetation sampling

After a period of eighteen months following the implementation of the treatments, coinciding with the second spring, surface soil composite samples (0 – 10 cm) for each treatment were collected, and the key soil properties of them were determined. In addition, composite samples were also taken from untreated polluted soil (PS) within the unvegetated soil patches, from reclaimed soil adjacent to them (RS), and from soils unaffected by the spill near the studied area (US), which were considered reference soils for the different degrees of pollution. Moreover, vegetation cover and plant species richness present in every reference soil (in a representative 4 m^2 surface) and treatment applied were measured to monitor the vegetation development in the plots. This was done using a 0.25 m^2 quadrat divided into 100 cells, which was placed on the surface of each of the studied soil, and all the cells with presence of an herbaceous species were counted, both for total vegetation cover and for the specific cover of each species individually. Also, richness, considered as the mean total number of different species encountered on the quadrat, was determined for each treatment. This procedure was performed in triplicate for each experimental plot and treatment. Moreover, to analyze the potentially toxic elements (PTEs) uptake by the main colonizing species in the plots, samples of *Spergularia rubra* (L.) J. Presl & C. Presl and *Lamarckia aurea* (L.) Moench were collected.

3.2.3. Analytical methods

3.2.3.1. Soil properties

Soil samples were air dried at room temperature and sieved (<2 mm). The main soil properties and constituents including pH, electrical conductivity, calcium carbonate content, and organic carbon content were analyzed following standard methods (MAPA, 1994). Soil pH was determined in a soil/water ratio 1:2.5 with a 914 pH/Conductometer Metrohm (Herisau, Switzerland); electrical conductivity (EC, dS m^{-1}) in a soil/water extract (1:5) using a Eutech CON700 conductivity-meter (Oakton Instruments, Vernon-Hills, IL, USA); calcium carbonate content (%$CaCO_3$) was measured by manometric method according to Barahona (1984); and organic carbon (%OC) was quantified by acid-oxidation method according to Tyurin (1951).

3.2.3.2. Total concentration and selective extractions of PTEs in soils

Total concentration of the main PTEs (Xx_T) present in the studied soils (Pb, As, Zn, Cu) were determined by X-ray fluorescence (XRF) with a NITON XL3t-980 GOLDD+ instrument (Thermo Fisher Scientific, Tewksbury, MA, USA) (n=3). The accuracy of the method was confirmed by analyzing a certified reference material (CRM052-050 RT-Corporation Limited, Salisbury, UK; n=6). The average recoveries for the studied elements were 108.0 ± 9% of the CRM. Also, selective extractions of PTEs were performed in all treatments to assess the risk of mobility and the bioavailability of these elements in soils. In this sense, a soil:water extract of 1:5 was prepared to obtain the PTEs water-soluble fraction (Xx_W) (Gomez-Eyles et al. 2011), since this extraction simulates the proportion of the elements that can lixiviate with rain water (Romero-Baena et al. 2021). Meanwhile, an extraction with 0.05 M EDTA (pH=7) was followed to obtain the bioavailable fraction (Xx_EDTA) for living organisms (Quevauviller et al. 1998). The PTEs concentrations for both extracted forms were measured by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) in a PerkinElmer NexION® 300D spectrometer (Waltham, MA, USA). The accuracy of the method for extracted forms was also confirmed by analyzing certified reference material SPS-SW1 Batch 133 (Spectrapure Standards, Manglerud, Norway; n=6). The recovery of the reference material for the studied elements by ICP-MS determination was 99.2 ± 0.8%.

3.2.3.3. PTEs concentration in plants

Collected plant samples were divided into shoots and roots to analyze them separately, washed with distilled water, and dried (70 °C for 48 h). Afterwards, all plant material was finely ground and digested in a microwave XP1500Plus (Mars®) in HNO_3:H_2O_2 (1:1) (Sah and Miller 1992), and the concentration of the main PTEs in each part of the plant was measured by ICP-MS (PerkinElmer

NexION® 300D spectrometer). The bioavailable (EDTA-extractable) PTEs fraction in soil samples was selected to calculate the bioaccumulation factor (BAF) for both parts, to assess the capacity of plants to uptake PTEs from the soil and either transport them to the shoots or accumulate them in roots. BAF, therefore, was calculated as the ratio between the PTEs concentration measured in plant (mg kg^{-1} dry weight) and the EDTA-extractable PTEs concentration in soils (mg kg^{-1} dry soil) (Anning and Akoto 2018; García-Carmona et al. 2019a), since this fraction represents PTEs bioavailable forms for plants (Kidd et al. 2007). Finally, the extent of elements migration from roots to shoots was estimated through the translocation factor (TF), calculated as the ratio between PTEs concentration in shoots to that in the roots of the plants (Zacchini et al. 2009; Boi et al. 2021). Plants with both factors > 1 for a given element are considered as accumulators and suitable for phytoextraction, while plants with both factors < 1 are considered as non-accumulators or pollutant-stabilizing, thus being suitable for phytostabilization (Buscaroli 2017).

3.2.4. Statistical analyses

Descriptive statistics of PTEs content in soils and plant material were calculated to check their normality, while homogeneity of variance was explored using Levene's test. The analysis of mean comparisons was performed by non-parametric Kruskal-Wallis test, in accordance with sample size (Theodorsson-Norheim 1986). Kruskal-Wallis post hoc test ($p<0.05$) was used to determine significant differences. Principal component analysis (PCA) was carried out to identify the relations between soil properties, PTEs fractions, and treatments. Also, to analyze the relation between PTEs concentrations in plants and their total and bioavailable concentrations in soils, Spearman's correlations were performed. All the statistical analyses were carried out with a confidence level of 95% using SPSS v.28.0 (SPSS Inc., Chicago, USA).

3.3. Results and discussion

3.3.1. Main soil properties related to pollution

The key soil properties of the different reference soils and under each of the treatments applied are shown in table 3.1. Soils with high levels of pollution remaining (PS) were mainly characterized by a strongly acidic pH (3.5), high EC (>3 dS m^{-1}), and low organic carbon content (<1%) due mainly to the lack of vegetation cover. In contrast, both the recovered soil (RS) and the unpolluted soil (US) were characterized by a less acidic pH, especially in US (>6), significantly lower EC (<1 dS m^{-1}), and significantly higher organic carbon content than PS (>2%). These results are consistent with the ones reported by Sierra-Aragón et al. (2019) in polluted soils within the same area. Regarding the treated soils, the pH in them was higher compared to PS and to RS in all cases, and even reached higher values under marble (M, MV) and gypsum (G, GV) treatments than those of US. However, EC did not experience a strong decline in the amended soils, and values remained significantly higher than those of RS and US. The organic carbon content in amended soils did not reach that of the RS and US either, although among treatments it was higher in soils where vermicompost was added, for the direct effect of this organic amendment (Adak et al. 2014; Shen et al. 2022). Overall, US and RS were more strongly associated to a higher content of OC while PS was inversely correlated to OC content and pH, and, as for treatments, the strongest correlation was that of M and MV with a more neutral pH, as well as with the highest content in calcium carbonate (Figure S3.1).

Table 3.1. Main soil properties (EC: Electrical conductivity; $CaCO_3$: Calcium carbonate equivalent content; OC: Organic carbon content) (n=6) related to the Aznalcóllar mining accident in the reference and treated soils.

	pH		EC (dS m^{-1})		$CaCO_3$ (%)		OC (%)	
	Mean	*sd*	Mean	*sd*	Mean	*sd*	Mean	*sd*
US	6.06 de	*0.28*	0.08 a	*0.06*	0.33 a	*0.14*	2.32 bc	*1.60*
RS	4.28 ab	*0.48*	0.98 b	*0.61*	0.52 a	*0.25*	3.68 c	*1.46*
PS	3.49 a	*0.23*	3.12 d	*0.63*	0.87 a	*0.06*	0.61 a	*0.09*
B	5.37 cd	*0.87*	2.35 cd	*0.30*	0.84 a	*0.32*	1.14 ab	*0.16*
BV	4.96 bc	*0.41*	2.15 c	*0.51*	0.70 a	*0.33*	1.62 ab	*0.38*
G	6.22 def	*0.29*	2.38 cd	*0.06*	1.11 ab	*0.39*	0.89 a	*0.11*
GV	6.41 ef	*0.19*	2.36 cd	*0.04*	0.78 a	*0.37*	1.67 ab	*0.26*
M	6.78 ef	*0.19*	2.29 c	*0.07*	2.76 bc	*2.34*	1.20 ab	*0.24*
MV	7.07 f	*0.07*	2.14 c	*0.26*	2.89 c	*1.23*	1.41 ab	*0.24*

US: Unpolluted soil; RS: Recovered soil; PS: Polluted soil; B: Biopile; BV: Biopile + vermicompost; G: Gypsum; GV: Gypsum + vermicompost; M: Marble; MV: Marble + vermicompost. *Sd*: standard deviation (n=6). Values followed by different letters are significantly different according to Kruskal-Wallis test (p<0.05).

3.3.2. Total, water-soluble and EDTA-extractable PTEs concentrations

When assessing the effectiveness of remediation measures and the risk to the ecosystem of the polluted soils treated, determining PTEs fractions is critical. Even after soil remediation and recolonization by vegetation, acid releases may occur, so that enough liming or other amendment material should be applied to buffer against these situations, which can represent a threat to future scenarios in the remediated soils (Wong et al. 1998). The total concentrations measured in the unpolluted soils (US) can be considered as the background levels for the potentially toxic elements (PTEs) present in the soils of the study area (Table 3.2). The mean total concentrations for Pb, As, Zn and Cu in US were similar to the background levels recorded in the same area both shortly after the accident (Simón et al. 1999) and by more recent studies (García-Robles et al. 2022), although Pb showed slightly higher values in this occasion, which may be explained by the natural variability of this element found in the natural soil. Recovered soils (RS) showed total PTEs concentrations approximately 5-fold higher than those of US for Pb, Zn and Cu, and almost 15-fold higher for As. In addition, RS showed the highest concentrations for Zn and Cu, while for As and Pb the highest concentrations were reached in PS, 10- and 23-times above background levels, respectively. When comparing the levels measured for these elements with the regulatory thresholds established by the Regional Government of Andalusia (BOJA 2015) [10,000 mg kg^{-1} for Zn, 595 mg kg^{-1} for Cu 36 mg kg^{-1} for As, and 275 mg kg^{-1} for Pb] the concentrations of As and Pb both in PS and RS widely exceeded these regulatory limits.

In relation to treatments, the dilution effect of the amendments over total PTEs concentrations was relevant for some elements, especially in biopile soils (B and BV). In this sense, total Pb and As concentrations were strongly reduced in B and BV compared to PS, showing concentrations similar to those in RS. Meanwhile, Zn levels in these treatments were intermediate compared to PS, while for Cu no changes were observed after the application of the treatments, indicating that the dilution effect for this element was not a determining factor. The rest of the amended soil treatments showed higher As and Pb total concentrations compared to RS, although still significantly lower than those in PS. Thus, PS was strongly related to high total concentrations of As and Pb, while RS did so for Zn and Cu total concentrations. This may be related to the association of Cu and Zn with the OC content, related to a specific binding by adsorption and complexation to soil organic matter (McLaren et al. 1981; Balasoiu et al. 2001; Yin et al. 2002), which led the RS, where the highest OC was found, to retain higher total concentrations of these elements. Moreover, the low total concentrations of Cu and Zn found in PS may be related to the increase in mobility of these elements with decreased soil pH (A.S. Wang et al. 2006; Zeng et al. 2011). Therefore, the acidic pH in PS could have led to a strong leaching of these elements at depths below the top 10 cm sampled.

Table 3.2. Total (T) PTEs concentrations (mg kg^{-1} dry soil) (n=6) in the reference and treated soils.

	Pb_T		As_T		Zn_T		Cu_T	
	Mean	*sd*	Mean	*sd*	Mean	*sd*	Mean	*sd*
US	100.6 a	*78.4*	19.5 a	*4.0*	74.2 a	*11.7*	44.2 a	*18.3*
RS	501.3 b	*142.3*	279.7 bc	*44.7*	416.7 c	*229.6*	216.8 b	*127.2*
PS	1003.4 c	*219.7*	451.8 f	*41.4*	215.9 ab	*82.5*	121.6ab	*29.2*
B	505.7 b	*134.9*	252.2 b	*84.4*	335.2 bc	*71.1*	103.7 ab	*35.3*
BV	536.4 b	*43.3*	286.1 bcd	*53.3*	372.0 bc	*77.1*	134.6 ab	*55.2*
G	560.1 b	*57.3*	374.1 cdef	*61.9*	263.6 bc	*87.5*	124.5 ab	*19.0*
GV	684.1 bc	*91.8*	407.4 ef	*53.4*	270.5 bc	*57.9*	132.5 ab	*32.1*
M	668.1 b	*238.2*	383.6 cdef	*58.9*	273.3 bc	*58.4*	126.7 ab	*21.7*
MV	680.1 b	*195.3*	392.8 def	*68.0*	283.6 bc	*60.2*	122.4 ab	*20.2*

US: Unpolluted soil; RS: Recovered soil; PS: Polluted soil; B: Biopile; BV: Biopile + vermicompost; G: Gypsum; GV: Gypsum + vermicompost; M: Marble; MV: Marble + vermicompost. *Sd*: standard deviation (n=6). Values followed by different letters are significantly different according to Kruskal-Wallis test (p<0.05).

With the aim to accurately evaluate PTEs toxicity in the soil, particular emphasis should be given to their solubility and bioavailability, which represent the fractions that are available for uptake by living organisms and that can spread within the soil and through the landscape, thus posing a potential toxicity risk to terrestrial ecosystems (Alibrahim and Williams 2016; Bagherifam et al. 2019). In this regard, water-soluble fraction of pollutants can be readily absorbed by plants and incorporated into the food chain by bioaccumulation, representing actual bioavailability and short-term metal dynamics. Moreover, Ghosh et al. (2004) found that water-soluble form of As was more toxic compared to total As and inflicted greater inhibitory effect on various microbiological parameters in polluted soils compared to other bioavailable forms. Consequently, water-soluble As may be indicative of As availability to microbial populations (Fernández et al. 2005). Concerning this fraction, the highest water-soluble concentrations of As and Pb were recorded in RS, while for Zn and Cu they were recorded in PS (Table 3.3), in contrast to what was observed for total concentrations of these PTEs. The high solubility of Zn and Cu under acidic conditions led to the high water-soluble concentrations found in PS. Meanwhile, higher As and Pb solubility in RS can be related to the high organic matter content in this soil, as well as to its specific composition, since these elements are usually related to the labile dissolved organic carbon (DOC) pool (Egli et al. 2010; Gangloff et al. 2014; Li et al. 2018). Thus, by different competing effects with these elements, organic matter in soil can lead to an increase in their availability by maintaining them in more soluble and labile forms (Sauvé et al. 2000; Sierra-Aragón et al. 2019).

Table 3.3. Water soluble (W) PTEs concentrations (mg kg^{-1} dry soil) (n=6) in the reference and treated soils.

	Pb_W		As_W		Zn_W		Cu_W	
	Mean	*sd*	Mean	*sd*	Mean	*sd*	Mean	*sd*
US	0.0067 c	*0.0015*	0.032 b	*0.009*	0.243 a	*0.043*	0.169 b	*0.026*
RS	0.0401 d	*0.0521*	0.096 c	*0.021*	18.193 bc	*8.790*	0.628 c	*0.256*
PS	0.0102 c	*0.0053*	0.051 bc	*0.035*	44.860 c	*17.813*	3.235 d	*1.414*
B	0.0041 ab	*0.0008*	0.019 a	*0.006*	22.774 c	*7.821*	0.161 b	*0.066*
BV	0.0044 b	*0.0016*	0.020 a	*0.005*	5.517 ab	*2.554*	0.165 b	*0.033*
G	0.0029 a	*0.0014*	0.020 a	*0.002*	0.294 a	*0.220*	0.093 a	*0.259*
GV	0.0058 bc	*0.0029*	0.042 bc	*0.008*	0.214 a	*0.118*	0.136 ab	*0.020*
M	0.0033 ab	*0.0016*	0.033 b	*0.017*	0.054 a	*0.033*	0.108 ab	*0.173*
MV	0.0028 a	*0.0016*	0.091 c	*0.052*	0.050 a	*0.029*	0.130 ab	*0.192*

US: Unpolluted soil; RS: Recovered soil; PS: Polluted soil; B: Biopile; BV: Biopile + vermicompost; G: Gypsum; GV: Gypsum + vermicompost; M: Marble; MV: Marble + vermicompost. *Sd*: standard deviation (n=6). Values followed by different letters are significantly different according to Kruskal-Wallis test (p<0.05).

Regarding treatments, Pb solubility strongly decreased in all treated soils, especially in limed soils, since the application of Ca-rich compounds has been found to be generally efficient for Pb immobilization (Kumpiene et al. 2008; Romero-Freire et al. 2015b). However, As solubility increased in carbonate soils and with higher organic carbon content, so that the highest As soluble concentrations between treatments were found under GV and MV. The increase in As mobility after the application of liming treatments and the increase of soil pH has been reported by previous studies (Simón et al. 2010; Romero-Freire et al. 2014). Also, an increase in As solubility induced by organic amendment, as in vermicompost treatments in our case, has also been pointed out (Karczewska et al. 2017; Aftabtalab et al. 2022). This could be associated with As competition with organic matter for sorption sites and metal oxide surfaces, which may represent a primary mechanism for As release and thus increasing its mobility (Redman et al. 2002; Bauer and Blodau 2006). On the other hand, the behavior of Zn and Cu solubility under treatments was very similar in both, and directly related to their strong negative correlation with pH (C. Wang et al. 2013; Laurent et al. 2020), showing Spearman's correlation coefficients with pH of -0.933 and -0.833 respectively (n=9). Thus, soils rich in $CaCO_3$ content were the most effective in decreasing their soluble concentrations, which were those amended with marble (M, MV) and gypsum (G, GV), the treatments with a higher pH. This is in accordance with previous studies that highlighted the effectiveness of soils rich in $CaCO_3$ in retaining these elements (Aguilar et al. 2004). On the contrary, the highest water-soluble concentrations for Cu and Zn were found under B and BV treatments, where the pH was neutralized in a lesser extent, and especially in the case of Zn (Figure S3.2), which reaches the highest mobility under acidic conditions and it is considered to present a higher mobility in soil with respect to Cu (Rocco et al. 2018; García-Carmona et al. 2019a).

EDTA-extractable fraction of PTEs is also used to assess their bioavailability in soils (Kidd et al. 2007; García-Carmona et al. 2019a). Unlike water-soluble fraction, EDTA-extractable fraction is regarded by different authors as a more reliable predictor of long-term availability of these elements in soils (Hurdebise et al. 2015). Moreover, among the different extraction methods frequently used for evaluating the bioavailable fraction of PTEs in soil (e.g. DTPA, EDTA, $CaCl_2$, and $NaNO_3$), EDTA was selected since it is considered more suitable for acidic soils (Hammer and Keller 2002; Feng et al. 2005), and it has a stronger extraction capacity than other extraction methods (Quevauviller et al. 1998; Han et al. 2020) This can be related to the EDTA high extraction potential, which may provide the maximum element extractability and, thus, indicate the higher levels of metal mobility (Labanowski et al. 2008). Furthermore, chelating agents such as EDTA are considered a more accurate indicator of metal availability to plants, as they can more effectively remove soluble metal-organic complexes that are potentially bioavailable (Bolan et al. 2008).

EDTA-extractable fraction in US showed the lowest concentrations for all PTEs except for Pb, where the highest concentration was found followed by

RS (Table 3.4). These values may be the result of an overestimation caused by the EDTA's significant binding capacity for Pb and its great efficiency in mobilizing this element from the soil (Shen et al. 2002; Santos et al. 2010). In addition, high Pb EDTA-extractable concentrations found in US and RS may be enhanced by the high organic carbon content in these soils coupled with EDTA capacity to effectively displace organically-bound fractions of metals present in soil through formation of strong chelates (Elliott and Shastri 1999; Nakamaru and Martín-Peinado 2017). The specific increase in Pb availability in soil due to the application of organic compounds has been previously proven (Angin et al. 2008). On the other hand, in PS the EDTA-extractable fraction was significantly the lowest for Pb, and for As and Cu were lower than in the amended soil treatments. Regarding the treatments applied to the amended soils, B and BV showed higher concentrations of Pb extractable with EDTA, driven by the higher organic carbon content in these treatments, while marble and gypsum treatments did so for As. Overall, vermicompost treatments showed higher concentrations of EDTA-extractable pollutants, which can be attributed to a positive correlation between organic matter content in soil and EDTA-extractable contents of PTEs, as previously reported by Zeng et al. 2011.

Table 3.4. EDTA extractable PTEs concentrations (mg kg^{-1} dry soil) (n=6) in the reference and treated soils.

	Pb_EDTA		As_EDTA		Zn_EDTA		Cu_EDTA	
	Mean	*sd*	Mean	*sd*	Mean	*sd*	Mean	*sd*
US	8.12 d	*1.51*	0.08 a	*0.02*	7.66 a	*1.48*	5.89 a	*1.18*
RS	4.22 c	*1.04*	0.63 ab	*0.27*	50.52 bc	*7.66*	46.71 d	*5.89*
PS	0.38 a	*0.16*	0.48 ab	*0.11*	51.90 bc	*2.16*	20.99 b	*4.68*
B	2.29 b	*0.74*	0.52 ab	*0.26*	58.43 c	*13.77*	25.97 bc	*5.75*
BV	1.39 ab	*0.37*	0.70 ab	*0.19*	45.90 bc	*10.17*	32.47 bc	*9.22*
G	0.37 a	*0.13*	0.95 b	*0.27*	42.73 bc	*12.40*	29.30 bc	*4.03*
GV	1.19 ab	*0.35*	2.92 d	*0.69*	50.54 bc	*14.84*	35.59 cd	*8.19*
M	0.60 a	*0.32*	1.06 b	*0.34*	36.88 b	*11.48*	30.38 bc	*6.47*
MV	0.78 a	*0.22*	1.99 c	*0.31*	59.98 c	*14.70*	33.87 c	*9.79*

US: Unpolluted soil; RS: Recovered soil; PS: Polluted soil; B: Biopile; BV: Biopile + vermicompost; G: Gypsum; GV: Gypsum + vermicompost; M: Marble; MV: Marble + vermicompost. *Sd*: standard deviation (n=6). Values followed by different letters are significantly different according to Kruskal-Wallis test (p<0.05).

3.3.3. Species richness and vegetation cover after remediation measures

The presence of vegetation in the studied soils eighteen months after the application of the treatments was evaluated compared to that present in the US. In this soil, a mean of about 12 different plant species was found growing on it (Figure 3.2a). Compared to US, species richness was slightly lower in RS, which could be related to the higher soluble concentrations found for the elements with highest mobility such as Zn and Cu, which in concentrations that exceed those required as nutrients may cause phytotoxicity to specific plant species (Nagajyoti et al. 2010). The high mobility of these elements could be, therefore, the main factor influencing the decrease in vegetation diversity and even, as in the case of PS, the responsible for the complete lack of vegetation on this soil, where the highest mobility for these elements was reached. In this regard, García-Carmona et al. (2019a) reported that the absence of vegetation in the soils that remain polluted in the studied area is mainly due to the high water-soluble concentration of Cu and Zn, rather than the high total concentration of As and Pb. Regarding the treated soils, species richness was half in the treatments with vermicompost addition compared to US, while for those without vermicompost it represented about just one-third. Meanwhile, both US and RS were totally covered by vegetation unlike PS, where vegetation growth was completely inhibited (Figure 3.2b). Under vermicompost treatments, vegetation cover was slightly lower if compared to US and RS, while for the treatments without vermicompost addition the vegetation cover represented less than half of that appearing when vermicompost was added, driven by the lower organic carbon content on these treatments. However, in general, all the treatments applied on PS resulted in enhanced soil properties and decreased soil toxicity, enabling the growth of vegetation to different extents, with the vermicompost treatments showing the greatest vegetation cover and species richness. Thus, vermicompost treatments were the most effective ones in terms of plant recolonization and development on the polluted soils, emphasizing that the addition of organic amendments in bare polluted soils is essential to facilitate the reestablishment and recolonization by the pioneer vegetation (Wong 2003). This vegetation development found on treated soils represents the spontaneous and natural response of the local vegetation to the changes produced in the soil by the amendments after eighteen months, so that the vegetation development in the treated soils could be expected to be similar to that found in the RS in the medium term. This could be assisted not only by the changes in soil properties induced by the treatments, but also by vegetation recolonization itself, since its presence produces many beneficial effects at the rhizosphere level and on the surrounding polluted soil. These can range from stimulation of microbial activity, improved aeration and stabilization of the soil to the reduction of PTEs concentrations and their stabilization (Erickson 1997; Davis et al. 2002). Moreover, the presence of vegetation may also facilitate other plant species to subsequently colonize the area through various mechanisms that reduce the potential transfer of elements from the soil to the plants, as the modification of rhizosphere pH or the exudation into the soil of organic acids that bind pollutants (Rutkowska et al. 2020).

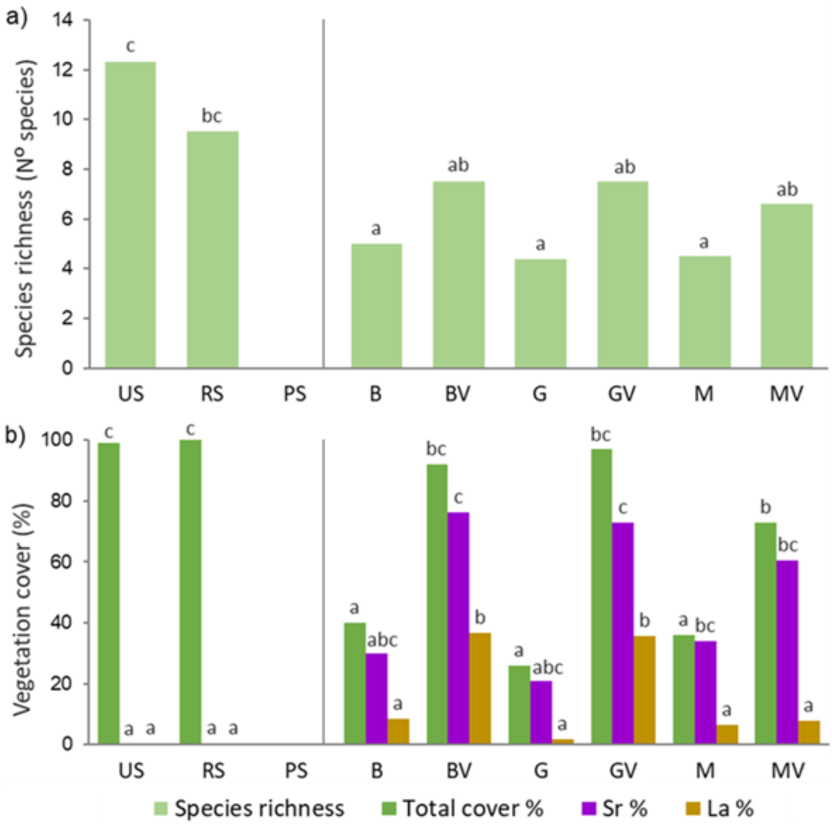

Figure 3.2. (a) Mean plant species richness present in each of the soils and treatments studied. (b) Total vegetation cover (%), and *Spergularia rubra* (Sr) and *Lamarckia aurea* (La) specific cover in each of the soils and treatments studied. US: Unpolluted soil; RS: Recovered soil; PS: Polluted soil; B: Biopile; BV: Biopile + vermicompost; G: Gypsum; GV: Gypsum + vermicompost; M: Marble; MV: Marble + vermicompost. Bars with different letters are significantly different according to Kruskal-Wallis test (p<0.05).

Frequently, in soils polluted by PTEs, their toxicity restricts the growth of all but the most tolerant plant species (Wong 2003). In our case, polluted soils did not show vegetation cover at all. However, eighteen months after the application of the amendments, mainly two species highly tolerant to the present PTEs concentrations appeared in all the treated soils: the herbaceous plants *Spergularia rubra* (L.) J. Presl & C. Presl and *Lamarckia aurea* (L.) Moench, being *S. rubra* the one with higher colonization percentage in all treatments (Figure 3.2b), present in about one third of the surface in treatments without vermicompost and in more than two thirds in those with vermicompost. Both species are frequently found in the soils polluted after the Aznalcóllar spill, particularly in those areas where PTEs concentrations remain higher, with acidic pH and high EC, and their presence being restricted mostly to them (Madejón et al. 2006; Montiel-Rozas et al. 2016; García-Carmona et al. 2019a). In this study, both species were dominant in the treated polluted soils where conditions (i.e., PTEs concentrations) remain more restrictive for vegetation growth. On the contrary, their presence was barely detected in US and RS, where a higher number of less pollution-tolerant species are present.

3.3.4. PTEs concentration and accumulation in plants

To fully evaluate the success of remediation strategies implemented on polluted soils, the determination of PTEs fractions in soil may not be sufficient to accurately predict the PTEs transfer risk to plants, since plant uptake not always correlates with them (Proto et al. 2023). Therefore, for a more accurate assessment of revegetation success, plant-based approaches should be used, either by carrying out plant bioassays or measuring PTEs concentrations in spontaneously growing vegetation in remediated soils, as this is a more reliable indicator of PTEs transfer potential in soils (Milton et al. 2002; Proto et al. 2023).

According to the predominant presence of *S. rubra* and *L. aurea* in the treated soils, their PTEs accumulation capacity was assessed (Table 3.5). Both species were able to accumulate high concentrations of PTEs both in their shoots and roots, which in most cases correlated significantly with the total and bioavailable concentration of the PTEs in the soil. In contrast, no direct correlation was observed with the soluble fraction of PTEs in soil, except for Zn concentrations (Table S3.2). In general, the concentrations measured in *L. aurea* were higher than in *S. rubra* for all elements except for Zn, which accumulated at a higher rate in *S. rubra*, and especially in the shoots of this species. The high Zn accumulation in *S. rubra*, and especially in its shoots, was previously reported by other authors (El Berkaoui et al. 2021). Meanwhile, *L. aurea* showed elevated PTEs accumulation for the other studied elements, highlighting the concentrations of Pb and As measured particularly in its roots. This confirms the high ability of this species to accumulate multiple PTEs, both in its shoots and roots, and an exceptional high capacity to accumulate specific elements in roots such as Pb (Condori 2004; Midhat et al. 2017).

Table 3.5. PTEs concentrations (mg kg^{-1} dry weight) (n=3) in aboveground part and in roots for the two main herbaceous species present in the different treatments applied after eighteen months.

	Pb_Shoot	Pb_Root	As_Shoot	As_Root	Zn_Shoot	Zn_Root	Cu_Shoot	Cu_Root
	Spergularia rubra							
US	3.5 a	16.0 a	0.6 a	0.9 a	104.4 a	72.1 a	6.8 a	8.0 a
RS	20.8 ab	24.7 ab	9.4 abc	17.3 bc	316.1 c	340.7 c	23.9 ab	30.1 b
PS	-	-	-	-	-	-	-	-
B	18.4 ab	24.0 ab	9.1 ab	10.2 ab	265.6 abc	411.3 c	27.1 b	18.8 ab
BV	35.1 bc	72.0 c	14.6 abc	24.4 c	302.8 bc	248.7 bc	28.1 b	33.0 b
G	37.0 bc	32.5 ab	23.2 c	19.3 bc	214.0 abc	158.0 ab	28.3 b	29.4 b
GV	42.6 c	63.6 c	17.5 bc	25.6 c	151.8 ab	127.3 ab	21.7 ab	30.1 b
M	21.3 abc	43.1 abc	8.2 ab	16.9 bc	171.5 abc	154.9 ab	19.8 ab	21.2 ab
MV	30.3 bc	50.4 bc	10.9 abc	14.4 bc	144.6 ab	150.8 ab	17.0 ab	20.2 ab
	Lamarckia aurea							
US	2.5 a	18.7 a	0.5 a	1.8 a	28.2 a	50.0 a	5.9 a	14.6 a
RS	37.8 b	65.1 abc	11.3 ab	27.3 abc	235.2 c	259.8 bc	20.7 ab	50.0 b
PS	-	-	-	-	-	-	-	-
B	29.6 ab	50.1 ab	13.5 ab	16.7 ab	159.4 abc	317.4 bc	26.4 ab	44.1 ab
BV	37.7 b	131.1 cde	16.4 ab	50.7 bcd	212.8 bc	235.4 bc	27.3 ab	52.1 b
G	43.2 b	99.2 bcd	25.1 b	49.8 bcd	146.7 abc	132.1 ab	26.5 ab	53.3 b
GV	36.7 b	231.0 f	17.0 ab	60.0 cd	78.7 ab	163.6 ab	24.4 ab	70.9 b
M	55.4 b	149.4 de	28.0 b	67.7 cd	139.7 abc	238.3 bc	42.5 b	67.7 b
MV	32.8 ab	168.9 ef	21.4 ab	87.9 d	81.3 ab	163.4 ab	26.3 ab	53.5 b

US: Unpolluted soil; RS: Recovered soil; PS: Polluted soil; B: Biopile; BV: Biopile + vermicompost; G: Gypsum; GV: Gypsum + vermicompost; M: Marble; MV: Marble + vermicompost. *Sd*: standard deviation (n=6). Values followed by different letters are significantly different according to Kruskal-Wallis test (p< 0.05).

In relation to plant indexes, the bioaccumulation factor (BAF) was calculated considering the PTEs bioavailable (EDTA-extractable) fraction in soil, since this fraction is considered more precise for this end (Losfeld et al. 2015). This is based in the fact that the danger of PTEs lies in their solubility and bioavailability rather than in their total concentrations, since they better represent the fractions that are available for uptake by living organisms and that can spread within the soil (Marguí et al. 2007; García-Carmona et al. 2019a). Moreover, total metal concentrations in soil are considered to correlate poorly with metal concentrations in plant tissues, since the amount of an element that is actually available to plants is different from that assessed by strong acid digestions (Buscaroli 2017). In this sense, BAF pointed out that *S. rubra* and *L. aurea* have a significant capability for accumulating Pb and As, both in their shoots and in their roots (Figure 3.3). According to the values obtained for these elements in both parts (BAF>10), we could consider that these two species act as hyperaccumulators of Pb and As (Rutkowska et al. 2020). Comparing these species to each other in terms of Pb and As accumulation, *L. aurea* had greater BAF than *S. rubra*, especially in the roots, highlighting that *L. aurea* is particularly tolerant to high levels of these elements, showing a higher ability to accumulate them in both the shoots and roots without sustaining toxicity, as remarked by previous studies (Midhat et al. 2017). Besides, the high values observed for Pb and As BAF in roots of *L. aurea* suggest that the translocation of these elements from soil to root was considerably high, so that *L. aurea* roots act as sink for Pb and As accumulation (Ng et al. 2016).

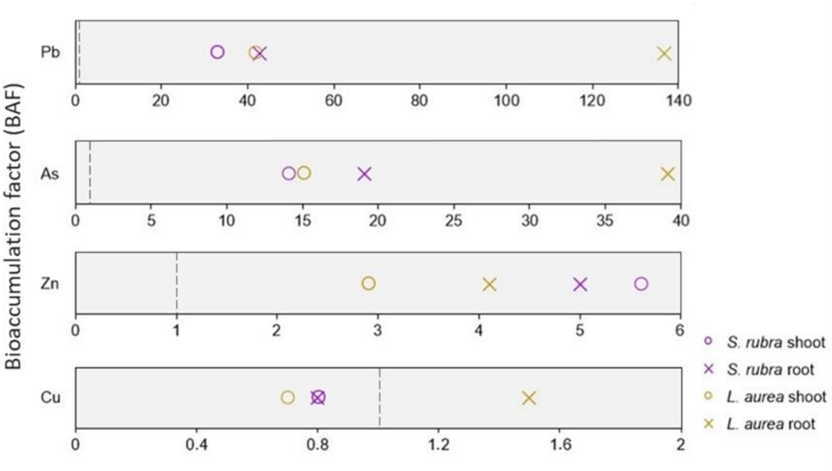

Figure 3.3. PTEs bioaccumulation factor (BAF) in aboveground part and in roots for the two main herbaceous species (*Spergularia rubra* and *Lamarckia aurea*) present in the different treatments applied after eighteen months. Dashed lines represent the threshold value 1 (BAF=1).

According to Zn, BAF measured for this element in both species was greater than one (Figure 3.3), although in a significantly lower degree than for Pb and As. Zn low accumulation (BAF<1) in different grass plants was observed even at increasing concentrations (Andrejić et at. 2018; Grassi et al. 2020), and even plants restricting Zn uptake at higher concentrations in the soil (Andrejić et al. 2018). Therefore, we could consider that although not at the same scale than Pb and As, the BAF>1 obtained for the studied species for Zn indicates that there is a considerable transfer of this element into the plant. Comparing both species, the higher accumulation of Zn showed by *S. rubra* (Figure 3.3 and 3.4) indicates that it is highly tolerant to this element (Gutiérrez-Ginés 2013), which could be one of the reasons of the higher abundance of this species compared to *L. aurea* (Figure 2b). In terms of Cu, neither *S. rubra* nor *L. aurea* showed a significant accumulation capacity for this element (BAF<1), which only slightly accumulated on *L. aurea* roots, and which is in accordance with the observed by García-Carmona et al. (2019a) in plants growing in residual polluted soils in the same area. The low Cu accumulation capacity in grass plants (BAF<1) was also reported in other studies (Bhatti et al. 2016; Satpathy et al. 2014).

The translocation factor (TF) calculated for the two selected species was lower than one in all cases, except very slightly for Zn in *S. rubra*, so that elements migration from the roots to the shoots of these species is not substantial (Figure 3.4). Nevertheless, TF in *S. rubra* for all elements was approximately two times higher than in *L. aurea*, showing that PTEs migration to *S. rubra* shoots is higher than that in *L. aurea*. Thus, the high capacity for pollutants concentration shown by *L. aurea* in its roots could reveal the mechanism that allows this species to thrive in the most heavily polluted soils and, consequently, be used as an accurate bioindicator of trace element availability in soils in this area, which is in accordance with Burgos et al. (2008b).

The natural settling of these two species, growing in the remediated soils after eighteen months, are good indicators of the success of the remediation treatments used (Álvarez-Rogel et al. 2021). In addition, although they were tolerant to the PTEs present in the soil and can accumulate some of them (BAF>1 for As, Pb and Zn), the absence of elements translocation from roots to shoots in the studied plants indicate that the selected cost-effective remediation techniques used, over time, may follow with passive enhancement, by modification of soil conditions by colonizing plants and being the first step in facilitating the growth of other species less tolerant to the stress (Navarro-Cano et al. 2018).

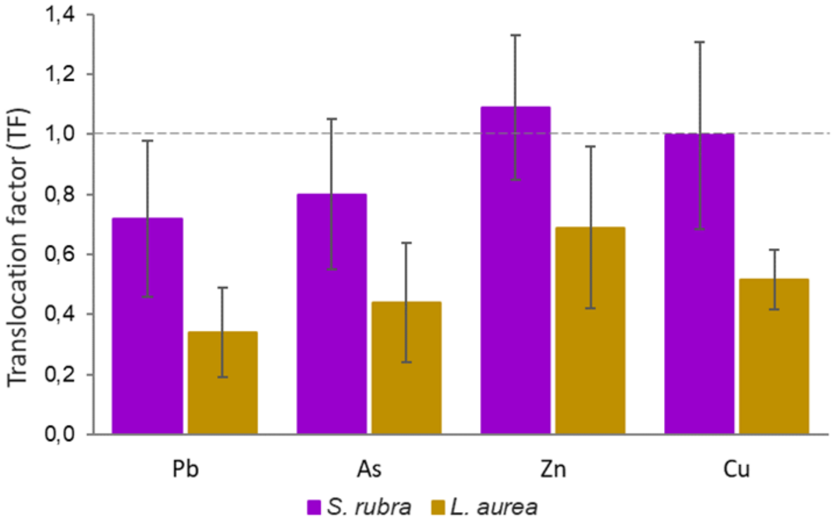

Figure 3.4. PTEs translocation factor (TF) for the two main herbaceous species (*Spergularia rubra* and *Lamarckia aurea*) present in the different treatments applied after eighteen months. Dashed line represents the threshold value 1 (TF=1).

3.4. Conclusions

The use of this novel cost-effective soil remediation strategy, implemented under field conditions for long-term soil remediation approach, has demonstrated significant improvements in key soil properties following treatments application, including elevated pH levels, augmented organic carbon content, and decreased salinity levels. Consequently, these treatments proved highly effective in fostering spontaneous vegetation colonization within severely degraded soil patches. Among the various remediation approaches, vermicompost amendment proved to be the most effective in promoting the vegetation recovery in the treated soils, leading to a greater diversity of plant species and increased vegetation cover in them.

The long-term field-based approach is crucial when evaluating the spontaneous recolonization of remediated soils by native vegetation, and the role of the actions implemented in achieving this. In this regard, two plant species relevant in the studied area and predominant in the treated soils after settling of treatments over time, *Spergularia rubra* and *Lamarckia aurea*, exhibited remarkable capacity in accumulating Pb and As, particularly in their root systems, indicating their high tolerance to the elevated pollutant concentrations prevalent in the study area. This points their role as key species, as they not only serve as pioneers in recolonizing the degraded soils but also facilitate the subsequent recolonization by other species less tolerant to the high pollution levels present in the studied area. Therefore, the success of the remediation strategy assessed may be promoted by the early recolonization of the soil by the high pollution-tolerant species, since their presence enhance further evolution in the soil physical, biological, and chemical conditions.

Supplementary Materials: Supplementary data to this article can be found online at https://doi.org/10.1007/s11104-024-06521-0

Chapter 4

Soil remediation approach and bacterial community structure in a long-term contaminated soil by a mining spill (Aznalcóllar, Spain)

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Abstract

The Aznalcóllar accident, which occurred in 1998, spilled 36 x 10^5 m^3 of pyritic sludge and 9 x 10^5 m^3 of acidic water around an area of 43 km^2 in the south of Spain. This spill is considered one of the most important metal-mining associated accidents worldwide. In this study, two soil remediation techniques were evaluated: the addition of marble sludge (liming treatment, M) and the mixing of recovered soils (RS) with contaminated soils (CT) (biopile treatment, B). Both M and B significantly reduce the solubility of Cu, Zn, As, and Pb mainly due to the increase in pH and organic matter content, respectively. Soil basal respiration rate and the seed germination and root elongation bioassay with *Lactuca sativa* were used to evaluate the toxicity of the potential pollution in the sampled soils. Both bioassays showed that the CT soils exhibited the highest toxicity with a significant reduction in the toxicity of the amended soils (M and B). The abundance and structure of microbial communities in the soils were determined by qPCR and Illumina 16S rRNA sequencing, respectively. The absolute abundances of total bacterial and archaeal populations, ammonium oxidizing bacteria, and denitrifiers in the CT soils were statistically lower than these found in the other three soils. Similarly, the structure of the bacterial community was highly different in the CT soils. Our results underline the persistence of the detrimental effect of pollutants in CT soils compared to the recovered (RS) and amended soils (M and B). We also highlight the uses of liming or biopile as remediation techniques as satisfactory tools to reduce the impact of heavy metals in the contaminated Aznalcóllar soils.

Keywords: soil pollution; toxicity bioassay; heavy metals; bioavailability; bacterial diversity; microbial communities

4.1. Introduction

On April 25, 1998, one of the most important accidents associated with metal mining worldwide took place in Aznalcóllar (Seville, Spain) (Nikolic et al. 2011). The breakage of the tailings dam of metal mine located on the Iberian pyrite belt produced a spill of 36 x 10^5 m^3 of pyritic sludge and 9 x 10^5 m^3 of acidic water into the basin of the Guadiamar river (Simón et al. 2001). The accident was caused by the excessive volume of waste deposited in the tailings dam, which had been expanded several times to increase its storage capacity, reaching between 21 and 27 m in height at the time of the accident.

The spill of the sludge reached 40 km while the acidic water even reached the vicinity of the Doñana National Park, 58 km from the tailings dam. In total, the affected area reached 43 km^2 (Grimalt et al. 1999; Ayala-Carcedo 2004) with a very heterogeneous sludge width and thickness along the Guadiamar basin, and a maximum depth of 2 m in some parts of the area affected (Cabrera et al. 1999; López-Pamo et al. 1999).

No data was found about the composition of the acidic water stored in the dam, but its composition is likely to be very similar to that which was characterized 12 km from the tailings dam a few hours after the spill (pH <5; 0.27 mg l^{-1} of As; 0.85 mg l^{-1} of Cd, 3.6 mg l^{-1} of Pb, and 463 mg l^{-1} of Zn) (Cabrera et al. 1999).

On the other hand, the sludge texture was silty loam texture (70% of silt content), and the structure was platy, pH was strongly acidic, without carbonates, and very low in organic matter content (Simón et al. 1999). The sludge was composed mainly of pyrite (68–78%, w/w) with minor proportions of sphalerite, chalcopyrite, galena, and arsenopyrite (heavy metal-bearing sulfides), as well as other minerals such as chlinochlore, quartz, calcite, and gypsum (Alastuey et al. 1999; López-Pamo et al. 1999). According to its mineralogical composition, the sludge presented high concentrations in As, Cd, Cu, Sb, Pb, Tl, and Zn, which were considered as being the main pollutants in the soils affected by the spill (Simón et al. 1999).

Due to the magnitude of the contamination, the Regional Government of Andalusia carried out the huge task of cleaning up and recovering the contaminated soils from 1998 to 2001. First, the sludge was removed with the use of heavy machinery, and then, the remaining soils were added with organic amendments and soils with high contents of iron-rich clays. Furthermore, a liming treatment with calcium carbonate was applied to the soils in different ratios depending on the soil properties and the residual concentration of pollutants (Aguilar et al. 2004a; Madejón et al. 2018). These actions significantly reduced the concentrations of soil pollutants, and the Regional Government of Andalusia finally declared the area affected by the spill as a protected area called Guadiamar Green Corridor (CMA, 2003).

Currently, the area is being considered as recovered. Although Pastor-Jáuregui et al. (2020a) reported that the concentrations of pollutants (mainly As and Pb) in the soils more strongly affected were higher than those measured in the nearby soils unaffected by the spill. However, Martín-Peinado et al. (2015) reported a low mobility and bioavailability of pollutants in Guadiamar Green Corridor soils that highlights the success of the soil remediation treatments applied, but with higher values of Cu and Zn than the recommendable amounts. Nevertheless, Aguilar et al. (2007) and Simón et al. (2008a) indicated that there might be areas which are still polluted due to traces of sludge that penetrated through the soil cracks and that were not removed in the cleaning tasks. Nowadays, this fact becomes clear by the existence of bare soil patches scattered along 18 km from the tailings dam. In these unvegetated soil patches, the soils are strongly acidic and poor in organic matter and carbonate contents (Martín-Peinado et al. 2015). According to García-Carmona et al. (2017), the unvegetated soil patches are an environmental risk, assessed by risk quotients (USEPA, 2007) for total concentration of As, Cu, and Pb, but only for Cu and Zn when considering the water-soluble concentrations. In any case, these unvegetated patches pose a high risk of dispersion of the contamination that must be addressed.

Over the last years a great number of soil remediation techniques have been developed becoming safer, cleaner, less cost effective, and more environmentally friendly (Kulshreshtha et al. 2014). García-Carmona et al. (2017) applied three "ex-situ" remediation techniques in soils sampled in five unvegetated patches in the Guadiamar Green Corridor (biopiles, landfarming, and composting) with the aim of reducing the mobility and bioavailability of soil pollutants, finding the biopiles technique to be the most effective technique for reducing soil toxicity. The success of the biopiles was related to the improvement of soil properties, mainly caused by the increase in pH due to a rise in $CaCO_3$ contents, and the reduction in the solubility of the potentially pollutant elements.

In this sense, Acevedo et al. (2005) described soil quality as a general improvement of physicochemical and biological parameters. Hence, the study of soil microbiota including abundance, diversity, and metabolism (M. Wang et al. 2013) could provide information about soil status (Chapman et al. 2007; Giacometti et al. 2013).

There are many techniques that provide information about soil microbiota. However, in recent years, molecular biological methods such as real-time polymerase chain reaction (PCR) or massive sequencing, have been used due to their capacity to provide more information (Hermansson et al. 2004). It has been proved that real-time PCR is an effective method for quantifying genes. The high sensitivity of this procedure enables the characterization of microbial communities with high specificity and accuracy (Zheng et al. 2020). On the other hand, conventional Sanger sequencing is being replaced by techniques such as "Next Generation Sequencing (NGS)". Thus, the use of the Illumina MiSeq platform as a massive sequencing technique is widely extended among the scientific community because it can produce large numbers of sequences in microbial community analyses (Jeon et al. 2015; Sun et al. 2018).

Therefore, the aim of this work is to assess the effectiveness of biopiles and liming as soil remediation techniques applied in field plots within the unvegetated soil patches. The degree of soil recuperation will be established according to the reduction in the solubility and bioavailability of the potentially pollutants elements as well as their toxicity through the use of bioassays and the analysis of the microbiological diversity in soils.

4.2. Materials and methods

4.2.1. Soil sampling

The area nearby the tailings dam in the Guadiamar Green Corridor is characterized by the existence of numerous randomly distributed unvegetated patches. The unvegetated patches are surrounded by successfully recovered soil with dense grass and scrub vegetation. For this study, three unvegetated patches of soils, where the remediation measures were not effective enough and which remained affected by residual contamination, were selected in November 2016. In each unvegetated patch, 3 experimental plots of 4 m^2 were established with two treatments and one control. The treatments consisted of a liming (M) using marble sludge from the cutting of the marble stone from the Macael quarry (Almería). The marble sludge was added in a dose of 5 kg m^{-2}. The second treatment was a biopile

(B) and consisted of mixing the contaminated soil with the adjacent recovered soil in a proportion of 50%. In limed and biopile plots, the uppermost 10 cm of soil were treated and a year and a half later, in May 2018, composite soil samples were taken by mixing 200 g of topsoil (0–10 cm) from each corner and center of a 1 m sided square located in the center of each treated plot. To assess the effectiveness of the amendment treatments, an undisturbed plot was defined and sampled in each unvegetated patch and used as a control (CT). Furthermore, recovered soil (RS), which was defined by the presence of dense vegetation, located near the selected unvegetated patches selected, was also sampled.

4.2.2. Soil analysis

Soil samples were dried and sieved (<2mm) prior to the analyses. Soil properties were determined according to standard methods (MAPA 1994). Soil granulometry was measured after the removal of organic matter with hydrogen peroxide and dispersion of samples with sodium hexametaphosphate. Soil pH was measured in a soil:water suspension of 1:2.5, and electrical conductivity (EC) was measured in a soil:water suspension of 1:5. Finely ground samples were used for the measurement of calcium carbonate contents by volumetric methods and for the quantification of the organic carbon (OC) by acid-oxidation method.

The concentrations of As, Pb, Cu, and Zn were also analyzed in finely ground samples by X-ray fluorescence (XRF) with a NITON XL3t-980 GOLDD+ instrument (Thermo Fisher Scientific, Tewksbury, MA, USA). The precision and accuracy were calculated the measurement of a certified reference material (CRM052-050 RT-Corporation Limited, Salisbury, UK).

Water soluble and ethylenediaminetetraacetic acid (EDTA) extractions were performed to assess the mobility and bioavailability of trace elements in soils according to Sposito et al. (1982) and Quevauviller et al. (1998). The soluble and bioavailable fractions were extracted with distilled water and 0.05 M EDTA (pH=7), respectively. Soil:liquid extract in a 1:5 proportion was used and As, Pb, Cu, and Zn concentrations were analyzed by inductively coupled plasma-mass spectrometry (ICP-MS) in a PE SCIEX ELAN-5000A spectrometer. For calibration, two sets of multi-element standards containing all the analytes of interest at five different levels of concentration were prepared using rhodium as an internal standard. All standards were prepared from ICP single-element standard solutions (Merck, Darmstadt, Germany) after dilution with 10% HNO_3. The accuracy of the method was confirmed by analyzing a Standard Reference Material SRM2711 Montana Soil (US NIST, 2003).

For the toxicity assessment of the potential pollution in the area, the bioassay with *Lactuca sativa* was used, according to OECD (2003) and USEPA (1996) recommendations. For each experimental unit, a saturated paste of the soil was obtained after 24 h of contact and vacuum pump extraction; after this, 5 mL of the extract were put in contact with 20 seeds of *Lactuca sativa* during 5 days of incubation at 25 ± 1 °C. After this period, the percentage of germination and root elongation were measured in both, the soil and control samples (distilled water). Values range from 0 (maximum toxicity) to 100 (no toxicity).

The basal respiration rate (ISO, 2002) was measured with SY-LAB equipment, μ-Trac 4200 model. Before starting the test, soil moisture was adjusted at 60% of water holding capacity. The equipment operates with a constant temperature of 30 °C and CO_2 production was determined from vials containing solution of potash (KOH 0.2%) for 96 h. The results were based on the variation of the electrical impedance produced over time by the reaction between the emitted CO_2 (heterotrophic soil respiration) and KOH.

4.2.3. Microbial analysis

4.2.3.1. DNA extraction and real-time quantitative PCR (qPCR) of the bacterial and archaeal 16S rRNA

The total DNA was extracted from 0.5 g of each sample which had previously been homogenized using a reproducible commercial kit for soil DNA extraction, the FastDNA SPIN Kit for soil, and the FastPrep 24-Instrument (MP Biomedicals, Germany) according to the manufacturer's protocol (Vela-Cano et al. 2019). The DNA obtained was eluted in 100 μl of DNase/Pyrogen-Free Water and stored at −20 °C until further use. DNA samples collected from the four studied areas were amplified by real-time quantitative polymerase chain reactions (qPCR). The qPCR analysis was chosen to determine the gene copy number of total bacteria, ammonia-oxidizing bacteria (AOB), denitrifying bacteria, and total archaea (Table S4.1). The analysis was performed using an Mx3000P qPCR system (Agilent Technologies), and real time PCR data was treated using a MxPro QPCR software version 3.0 (Stratagene, USA) (Gallardo-Altamirano et al. 2018). The qPCR calibration curves were constructed with the plasmid standard using serial ten-fold dilutions (10^2–10^8) as described Maza-Márquez et al. (2016) and all of them had a correlation coefficient r^2>0.99 in all the assays and the efficiency of PCR amplification ranged between 90 and 100%.

The qPCR reaction mixtures were made in a total volume of 25 mL (Muñoz-Palazón et al. 2020) containing 2.5 µl of 10x Taq Buffer (with $MgCl_2$), 0.5 µl of dNTPs (10 mM), 0.15 µl of each primer (10 µM), 0.0625 µl of bovine serum albumin (BSA, 20 mg ml^{-1}), 0.125 µl SYBR green diluted in DMSO (20x), and 0.125 µl of DNA polymerase (50 U $µl^{-1}$). All quantitative amplifications were performed in triplicate. The cycling conditions applied to each primer pair are summarized in Table S4.2.

4.2.3.2. Illumina Miseq sequencing and data processing

DNA extracts were subjected to Illumina sequencing at the Department of Gastroenterology, Hepatology, and Infectious diseases in the University Hospital of Magdeburg (Otto-von-Gericke University) for partial 16S rRNA sequencing. The amplification was performed using the prokaryote-specific primers 341F and 534R. The raw data of sequencing were processed using the mothur v1.43.0 software according to the Mothur MiSeq-pipelines (Schloss et al. 2009). Default settings were used for quality control, primer trimming, filtering, preclustering, and chimera detection. The Silva SEED v132 database was used for alignment and the core sequence positions from 40 to 854 bases were maintained to obtain a tighter alignment of the sequences. Operational taxonomic units (OTUs) were assigned at the 97% cutoff level and singleton OTUs were removed for later analysis. Finally, the consensus sequences of each OUT were taxonomically classified according to the Silva SEED database. The alpha diversity indices were calculated according to Hill et al. (2003).

4.2.3.3. 16S rRNA sequence accession number

The 16S rRNA sequences reported in this work have been deposited in GeneBank under the accession number SUB8135263.

4.2.4. Statistical analysis

The non-parametric Kruskal-Wallis test (Conover-Iman post hoc test; $p<0.05$) was chosen for the analysis of the mean comparison due to sample size (Theodorsson-Norheim 1986). Spearman's correlations were performed to analyze the influence of soil properties on the selective extractions of metals. All these analyses were performed with a confidence level of 95% by using the corrplot v0.84 package in Rstudio v.3.4.1 (Rstudio, Boston, MA, USA). A heatmap of structures of bacterial communities at the genus level was made using the average clustering method in R studio. The PC-ORD software v6.08 (MjM Software, Oregon, USA) was used for non-metric multidimensional scaling (NMS) analysis to determine the assemblages across samples of the gene abundance and bacterial biodiversity data; afterwards, the physiochemical data were coupled to the NMS analyses to determine the relationships between biotic and abiotic variables. Correlations among soil properties, microbial abundance, and diversity data to the soil respiration rate, root elongation, and germination rate were also determined.

4.3. Results

4.3.1. Soil properties and pollutant concentrations

The soils studied were placed in the upper sector of the Green Corridor of the Guadiamar River, next to the tailings dam and they are characterized by high sand contents (>50%) and are thus classified as having a sandy loam texture. The control soil, which remains contaminated and unvegetated, compared to the recovered soil had a 7 times lower organic matter content, half the cation exchange capacity, a 7 times higher salinity, and a strongly acidic pH, compared to a slightly acidic pH in the recovered soil (Table 4.1). In the amended soils, the organic carbon content was almost double in the biopile soils compared to the limed soils. However, the limed soils had a neutral pH due to their higher calcium carbonate contents, although the electrical conductivity and the capacity of change were similar in both amended soils.

Table 4.1. Main soil properties (n=3) of the limed soil (M), biopile soil (B), control soil (CT) and recovered soil (RS).

	Clay	Silt	Sand	SOC	CIC	pH	EC	$CaCO_3$
M	14.4 *b*	29.9 *bc*	55.7 *a*	4.9 *b*	6.4 *c*	6.9 *a*	1.9 *b*	4.4 *a*
B	17.7 *a*	24.4 *a*	58.0 *a*	8.8 *a*	8.0 *b*	5.6 *b*	2.2 *b*	1.0 *b*
CT	9.6 *c*	38.4 *c*	52.0 *a*	5.0 *b*	6.6 *c*	3.5 *c*	2.8 *a*	0.6 *b*
RS	11.8 *bc*	32.8 *bc*	55.4 *a*	34.8 *a*	13.1 *a*	6.6 *b*	0.4 *c*	0.9 *b*

SOC: soil organic carbon (g kg^{-1}); Clay, Silt and Sand (%); CIC: cation exchange capacity ($cmol_{(+)}$ kg^{-1}); EC: electrical conductivity (dS m^{-1}); $CaCO_3$: percentage of calcium carbonate. Lowercase letters represent significant differences among soils (Kruskal-Wallis (Conover-Iman post hoc test; p<0.05)).

4.3.2. Pollutant concentration and availability

The total Cu and Zn contents were significantly higher in the recovered soil, while As and Pb concentrations were higher in the control soil (Table 4.2). In the soils amended with marble sludge, the total contents of the analyzed elements were more like those of the control soil, while the biopile soils showed a total concentration of metals more like that measured in the recovered soil, due to the mixture of the recovered soil with the contaminated unvegetated soil. According to Spearman's correlation analysis (Figure S4.1), total Cu and Zn concentrations on the one hand, and As and Pb on the other were positively correlated. However, total As and Pb concentrations were negatively correlated to Cu and Zn total concentrations, but only statistically significance was reached in the case of Pb with Cu and Zn.

Table 4.2. Trace elements concentrations (mg kg^{-1} dry soil) (n=3) for the different soil fractions in limed soil (M), biopile soil (B), control soil (CT) and recovered soil (RS).

	Cu_T		Zn_T		As_T		Pb_T	
	Mean	sd	Mean	sd	Mean	sd	Mean	sd
M	133.4 *c*	9.38	275.4 *c*	26.98	327.9 *ab*	7.63	601.2 *b*	17.13
B	165.6 *ab*	22.99	407.7 *b*	38.06	305.8 *b*	51.72	555.9 *b*	103.41
CT	141.6 *bc*	11.74	357.6 *b*	68.38	462.8 *a*	107.28	751.1 *a*	43.99
RS	256.9 *a*	69.91	898.8 *a*	134.70	299.9 *b*	44.28	397.1 *c*	11.66
	Cu_W		Zn_W		As_W		Pb_W	
	Mean	sd	Mean	sd	Mean	sd	Mean	sd
M	0.18 *c*	0.035	0.15 *c*	0.028	0.05 *b*	0.004	0.01 *c*	0.007
B	0.53 *b*	0.339	1.01 *b*	1.253	0.06 *b*	0.012	0.03 *bc*	0.014
CT	8.28 *a*	2.868	16.66 *a*	2.368	0.18 *ab*	0.019	0.07 *a*	0.013
RS	0.70 *b*	0.121	0.71 *b*	0.341	0.24 *a*	0.068	0.04 *b*	0.006
	Cu_EDTA		Zn_EDTA		As_EDTA		Pb_EDTA	
	Mean	sd	Mean	sd	Mean	sd	Mean	sd
M	29.10 *c*	2.775	29.88 *c*	6.592	0.55 *a*	0.364	0.38 *b*	0.271
B	39.82 *b*	3.469	89.02 *b*	27.840	1.25 *a*	0.808	3.28 *a*	0.835
CT	36.79 *bc*	5.280	181.51 *a*	24.493	0.47 *a*	0.119	0.25 *b*	0.160
RS	77.78 *a*	4.282	135.03 *b*	17.581	1.35 *a*	0.237	4.14 *a*	1.716

T: total; W: water soluble; EDTA: bioavailable fraction extracted with EDTA; sd: standard deviation (n=3). Lowercase letters represent significant differences among soils (Kruskal-Wallis (Conover-Iman post hoc test; p<0.05)).

The soluble concentrations for the four elements analyzed were significantly higher in the control soil compared to the liming or biopile soil treatments. They were also higher with respect to the recovered soil, except in the case of water soluble As, where the highest concentration was measured in the recovered soil. Limed soils significantly reduced the water solubility of Cu and Zn compared to biopile soils. However, no significant differences were found for As and Pb soluble concentrations. Soil properties such as pH and calcium carbonate contents determined the solubility of the four elements in the soil. Cu, Zn, As, and Pb concentrations were statistical and inversely correlated to the carbonate content of the soils (Figure S4.1). The pH was inversely correlated to Cu, Zn, and Pb as well, but no statistical significance for soluble As was reached. No significant correlations were found between the solubility of the four elements analyzed and the rest of the measured soil properties (organic carbon, electrical conductivity, texture fractions, and cation exchange capacity).

The Cu, As, and Pb EDTA extractable concentrations were significantly higher in the recovered soil compared to the control soil. Only the Zn extracted with EDTA was significantly higher in the control soil. Liming was the soil amendment that most significantly reduced the EDTA extractable concentration, while biopile soils showed similar concentrations to those of the recovered soil, except for the case of Cu concentrations. The bioavailability of Zn was inversely correlated to the calcium carbonate content and soil pH (Figure S4.1). However, the soil organic carbon content was positively correlated to Cu and Pb. The strong correlation between the bioavailable fractions of Cu and Pb with the soil organic carbon content was also shown by their significant correlation with the cation exchange capacity of the soil.

4.3.3. Toxicity bioassays

In the soil toxicity tests, only the control soil showed a toxic effect in both the germination and root elongation of the lettuce seed (*Lactuca sativa*). While the recovered soil and all the amended soils showed a germination rate of 100%, the control soil's germination rate was only 38% (data not shown). In the contaminated soils with added marble sludge (M), a reduction in root elongation was observed (Figure 4.1); furthermore, the toxicity effect was significantly lower than in the control soils. In contrast, both biopile soils (B) and recovered soils (RS) underwent a hormesis effect, and a root elongation greater than that measured in the test blank with distilled water was recorded.

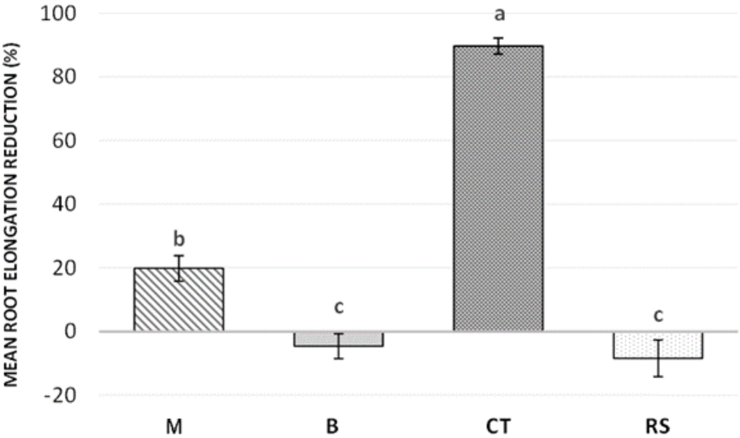


Figure 4.1. Percentage reduction in *Lactuca sativa* root elongation. Limed soil (M), biopile soil (B), control soil (CT), and recovered soil (RS). Lowercase letters represent significant differences among soils (Kruskal Wallis test p<0.05). Whiskers show the standard deviation.

Significant differences were found in microbiological activity as a function of the basal respiration rate measured in the soils (Figure 4.2). The basal respiration rate was 5-fold and 4-fold higher in the recovered soil (RS) and in amended soils than in the control soil, respectively. However, the respiration rates in the amended soils (M and B) were similar and did not exhibit any statistically significant differences between them.

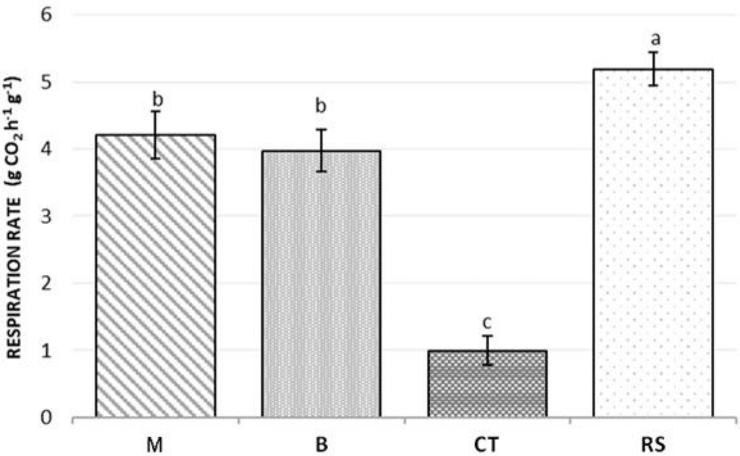


Figure 4.2. Basal soil respiration bioassay rates. Limed soil (M), biopile soil (B), control soil (CT), and recovered soil (RS). Results show average respiration rates (n=3) ± standard deviation. Lowercase letters represent significant differences among the soils (Kruskal Wallis test p<0.05). Whiskers show the standard deviation.

4.3.4. Microbial analysis

4.3.4.1. Abundance of target genes in soil samples

Total bacteria, total archaea, and two of the microbial groups involved in the soil N cycle (AOB and denitrifying bacteria), were studied to check the changes in the abundance of genes among the different soils.

The soil remediation treatments applied influenced the abundance of all the studied microbial communities, according to the copy numbers determined by qPCR. The results obtained are shown in Figure 4.3.

The data showed changes in soil microbial abundance when comparing the different gene copy numbers of the soils. The numbers of DNA copies g^{-1} in the RS soil were the highest in all the studied microbial groups, and the lowest corresponded to CT soil. In fact, it was evident that the applied treatments influenced the soils, since the obtained values in M and B were higher than in CT in all cases. The microbial abundances were not statistically different between limed and biopile treated soils, except for the AOB population that was higher in m compared to B soil. Therefore, the differences between the values of treated soils (M and B) compared to values of CT are remarkable and reflects the effectiveness of the actions carried out in these areas.

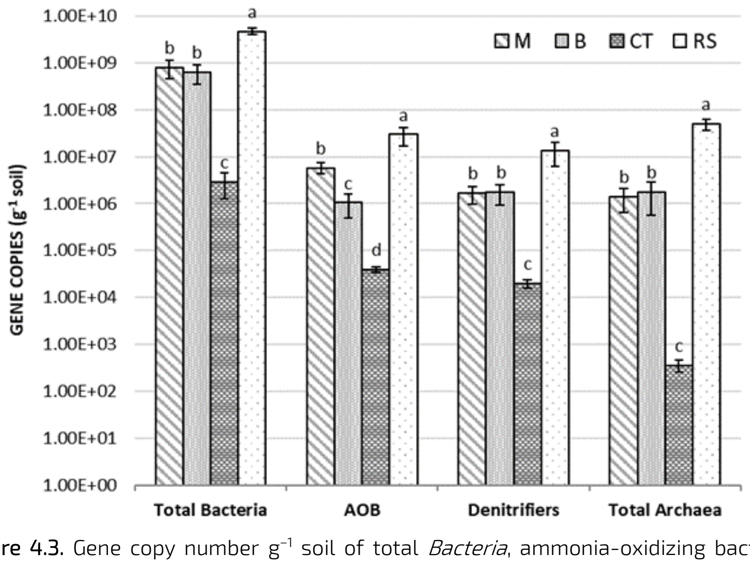


Figure 4.3. Gene copy number g^{-1} soil of total *Bacteria*, ammonia-oxidizing bacteria (AOB), denitrifying bacteria, and total *Archaea* in limed soil (M), biopile soil (B), control soil (CT), and recovered soil (RS). The results of two independent determinations (n=3) are shown. Lowercase letters represent significant differences among the soils (Kruskal Wallis test $p<0.05$).Whiskers show the standard deviation.

4.3.4.2. Structure and diversity of bacterial and archaeal populations

Prokaryotic community structures retrieved from the different soils used in this work were determined by Illumina sequencing of partial 16S rRNA gene. There were 510,406 raw sequences. After filtering, chimera analysis, and misaligned sequences filtering, there were 269,785 sequences (228,701 unique sequences). The clustering of the sequences into OTUs resulted in 48,775 different OTUs. Finally, the elimination of singletons led to 10,234 OTUs (231,244 sequences).

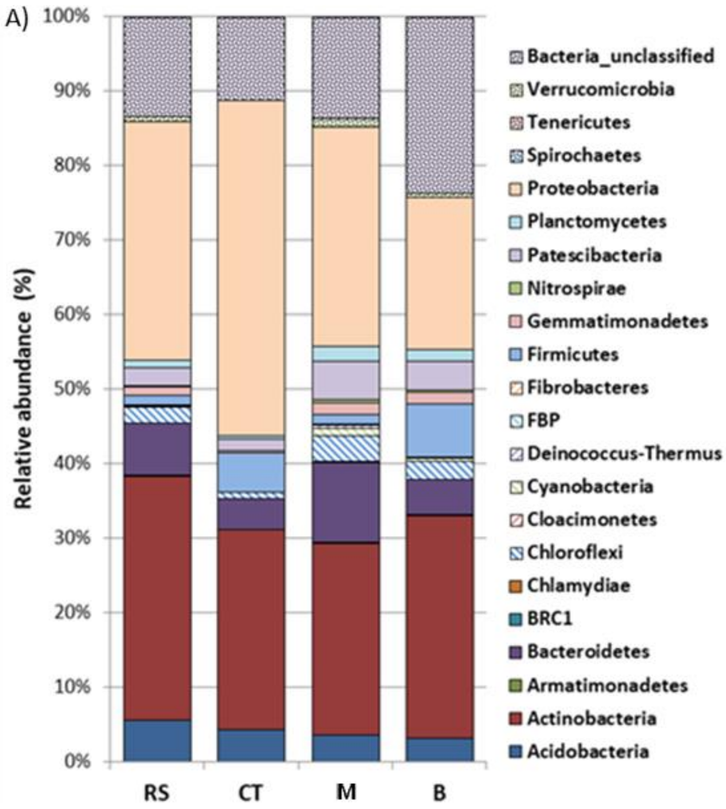
The OTU consensus sequences were classified into 72 OTUs belonging to the domain *Archaea*, 10,129 to *Bacteria*, 8 were classified as chloroplasts, and 25 were not classified.

The OTUs number was highest for M and B samples (with no differences between them), the RS soil had intermediate values, and the CT samples presented the lowest values (Table S4.3). While the Simpson index did not vary significantly among the soils, the Shannon index values were lower for CT samples than for the other 3 soils (Table S4.3).

The archaeal community comprised 2 phyla (*Euryarchaeota* and *Thaumarchaeota*) and the group of archaeal unclassified sequences (Table S4.4). The phylum *Thaumarchaeota* constituted the more abundant fraction of the entire *Archaea* (98.54±0.83). Eight different archaeal genera were found in the soil samples (Table S4.5) with no differences among the samples according to the Kruskal-Wallis and Conover-Iman tests for a given genus.

On the other hand, the bacterial community was composed by 21 different phyla and the group of unclassified bacteria (Figure 4.4A). Altogether, these groups were (in decreasing order of abundance) *Proteobacteria*, *Actinobacteria*, Bacteria_unclassified, *Bacteroidetes*, *Firmicutes*, *Acidobacteria*, *Patescibacteria*, *Chloroflexi*, *Planctomycetes*, *Gemmatimonadetes*, *Verrucomicrobia*, *Cyanobacteria*, *Nitrospirae*, *Deinococcus-Thermus*, *Armatimonadetes*, *Fibrobacteres*, *Chlamydiae*, BRC1, *Spirochaetes*, *Tenericutes* and *Cloacimonetes*. Significant differences among samples were only observed for *Bacteroidetes*, *Verrucomicrobia*, *Cyanobacteria*, *Nitrospirae*, *Deinococcus-Thermus*, *Fibrobacteres*, *Chlamydiae*, BRC1 and *Tenericutes* (Table S4.6). The relative abundance (RA) of phyla *Acidobacteria*, *Actinobacteria*, *Armatimonadetes*, *Chloroflexi*, *Cyanobacteria*, *FBP*, *Firmicutes*, *Gemmatimonadetes*, *Patescibacteria*, *Planctomycetes*, *Proteobacteria*, *Spirochaetes* and *Tenericutes* were not statistically different among samples and the remaining phyla were more abundant in M samples compared to the other 3 soils, except the phyla *Verrucomicrobia* that was the highest in RS samples.

Similarly, there were a total of 465 different groups classified at the genus level; of them 40 were considered dominant genera (RA>0.5%) that represented 75% of the bacterial sequences. An asterisk was added at the end of the name of the bacterial groups that were not classified at the genus level to facilitate the reading of the text. The RA of the majority of the genera are shown in Figure 4.4B and Table S4.7. The abundance of groups Micrococcaceae* and Chloroflexi* were statistically higher in M samples and similar among the other soils. *Blastococcus*, Intrasporangiaceae*, Microscillaceae*, and Rhodobacteraceae* were higher in M and B followed by RS and CT samples, which had similar relative abundances between them (Table S4.7). The RA of *Solirubrobacter* and Bacillaceae* were significantly higher in RS and B samples and similar between CT and M samples. The abundance of *Cutibacterium* was higher in CT and lower in the remaining soils.



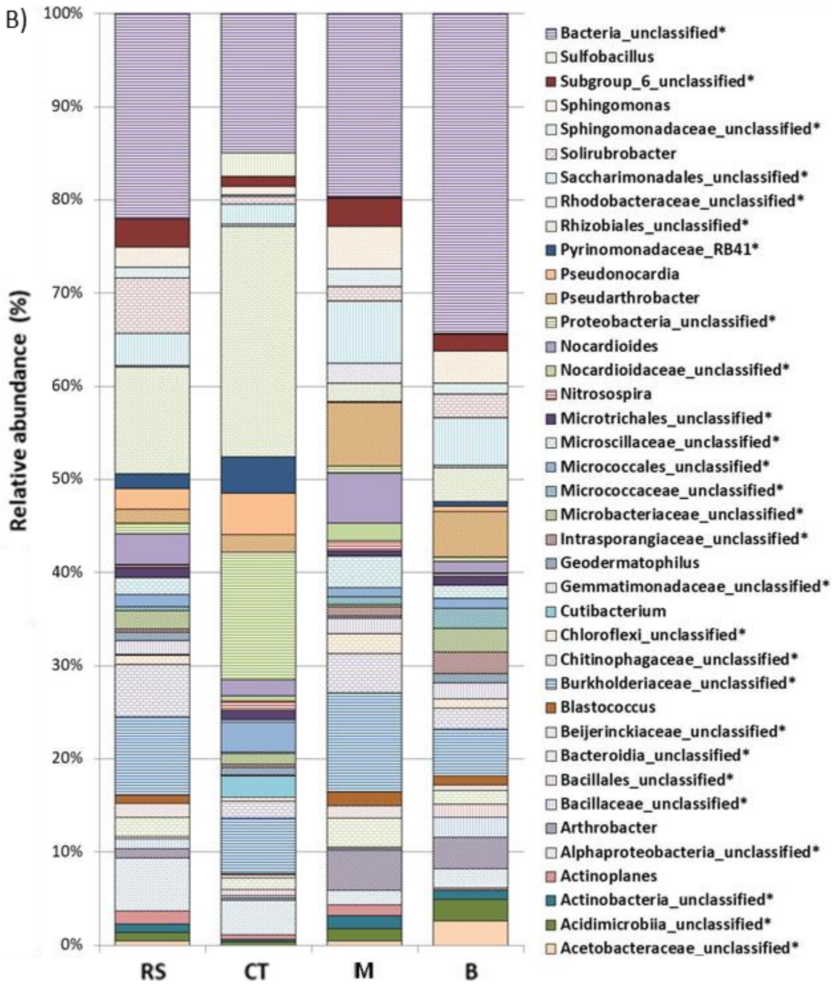


Figure 4.4. Average abundance (n=3) of bacterial phyla (A) and dominant genera (RA>0.5%) (B) by Illumina high-throughput sequencing for the different soil fractions in limed soil (M), biopile soil (B), control soil (CT), and recovered soil (RS). *: not classified into the genus level.

The highest RA of Actinobacteria* was found in RS and B soils followed by M and, finally, CT samples. The abundance of Sphingomonadaceae* was higher in M and B, and lower in CT, with no differences among RS and the other 3 soils. No statistical differences in the RA among soils were found in the remaining dominant genera.

Figure S4.2 shows the hierarchical clustering of the relative abundances of the main genera (RA>0.5%) found in the 12 amplicons here analyzed. The CT samples clustered apart from the remaining samples, and they were highly different among them. The CT1 sample was characterized by a dominance of Microbacteriaceae*, Bacillales*, Microtrichales*, and *Sulfobacillus*. The CT2 sample was over-represented by *Cutibacterium*, Alphaproteobacteria*, *Geodermatophilus*, Micrococcaceae*, *Pseudonocardia*, Pyrinomonadaceae_RB41, and Actinobacteria* cluster 1. Finally, the CT3 samples had the highest relative abundances of Rhizobiales* and Proteobacteria*. The remaining samples clustered together in the same branch of the hierarchical analysis. No over-representations of genera were detected indicating that the bacterial structures were very similar in the RS, M, and B samples.

4.3.4.3. Interaction among bacterial and archaeal communities, soil properties and pollutant concentrations

The NMS ordination of the abundances of total *Bacteria* and *Archaea*, AOB and *nos*Z-bearing showed shifts in the gene copy numbers among soils (Figure 4.5). The CT samples were placed independently in the NMS ordination. The remaining samples were grouped together without a clear independence among them. Whereas RS, M, and B samples were characterized by high copy numbers per gram of soil of total *Bacteria* and *Archaea*, AOB and denitrifiers, the CT samples presented lower values for these populations. According to Table S4.8, several correlations were observed among the physicochemical properties and population abundances.

Similarly, the NMS analysis of the RA of the dominant bacterial genera (RA>0.5%) and the shifts in the physicochemical parameters are shown in Figure 4.6. The 12 amplicon libraries from the 4 soils were distributed differently in the NMS space. Similarly to what was observed in the NMS analysis of the abundance the RS, M, and B grouped nearby, and the CT samples clustered independently on the 2-D space. These results were also in agreement with those observed for the heatmap (Figure S4.2). The linking of the physicochemical properties to the NMS revealed that several properties influenced the RA of the dominant genera (Table S4.9). A similar level of positive pairwise correlations (221 of 800) and negative correlations (189 of 800) were found. In general, higher levels of EC, $CaCO_3$, silt, Cu_T, As_T, Pb_T, Cu_W, Zn_W, As_W, Pb_W, and Zn_EDTA exercised mostly a stimulating effect on the dominant genera, whereas pH, SOC, clay, sand, CIC, Zn_T, Cu_EDTA, As_EDTA, and Pb_EDTA were more related to a decrease of the RA of the dominant genera.

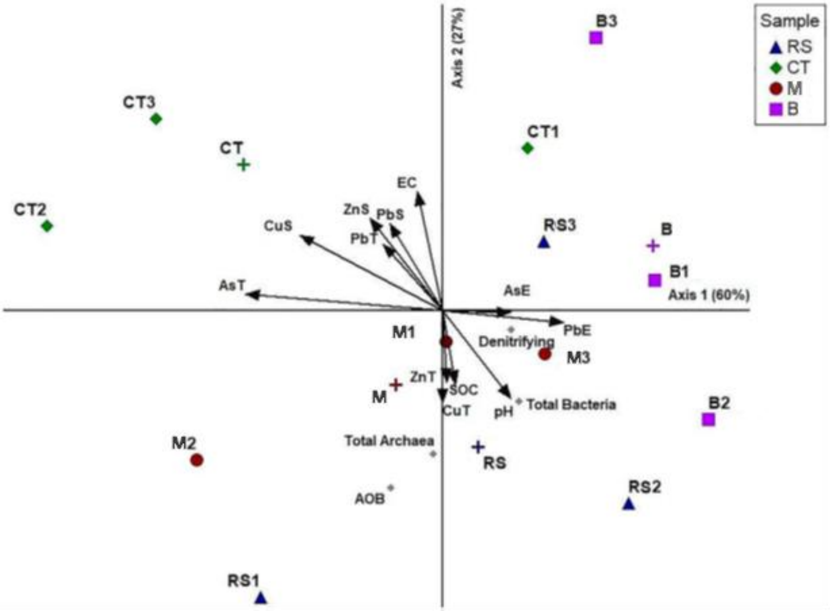


Figure 4.5. Non-metric multidimensional scaling analysis (NMS) of the ordination of the soil samples according to the copy numbers of 16S rRNA of *Bacteria* (total bacteria) and *Archaea* (total archaea) and functional marker genes bacterial *amoA* (AOB) and *nosZ* (denitrifiers) genes per gram of soil represented by grey diamonds. Physicochemical properties (pH; SOC: soil organic carbon (g kg^{-1}); EC: electrical conductivity (dSm^{-1}) and trace elements (CuT, PbT and AsT: total Cu (Cu_T), CuS, ZnS and PbS:water soluble Cu (Cu_W), Zn (Zn_W), Pb ((PB_W); AsE and PbE: bioavailable EDTA As (As_EDTA) and Pb (Pb_EDTA) with a magnitude higher than 0.5 are shown. Recovered soils (RS) - blue triangles; control soils (CT) - green diamonds; limed soils (M) - red circles; biopile soils (B) - purple squares. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

4.3.4.4. Linking the physicochemical properties and biotic variables to basal respiration, root elongation, and germination rates

Finally, Spearman's correlation analyses were employed to explore the relationships among basal respiration, root elongation, and germination rate, physicochemical soil properties, pollutants fractionation, and biotic variables (Figure S4.3, Table S4.10). There were several correlations among the toxicity bioassays and microbiological activity and the remaining variables determined in this study. The results of the bioassays were positively correlated to pH, organic carbon content, and cation exchange capacity. However, electrical conductivity was negatively correlated with all bioassays, with this parameter being significantly lower in RS where bioassays showed higher basal respiration rate and root elongation. There were also large number of negative correlations between potentially harmful elements and the results of the bioassays, especially with the germination rate. On the other hand, bioassays data had a positive correlation with the abundances and diversity of microbial populations and, also, to the RA of the dominant bacterial genera with the exception of *Cutibacterium* which, in higher abundances, had a negative effect on root elongation and basal respiration.

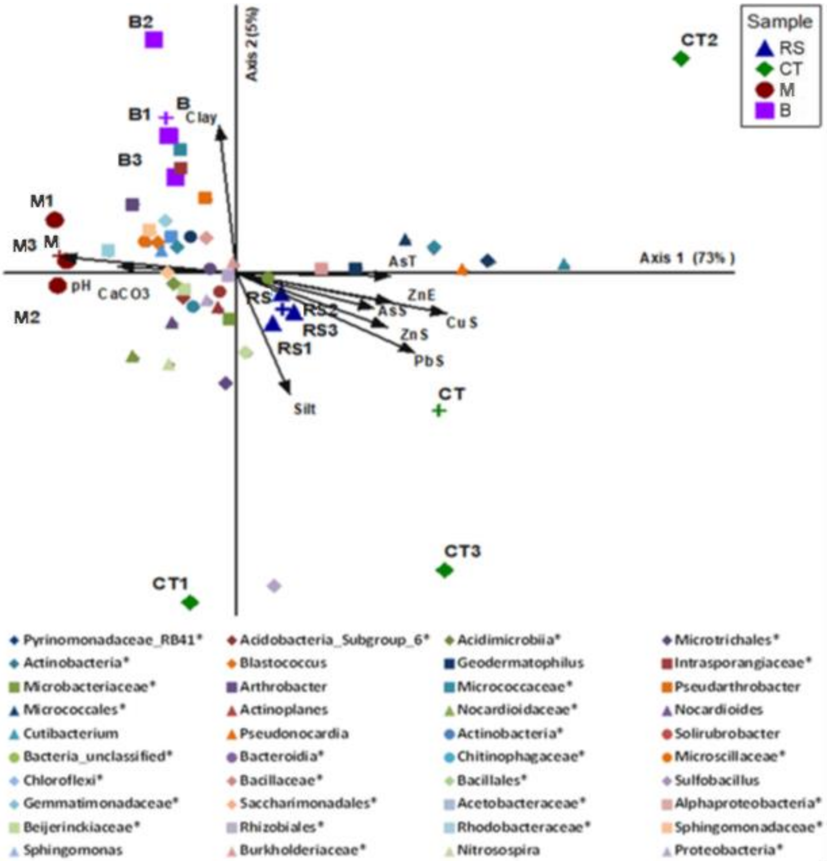


Figure 4.6. Non-metric multidimensional scaling analysis (NMS) of the ordination of the soil samples according to the relative abundances of dominant bacterial genera (RA>0.5%). Physicochemical properties (pH; $CaCO_3$ (%); Silt (%); Clay (%)) and trace elements (PbS, ZnS and AsS water soluble Pb (Pb_W); Zn (Zn_W) and As (As_W); AsT: total As (As_T); ZnE: bioavailable EDTA Zn (Zn_EDTA) with a magnitude higher than 0.5 are shown. Recovered soils (RS) - blue triangles; control soils (CT) - green diamonds; limed soils (M) - red circles; biopile soils (B) - purple squares. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

4.4. Discussion

4.4.1. Pollutant behavior and soil properties

The accident that occurred in the Aznalcóllar mine (1998) resulted in huge soil contamination, both in its extent and in the concentration of potentially harmful elements. Despite the recovery tasks carried outby the Regional Government of Andalusia and two decades later, there are still randomly scattered areas where the high contamination of the soil persists, and these can be identified by the absence of vegetation. According to Romero-Freire et al. (2015b), these areas with contaminated bare soil areas represent approximately 7% of the 43 km^2 area affected by the spill. In these contaminated bare soils, Cu, Zn, As, and Pb are the elements present in high concentrations, which may represent an environmental risk, due to their solubility and long-term bioavailability. Moreover, a spread of pollutants from the most polluted soils to the least polluted ones can occur, likely related to surface runoff water described in the area as being due to the torrential nature of rainfall associated with the Mediterranean climate (Simón et al. 2005a). This is in agreement with García-Carmona et al. (2019a) who reported in the contaminated bare soil concentrations of Cu, Zn, As, and Pb that exceeded 3, 2, 19, and 10-fold values respective to the background values established in the area by Simón et al. (1999). However, our results show even higher total, soluble, and bioavailable (EDTA-extracted) concentrations (Table 4.2) than those reported by García-Carmona et al. (2019a) and Sierra Aragón et al. (2019). These variations reveal the high heterogeneity of both the initial contamination (Cabrera et al. 1999; López-Pamo et al. 1999) and the effectiveness in the sludge remotion tasks and in the addition of the amendments (Simón et al. 2008a).

Pastor-Jáuregui et al. (2020a) monitored the Guadiamar Green Corridor soil contamination and observed that 13% and 70% of the studied soils exceeded the guideline values proposed for soil protection established by the Regional Government of Andalusia for Pb (275 mg kg^{-1}) and As (36 mg kg^{-1}), while Cu and Zn remained below their threshold values (595 mg kg^{-1} for Cu; 10,000 mg kg^{-1} for Zn). Similarly, in the soils analyzed in this work, the total concentrations of As and Pb exceeded 13 and 3 times the guidelines values for Andalusia, but Cu and Zn remained below their thresholds. This is due to the higher solubility of Cu and Zn under acidic conditions with a strong leachate of both elements through the soil by infiltration waters and precipitation at depth, reducing their concentration in the topsoil, while the lower solubility of As and Pb involves their accumulation in the topsoil (Kraus and Wiegand 2006; Simón et al. 2008a).

It is well known that the toxicity of potentially harmful elements in the soil is controlled by their bioavailability instead of their total concentrations. Water soluble pollutants can be freely taken up and get to the endpoints where they may pose a risk to target organs of living organism (Bagherifam et al. 2019) or they can be bioaccumulated in plants, posing a risk due through their potential incorporation into the food chain. For example, pollutants that are encapsulated, insoluble, or strongly bound to solids may not be prone to biological uptake or cause a biological response, while chemicals that are dissolved may be readily available (Kuppusamy et al. 2017).

Soil properties are the main factors affecting metal bioavailability in the environment (Sheppard and Evenden 1988). The solubility of Cu, Zn, As, and Pb significantly decreased in the amended soils (M and B) compared to the control soil (CT) and the recovered soil (RS) (Table 4.2). The decrease in the solubility of the studied elements is correlated with the increase in the pH of the amended soils. This increase in the soil pH is due to the mixing of the CT soil (pH=3.5) with the RS soil (pH=6.6) in B soils, whereas in limed soils with marble sludge it is due to the alkaline reaction caused by the addition of calcium carbonate to the soil solution.

The water solubility of Zn in soils is strongly related to soil pH, reaching a greater mobility under acidic conditions, while slightly acidic to neutral soil conditions enhance precipitation as insoluble forms (Rieuwerts et al. 1998). Although some authors have reported that the solubility of Cu is less sensitive to soil pH changes than that of Zn (Rieuwerts et al. 1998), a strong inverse correlation has been found between pH and water soluble Cu concentrations in our results (Figure S4.1), which is in agreement with those reported by Simón et al. (2005a), Martín-Peinado et al. (2015), and García-Carmona et al. (2019b).

According to Bur et al. (2012), Ming et al. (2012), and Romero-Freire et al. (2015b), among others, Pb solubility is highly influenced by the soil pH and calcium carbonate content, and therefore it has been reported that this influence increases with increasing Pb concentration in soils. Our results show that Pb solubility is inversely correlated (p<0.05) to both soil pH and calcium carbonate (Figure S4.1), which explains the lower Pb soluble concentration in limed and biopile soils compared with the control and recovered soils.

As is described as one of the few metalloids that can move within a pH range from 6.5 to 8.5 under oxidizing soil conditions (Dzombak and Morel 1990). However, in our soils, soluble As concentration is inversely correlated to soil pH and calcium carbonate content (Figure S4.1), leading to a reduction in the concentration of soluble As in the amended soils, where the pH ranges from 5.6 to 6.9 (Table 4.1). The increased solubility of arsenic when the soil pH exceeds normal values may be due to its desorption to iron oxides and/or organic matter (Wang and Mulligan 2006; Klitzke and Lang 2009), but our soils have a low organic matter content, and only limed soils are pH neutral. In fact, Simón et al. (2010) indicated that it is necessary to control soil liming as an amendment to control the mobility of metals in contaminated soils when arsenic is present, and a neutral or slightly alkaline pH below 6.5 should be maintained.

The water soluble metal fraction represents their actual bioavailability, while different authors use the fraction extracted with EDTA as an indicator of the long-term availability of metals (Labanowski et al. 2008; Hurdebise et al. 2015). The limed soils are those where the highest reduction in the EDTA extractable concentration of all four elements analyzed was measured. The addition of carbonates in soils affected by the oxidation of pyrite tailings increases the pH of the soils and encourages the co-precipitation of metals as complex iron sulphates and hydroxysulphates, especially at slightly acidic pH values, around 6.3 (Simón et al. 2010). This occurs with greater intensity in the case of Cu and Zn according to Simón et al. (2005a), who worked with soils contaminated by the Aznalcóllar mine spill. This explains the significant inverse correlation found between the content of calcium carbonate and the EDTA extractable concentration of Zn, although it does not reach the statistical significance in the case of Cu. However, EDTA extractable Cu, As, and Pb are significantly correlated to the cation exchange capacity of the soils and with the soil organic carbon. The cation exchange capacity is closely related to the organic matter of the soil, and the latter is recognized as one of the most important soil parameter governing Cu, As, and Pb bioavailability according to numerous authors (Coppola et al. 2010; Kabata-Pendias 2010; Minkita et al. 2013; Romero-Freire et al. 2014 and 2015b).

4.4.2. Microbial abundance and diversity in heavy metal contaminated soils

It is well known that an excess of heavy metals in soils interferes with soil microbial activity, plant growth and the metals can become concentrated in food chains, and finally affect the health of animals and humans (Torsvik et al. 1996; Swati and Hait 2017) due to their accumulation in cells (Abdu et al. 2017; Bazzi et al. 2020).

In a contaminated soil, microbial biomass (Shukurov et al. 2014) and the taxonomic diversity of soil communities (Stefanowicz et al. 2008) can both be reduced. This can affect a variety of soil microbial processes, and so disturb their nutrient cycling and capacity to perform key ecological functions, such as mineralizing organic compounds and synthesizing organic substances (Giller et al. 1998; Moreno et al. 2009). Additionally, they can be used as soil quality indicators in ecological risk assessments as key endpoints to follow their toxicity over time (White et al. 1998; Frey et al. 2006).

The treatments applied to the soils influenced the abundance of the studied microbial groups (Figure 4.3). In general, there were higher gene copies in the RS soil, followed by the M and B soils and finally the non-amended soil. Except for the CT soils, the gene abundances fell within the results previously described in riverine soils from North Dakota (Ligi et al. 2014) and agricultural soils from Scotland (Yao et al. 2013).

Soil microorganisms participate in different soil biochemical processes, which mainly include organic matter decomposition nitrogen metabolism, and the maintenance of soil structure, and they can be used as bioindicator of the soil quality (Giller et al. 1998). An adequate abundance of them is crucial to the functioning of any terrestrial ecosystem. Therefore, our results indicated that the different amendment treatments restored the quality of the soil as both the abundance of total *Bacteria* and *Archaea* and the populations involved in the N-cycle were restored to an optimal level similar to the values found in the RS soil (Figure 4.3).

Previous studies in sites contaminated with heavy metals have focused on the impact of different kinds of amendments on soil microorganisms. Heavy metals contamination can negatively affect both microbial abundance and microbial community structure and diversity (Deng et al. 2015). Similarly, Wang et al. (2018) found that the presence in higher concentration of some heavy metals such as Cd resulted in the inhibition of the AOB populations in an experimental field of Taian (China).

In general, the bacterial structure at the phylum level (Figure 4.4A and Table S4.6) was in agreement with those previously described in grasslands (Zhang et al. 2017; Zhao et al. 2019) and paddy soils (Wang et al. 2017). Despite that the structure of both archaeal and bacterial community structures in the non-recovered soil (CT) greatly differed from those observed in the other soils (RS, M, and B), at the phylum level the bacterial community structure was recuperated 20 years after the Aznalcóllar mine accident. Pérez-de-Mora et al. (2006b) studied the effects of different organic (municipal waste compost, biosolid compost, leonardite, and litter) and inorganic amendments (sugar beet lime) in the same site and concluded that the use of these substances was an appropriate method to increase soil pH and decrease heavy metal solubility, which improved soil microbial diversity.

Kuppusamy et al. (2016) found that *Proteobacteria* thrived in soils contaminated with polycyclic aromatic hydrocarbons (PAHs) and heavy metals suggesting that they are well-adapted to these stresses. A higher level of *Proteobacteria* was found in the CT soils studied here but the difference was not statistically higher than the values observed for the other soils (Table S4.6). Hence, in general the liming (M) and biopile (B) treatments produced changes in the microbial structure making it in some way more similar to the observed in the recovered soil.

At the genus level (Figs. 4.4B and S2 and Table S4.7), the structures of the bacterial community among soils were more different than at the phylum level. The bigger differences were observed in the CT soils that clustered apart from the other soils. This suggests that the heavy metal contamination resulted in a disturbance of bacterial diversity that needed more time to reach the structure of the RS soils. The genera *Bacillus*, *Staphylococcus*, *Planococcus*, and *Vibrio* have been proposed as heavy metal contamination resistant in marine sediments (Kannan et al. 2007; Zampieri et al. 2016). However, in this study (Table S4.7) these genera were a minority indicating that they are not pivotal players in the bacterial structure in the Aznalcóllar soils. It could mainly be because these populations were not hugely abundant before the Aznalcóllar accident.

It is important to note that at the genus-level a great fraction of sequences was not classified within a true taxonomic genus (Table S4.7). In this sense, Li et al. (2020) found similar results in farmland soils contaminated with different heavy metals as the ones observed here. This suggests that the diversity of soils contaminated with different heavy metals is not well known and more advances are necessary to understand how that type of pollution affects the microbial biodiversity in soils.

4.4.3. Relationship between bacterial community and environmental factors

In the present study, the abundance and composition of microbial communities were influenced by several physicochemical factors as revealed by NMS analysis (Figs. 4.5 and 4.6). Based on NMS, both abundance and composition showed a distinguishable pattern among treatments, with the CT soils placed independently in both NMS. These results were in agreement with the results above described. Therefore, microbial community abundance and structure were strongly impacted by the amendment treatment.

The amendments applied to the contaminated soils produced an improvement in soil quality: an increase in pH, organic matter, and cation exchange capacity, together with a decrease in the salinity and the solubility of Cu, Zn, As, and Pb, stimulating the abundance of total bacteria and archaea and AOB populations (Figure 4.5 and Table S4.8). On the other hand, different genus-like groups were found in increased concentrations when a higher level of heavy metal was found and, subsequently, it can be proposed that these bacterial groups are resistant to heavy metal contamination in the Aznalcóllar soils (Figure 4.6 and Table S4.9). In the current study, Acetobacteraceae*, Actinobacteria*, *Arthrobacter*, *Blastococcus*, Chloroflexi*, Gemmatimonadaceae*, Microscillaceae*, Sphingomonadaceae*, and *Sphingomonas* were positively correlated to some heavy metal contents and, consequently, they were the more adapted groups to heavy metals. On the other hand, As and Pb EDTA fractions presented a higher number of negative correlations suggesting that the long-term bioavailable As and Pb were more toxic than other types of fractions or metals.

4.4.4. Effects of remediation treatments on toxicity bioassays

The recovery of contaminated soils is essential to avoid the degradation of the environment, and limit the exposure of humans and other living organisms to potentially harmful compounds (Mulligan et al. 2001; Pavel and Gavrilescu 2008). Residual contamination and toxicity by heavy metals in polluted soils are generally evaluated using bioassays (García-Carmona et al. 2017).

Bioassays measure the response of organisms exposed to contaminated soil and are indicative of their toxicity, which depends on their bioavailability (Petänen et al. 2003).

In the toxicity bioassays performed, it was expected that each organism would respond differently according to their own sensitivity and to different pollution levels (Martín-Peinado et al. 2012). In this study, toxicity bioassays have shown different responses according to the amendment applied (Figs. 4.1 and 4.2). The amendments applied to soils have strongly modified pollutants concentrations, with a significant influence in water soluble and long-term availability (EDTA extract) concentrations (Table 4.2). However, although biopile soils have shown a significant smaller decrease in soluble Cu, Zn concentration, and the long-term availability Cu, Zn, As, and Pb, compared with limed soils; the latter were more sensitives to the root elongation of lettuce seed, but no differences were found in lettuce seed germination tests. According to Bagur-González et al. (2011), the *Lactuca sativa* root elongation bioassay is more sensitive and a better estimator of soil phytotoxicity than seed germination. Our results from the root elongation bioassay (Figure 4.1) are consistent with those reported by García-Carmona et al. (2017), obtaining elongation values in biopile soil 1:1 (contaminated bare soil:recovered soil) in in vitro experiments very similar to those from the recovered soil.

Apart from the total Cu and EDTA soluble Pb, the remaining heavy metal contents were negatively correlated with both root elongation and germination rates (Figure S4.3), confirming the previously described negative effect of heavy metals on plant development (Park et al. 2016; Lyu et al. 2018). On the other hand, higher levels of abundance and diversity were linked with greater root elongation. Different mechanisms have been proposed as traits that promote root elongation and germination rate such as high chitinase, protease, antifungal activities, or indole acetic acid production (Afzal et al. 2017; Danish and Zafarul-Hye 2019). Similarly, the high presence of each one of the dominant bacterial genera were positively correlated to both root elongation and germination rate suggesting their potential role as plant growth promoting rhizobacteria (PGPR) (Figure S4.3 and Table S4.10). An exception was the RA of *Cutibacterium* which was negatively correlated to plant growth capacity. This genus is mainly composed by the *C. acnes* specie (formerly known as *Propionibacterium acnes*) that turn into opportunistic pathogens of *Acne vulgaris* when they acquire some lineage-specific genetic elements (Scholz and Kilian 2016). Although these bacteria could be provided to some PGPR traits (Wani and Gopalakrishnan 2019) in the current work, they were negatively linked with root elongation. To the best knowledge, this is the first work that highlights their detrimental role in plants as is also observed for humans.

On the other hand, the basal soil respiration bioassay uses the soil heterotrophic respiration rate as a soil quality indicator related with soil microbiological activity (Romero-Freire et al. 2016b). According to our results, the amendments applied to the contaminated soil gave rise to a significant increase in soil microbiological activity determined as a basal respiration rate (Figure 4.2). This is related to a decrease in the availability of contaminants in the amended soil, as well as the improvement of the soil properties, especially due to the increase in the pH of the soil as a result of the liming, and the increase in soil organic carbon of the biopile soils as was described earlier. Organic matter can decrease the availability of metals in the soil (Kabata-Pendias 2010; Alloway 2013) and allows greater microbial development and greater basal soil respiration (Nwachukwu and Pulford 2011). Moreover, higher organic matter content usually implies greater availability of nutrients coming from the mineralization of organic compounds for coping with the stress related to metal pollution, allowing a higher microbial growth rate (Romero-Freire et al. 2016b). However, in both biopile and limed soils, soil pH and calcium carbonate contents control the metal bioavailability and its toxicity, and according to Azarbad et al. (2013) this has an effect on the soil-respiration response. In this sense, Romero-Freire et al. (2016b) reported that among different soils artificially contaminated with metals, those with higher calcium carbonate contents or with neutral to alkaline soil pH had a lower reduction in soil respiration rate compared to the same uncontaminated soils.

Similarly to the results observed for the root elongation and germination rate, high levels of heavy metals inhibited the soil microbiological activity and, on the other hand, higher bacterial abundance and diversity stimulated the soil respiration (Figure S4.3). Therefore, the properties that stimulated the root elongation and soil respiration were the same, highlighting the link between the pollutant's concentrations, bacterial communities, and the toxicity bioassays.

4.5. Conclusions

Two decades after the spill of pyritic sludge and acidic waters which occurred in the Aznalcóllar mine in 1998 polluting the Guadiamar river basin, soils with high levels of contamination, identified by the lack of vegetation, still persist in the affected area. A restoration of these contaminated soils needs to be addressed in order to prevent a continuous spread of the contamination using effective, low-cost, and environmentally friendly soil restoration techniques. The addition of the residue from the cutting of the marble stone to the contaminated soil or the mixture of the contaminated soils with nearby recovered soils have resulted in a decrease in the bioavailability of the potential harmful elements, significantly reducing the soil toxicity and improving the soil quality. A year and a half after the application of the liming and biopile restoration techniques, an increase of the pH, the organic matter content, and the cation exchange capacity occurred as well as a reduction of soil salinity. The decrease in the solubility of heavy metals in the amended soils, and hence in the soil toxicity, is mainly related to the increase in pH and organic matter in limed and biopile soils, respectively.

Microbial community abundance and structure were strongly impacted by the amendment treatments. Bacterial structure at the genus level was highly different among soils suggesting that this taxonomy level is the optimal to obtain a better demonstration of the disturbance posed by heavy metals to soil bacterial communities. Acetobacteraceae*, Actinobacteria*, *Arthrobacter*, *Blastococcus*, Chloroflexi*, Gemmatimonadaceae*, Microscillaceae*, Sphingomonadaceae*, and *Sphingomonas* were more abundant in the contaminated soil compared to the amended and recovered soils and, therefore, they were well adapted groups to heavy metals in Aznalcóllar contaminated soils.

The uses of liming or biopile were satisfactory remediation techniques to reduce the disturbance of heavy metals in the ecosystem. Taking all these results into account, this study highlights the narrow relationships among soil properties, microbial activity, and toxicity bioassays as tools for the evaluation of soil quality and for the planning of restoration measures in soils contaminated by heavy metals.

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Chapter 5

Integrating waste valorization and symbiotic microorganisms for sustainable bioremediation of metal(loid)-polluted soils

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Abstract

Remediation strategies for metal(loid)-polluted soils vary among the wide range of approaches, including physical, chemical, and biological remediation, or combinations of these. In this study, we assessed the effectiveness of a set of soil remediation treatments based on the combined application of inorganic (marble sludge) and organic amendments (vermicompost, and dry olive residue [DOR] biotransformed by the saprobic fungi *Coriolopsis rigida* and *Coprinellus radians*) and inoculation with arbuscular mycorrhizal fungi (AMFs) (*Rhizophagus irregularis* and *Rhizoglomus custos*). The treatments were applied under greenhouse conditions to soil residually polluted by potentially toxic elements (PTEs) (Pb, As, Zn, Cu, Cd, and Sb), and wheat was grown in the amended soils to test the effectiveness of the treatments in reducing soil toxicity and improving soil conditions and plant performance. Therefore, we evaluated the influence of the treatments on the main soil properties and microbial activities, as well as on PTE availability and bioaccumulation in wheat plants. Overall, the results showed a positive influence of all treatments on the main soil properties. Treatments consisting of a combination of marble and organic amendments, especially biotransformed DOR amendments, showed the greatest effectiveness in improving the soil biological status, promoting plant growth and survival, and reducing PTE availability and plant uptake. Furthermore, AMF inoculation further enhanced the efficacy of DOR amendments by promoting the immobilization of PTEs in soil and stimulating the phytostabilization mechanisms induced by AMFs, thus playing an important bioprotective role in plants. Therefore, our results highlight that biotransformed DOR may represent an efficient product for use as a soil organic amendment when remediating metal(loid)-polluted soils, and that its application in combination with AMFs may represent a promising sustainable bioremediation strategy for recovering soil functions and reducing toxicity in polluted areas.

Keywords: soil pollution; soil bioremediation; waste valorization; biotransformed dry olive residue; arbuscular mycorrhizal fungi; phytostabilization

5.1. Introduction

Soil, which is an essential element of ecosystems, has great regulatory potential to respond to all types of pollution and protect other ecosystem elements such as water, air, and living organisms (Adhikari and Hartemink 2016). However, the resilience and protective capacity of soil are finite, and intense or continuous degradation processes over time can exceed its buffering capacity and prevent the performance of one or more of these functions, leading to serious damage to ecosystems and humans (Lal 1997; Seybold et al. 1999). Soil pollution has become an increasingly pressing issue in recent decades, with the main cause being associated with human activities, such as mining, which have resulted in the accumulation of pollutants in soils to levels that may be of concern (Hu et al. 2013; Cachada et al. 2018). Active soil remediation is required in scenarios involving extensive and severe pollution.

The revalorization of waste materials through their use as soil amendments can represent an effective, low-cost, and ecological solution to remediate polluted soils (González-Núñez et al. 2015), as it converts waste into essential resources, thereby improving soil properties. This may simultaneously help mitigate a major global problem representing the increasing generation of waste from human activities, which constitutes a serious environmental and management challenge (Abdel-Shafy and Mansour 2018; Ferronato and Torretta 2019). Techniques based on the addition of inorganic and organic amendments to facilitate the natural remediation processes of soils damaged or polluted with potentially toxic elements (PTEs) represent a viable and widely recognized strategies for soil remediation (Bolan and Duraisamy 2003; Guo et al. 2006; Raklami et al. 2021).

A wide variety of materials and by-products have been used as soil amendments (Adriano et al. 2004; Pérez-de-Mora et al. 2006a). Marble-stone waste sludge generated during processing has been used and tested by several authors, showing promising results as an amendment for assisting the natural remediation of heavily polluted acidic mine soils and for reducing PTE toxicity risks (Pérez-Sirvent et al. 2007; Fernández-Caliani and Barba-Brioso 2010; González et al. 2017). Considering that marble is the most widely produced natural stone in the world and that at least 20-30% of marble is turned into powder during cutting, large quantities of this low-cost waste material are produced annually and must be sustainably managed (Alyousef et al. 2019). Moreover, organic wastes, such as compost or those produced by the agri-food industry, can be used as soil amendments owing to their high organic matter content, which enhances the soil pH and microbial structure as well as reduces PTE bioavailability in metal-polluted soils, thereby restoring soil quality and functionality (García-Sánchez et al. 2015, 2019). Among the different organic amendments used in recovering metal-polluted soils, the promotion of dry olive residue (DOR) is of great interest because this residue is produced by the olive oil industry in very large quantities, which is a major concern for this sector, especially in Mediterranean countries where this industry is of great importance at social and economic levels (Tortosa et al. 2012). However, the direct application of DOR to soil can also increase metal availability and negatively impact soil microorganisms and plant growth owing to its phytotoxicity, which is mainly caused by the high presence of phenolic compounds and other substances such as fatty acids (García-Sánchez et al. 2012; Siles et al. 2014a). Therefore, transformation of DOR prior to its application to soil is required. The inoculation with saprobe fungi, which are able to both stabilize waste and degrade phytotoxic compounds such as phenols, is a rapid and effective technique for reducing the phytotoxic effects of DOR, which facilitates its use as an organic amendment (Sampedro et al. 2005, 2009; Aranda et al. 2006). The application of DOR mycoremediated by these fungi has positive effects on plant growth, increases microbial diversity, and decreases metal bioavailability (Siles et al. 2014b; Hovorka et al. 2016; Reina et al. 2017). However, García-Sánchez et al. (2017) highlighted the need for further research on the potential remediation effectiveness of biotransformed DOR in polluted soils, as well as on the combined application of this material with other element-stabilizing materials, as its potential effect on the mobility and plant availability of risk elements has not been widely investigated.

The application of arbuscular mycorrhizal fungi (AMFs) is another strategy for the remediation of soils polluted by PTEs, because AMFs can provide plant protection against adverse elements through their immobilization, extraction, and concentration in their tissues (Arriagada et al. 2009; Meier et al. 2012; Leung et al. 2013). AMFs occur extensively in polluted soils, and evidence suggests that they improve plant nutrition and tolerance to excess trace elements (Janoušková et al. 2005; Cabral et al. 2015). In particular, the toxicity of PTEs in plants and the environment may be mitigated by the high metal sorption capacity of mycorrhizal mycelia and their ability to transfer and sequester excess elements into vacuoles and root cell walls, along with the retention of metal ions in chemically inactive complexes (Colpaert et al. 2011;

Shi et al. 2019). In addition, the combination of AMFs with organic amendments may facilitate the successful establishment of the soil microbial community and full restoration of soil function, while also improving AMF PTE stabilization (García-Sánchez et al. 2017, 2019). Consequently, AMFs represent an important tool used for the recovery of polluted sites (Leung et al. 2006; Cabral et al. 2015), and bioremediation approaches based on microorganisms should be considered, as they may complement or replace conventional methods by enhancing the effectiveness of the processes involved (Sepehri et al. 2020).

We hypothesized that the combined application of amendments and AMF inoculation is a promising remediation strategy for the recovery of soil function in PTE-polluted areas. Therefore, the general objective of this study was to evaluate the effectiveness of soil remediation treatments based on inorganic (marble sludge) and organic (vermicompost and DOR amendments biotransformed by the saprobic fungi *Coriolopsis rigida* and *Coprinellus radians*) combined with the inoculation of AMFs (*Rhizophagus irregularis* and *Rhizoglomus custos*) applied to metal(loid)-polluted soil (Pb, As, Zn, Cu, Cd, and Sb).

5.2. Materials and methods

5.2.1. Soil sampling and materials

Residual polluted soil from the Aznalcóllar metal mining spill affected area (Seville, SW Spain) was sampled for use in the experimental set up (37°29'36"N, 6°13'14"W). The spill accident occurred in 1998 after the collapse of the tailings dam of the mine, which produced a spillage of approximately 4,5 x 10^6 m^3 of acidic water and toxic tailings containing high concentrations of PTEs), affecting an area of over 43 km^2 (Grimalt et al. 1999; Simón et al. 1999). A detailed map of the sampling locations is available in Figure S5.1. The area is characterized by heterogeneously distributed unvegetated soil patches of different sizes with high concentrations of several PTEs (especially Pb, As, Zn, Cu, Cd, and Sb). Composite soil samples were obtained from these unvegetated patches by mixing 200 g of topsoil (0-10 cm) from each corner and the center of a square of 1 m per side. Individual soil samples were thoroughly mixed and homogenized to obtain a single representative soil sample. Finally, the soil was air-dried at room temperature in the laboratory, sieved (<2 mm), and stored until its use.

Polluted soil was amended with inorganic and organic waste materials. Marble sludge, from the mining area of Macael (Almería, SE Spain), which consisted of waste material from the cutting and polishing of marble stones, was used as an inorganic amendment. Two organic amendments were used: commercial vermicompost provided by Lombricor SCA (Algallarín, Córdoba, Spain), and DOR, which was used as an organic amendment after transformation with two saprobic fungi. This last amendment was supplied by the olive oil manufacturer Aceites Sierra Sur S.A. (Granada, Spain), sieved, autoclaved over three cycles (121 ºC for 20 min), and stored at 4 ºC prior its biological transformation.

5.2.2. Biological transformation of DOR (mycoremediation)

The DOR used as an organic amendment was transformed by two different saprobic fungal species: *Coriolopsis rigida* (EEZ-92), and *Coprinellus radians* (EEZ-84). The fungi were pre-cultured on a 2% malt extract agar plates for two weeks at 28 ºC to maintain a fresh inoculum. Fungal transformation of the DOR was performed under solid-state fermentation conditions to accelerate the ability of the fungi to undergo DOR transformation, as described by Reina et al. (2013). For this purpose, the fungi were first pre-cultured in barley-based media (18 g of barley seeds and 30 mL of sterile water) for one week. Subsequently, sterilized DOR (50% w/w) was added to a barley-seed media inoculated with fungi, moistened with sterile water, and incubated at 28 ºC for four weeks. Non-inoculated barley-based media with sterilized DOR were also incubated and used as controls. The incubation was heat-inactivated by autoclaving and the DOR samples were sieved (<5 mm). Finally, the remaining barley seeds were manually removed, and the samples were manually homogenized and stored at 4 ºC.

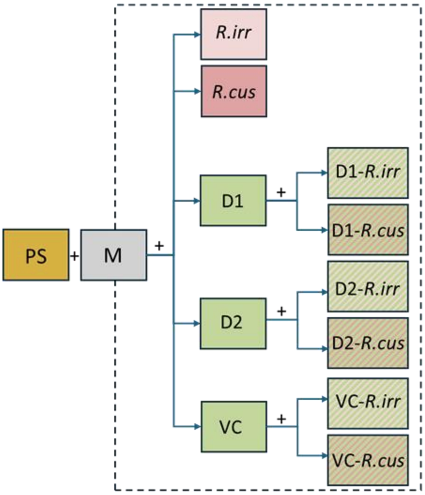
5.2.3. Arbuscular mycorrhizal fungi (AMFs) inocula

The AMFs used in this experiment were *R. irregularis* DAOM 197198 (*Glomus irregulare* DAOM 197198), formerly known as *Glomus intraradices*, and *R. custos* MUCL47214. Both are known for their ability to support plant survival and development in mining soils (Silva-Castro et al. 2022). The model fungus *R. irregularis* was used as the control. *R. custos* was originally isolated from the banks of the Tinto River near Nerva (Huelva, southern Spain), a mining area located in the Iberian Pyrite Belt, which also comprised our study site, where high concentrations of PTEs occur naturally (Cano et al. 2009). AMF inoculation was carried out by adding a 1-cm^3 cube of AM-in-vitro-issued inoculum (Cano et al. 2008) containing high amounts of AM propagules (spores, active extraradical hyphae, and aseptic root pieces colonized by AM intraradical mycelia) from the different AM used. AM inoculation was performed during seedling transplantation, and special care was taken to ensure that fungal propagules were in close contact with the roots, thus inducing rapid colonization.

5.2.4. Experimental design and set up

A model pot experiment was set up to evaluate the role of the selected amendments and AMF and their different combinations, as a potential strategy for immobilizing PTEs present in soil and for improving soil properties and quality. The experiment was set up in a series of identical polypropylene pots, each with a volume of 0.3 L. Approximately 300 g of polluted soil was placed in each pot. The experimental design consisted of a randomized factorial system with three variation factors. The first experimental factor consisted of two levels: polluted-soil application with either the inorganic amendment (marble sludge) or its application with both the inorganic amendment and an organic amendment or AMF. The second factor was the organic amendment type and comprised three levels: DOR mycoremediated by *C. rigida*, DOR mycoremediated by *C. radians*, and vermicompost. The third factor included AMF inoculation comprising two levels: soil non-inoculated and soil inoculated with the two AMF (*R. irregularis* and *R. custos*). In total, thirteen treatments (n=13), resulting from the combinations of the polluted soil with the different amendments and AMF (Figure 5.1), were established and tested as follows: 0) polluted soil (PS); 1) polluted soil + marble sludge addition (M); 2) M + inoculation with *R. irregularis* (*R.irr*); 3) M + inoculation with *R. custos* (*R.cus*); 4) M + DOR mycoremediated by *C. rigida* (D1); 5) M + DOR mycoremediated by *C. radians* (D2); 6) M + vermicompost (VC); 7) M + D1 + inoculation with *R. irregularis* (D1-*R.irr*); 8) M + D1 + inoculation with *R. custos* (D1-*R.cus*); 9) M + D2 + inoculation with *R. irregularis* (D2-*R.irr*); 10) M + D2 + inoculation with *R. custos* (D2-*R.cus*); 11) M + VC + inoculation with *R. irregularis* (VC-*R.irr*); 12) M + VC + inoculation with *R. custos* (VC-*R.cus*). Five replicates were performed for each treatment.

The inorganic and organic amendments were applied and manually mixed with the soil. The marble and vermicompost amendments were applied at a rate of 5% (v/v) following previous studies (Paniagua-López et al. 2023), whereas the DOR amendments were applied at a rate of 8% (v/v) according to preliminary tests. The soil moisture was brought to 60% of the soil water-



	ACRONYM	TREATMENT DESCRIPTIONS
0	**PS**	Polluted soil (control -)
1	**M**	Marble sludge addition (control +)
2	***R.irr***	Inoculation with *R. irregularis*
3	***R.cus***	Inoculation with *R. custos*
4	**D1**	DOR mycoremediated by *C. rigida*
5	**D2**	DOR mycoremediated by *C. radians*
6	**VC**	Vermicompost
7	**D1-*R.irr***	D1+inoculation with *R. irregularis*
8	**D1-*R.cus***	D1+inoculation with *R. custos*
9	**D2-*R.irr***	D2+inoculation with *R. irregularis*
10	**D2-*R.cus***	D2+inoculation with *R. custos*
11	**VC-*R.irr***	VC+inoculation with *R. irregularis*
12	**VC-*R.cus***	VC+inoculation with *R. custos*

Figure 5.1. Flow diagram of treatments used and descriptions of their compositions and acronyms. DOR (dry olive residue).

holding capacity. A 10-day-old wheat plant (*Triticum aestivum* L.) was planted in each pot. The experiment was carried out for 45 d under greenhouse conditions (supplementary light at 25/19 °C and relative humidity at 50%), and the plants were regularly watered to maintain the same initial moisture conditions.

After 45 d, soil samples from each pot were collected individually, sieved (<2 mm), homogenized, and subdivided into three subsamples. The first subsample was air-dried at room temperature and used for chemical analyses, the second was maintained at 4 ºC for the analysis of the soil enzymatic activity, and the third was finely ground and used for the determination of the PTE concentrations. Wheat plants were individually harvested, divided into roots and shoots, and then divided into different subsamples to determine the plant biomass, percentage of root mycorrhization, and PTE concentrations in each plant part after homogenization.

5.2.5. Analytical methods

5.2.5.1. Soil analysis

The main chemical properties of the soil in each sample were characterized. Soil pH was determined at a soil:water ratio 1:2.5 with a 914 pH/Conductometer Metrohm (Herisau, Switzerland); a soil:water extract (1:5) was prepared to determine the electrical conductivity (EC, dS m^{-1}) using a

Eutech CON700 conductivity meter (Oakton Instruments, Vernon-Hills, IL, USA); organic carbon (%OC) was determined according to Tyurin (1951); available phosphorous was analyzed according to Olsen and Sommers (1982); and total nitrogen (%N) was analyzed by dry combustion using an elemental analyzer TruSpec CN LECO® (St. Joseph, MI, USA).

Dehydrogenase activity was quantified using a method by Camiña et al. (1998). To determine urease activity, a method described by Kandeler and Gerber (1988) was adopted. Phosphatase and β-glucosidase activities were assessed following methods described by Eivazi and Tabatabai (1977, 1988), respectively.

5.2.5.2. Total and bioavailable concentrations of PTEs in soil samples

The total concentrations of the main PTEs present in the studied soil (Pb, As, Zn, Cu, Cd, and Sb), expressed in mg kg^{-1}, were determined by X-ray fluorescence with a NITON XL3t-980 GOLDD + Analyzer (Thermo Fisher Scientific, Tewksbury, MA, USA). The precision and accuracy of the method were confirmed by analyzing a certified reference material (CRM052-050 RT-Corporation Limited, Salisbury, UK; n=6). The average concentrations of the studied elements ranged from 90% to 110% of the reference material. Moreover, selective extraction of PTEs was performed for all treatments to assess the bioavailability of these elements in the soils. The bioavailable fraction was extracted using 0.05-M EDTA (pH=7) at a soil:solution ratio of 1:10 (w/v) and shaken for 1 h at room temperature, as described by Quevauviller et al. (1998). The PTE concentrations for this extracted form were measured by Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) in a PerkinElmer Avio® 500 spectrometer (Shelton, CT, USA).

5.2.5.3. Plant analysis

After harvesting, the plants were washed, divided into roots and shoots, dried (70 °C for 24 h), and used for the different determination methods. Plant biomass was recorded and expressed as the root and shoot dry weights. The percentage of mycorrhization in the roots was estimated according to the staining methodology described by Phillips and Hayman (1970), and colonization was quantified using a method by Giovannetti and Mosse (1980). Additionally, dry plant materials were finely ground and used to measure the concentrations of PTEs in both the roots and shoots. Acid digestion was performed in a Mars® XP1500 Plus microwave in a HNO_3:H_2O_2 (1:1) mixture for

1 h at 200 °C and 800 W. Thereafter, PTE concentrations were measured using an Avio® 500 ICP-optical emission spectrometer (Shelton, CT, USA).

The bioaccumulation factor (BAF) in the shoots and bioconcentration factor (BCF) in the roots were calculated to determine the uptake of PTEs by wheat plants under each of the applied treatments, which allowed for the determination of whether the PTEs were transported to the aboveground part of the plants or accumulated in the roots. For this purpose, the bioavailable (EDTA-extractable) PTE fraction in the soil samples of each treatment was selected, and both the BAF and BCF were calculated as the ratio between the PTE concentration measured in each part of the plant (shoot or root) (mg kg^{-1} dry weight) and EDTA-the extractable PTE concentration in the soils (mg kg^{-1} dry soil). Finally, the translocation factor (TF) within the plant was estimated as the ratio between the concentration of PTEs in shoots and their concentration in roots, to determine the degree of element migration from the roots to the aboveground part of the plants.

5.2.5. Statistical analysis

Mean values and the corresponding standard deviations of the parameters were calculated for each sample set. After verifying that the data fit a normal distribution, differences between the individual means of the samples were analyzed by one-way analysis of variance as well as post-hoc analysis using Duncan's test. Levene's test was used to check for homogeneity of variance. All analyses were performed at a 95% confidence level using SPSS v.28.0 (SPSS Inc., Chicago, USA). Principal component analysis (PCA) was performed to determine the relationships between the treatments and the PTE concentrations in both the soils and plants by using the Canoco 5.04 software. When necessary, that is, to meet normality assumptions, the data were log-transformed.

5.3. Results and discussion

5.3.1. Effect of amendment and AMF treatments on soil properties

5.3.1.1. Description of soils and amendments

The main chemical properties of the polluted soil and those of the inorganic and organic amendments used in the experiment, were analyzed (Table S5.1). The control polluted soil (PS) was characterized by being highly acidic (pH 3.4) and having a high EC (3.5 dS m^{-1}), as previously discussed in studies carried out in the same study area (Romero-Freire et al. 2016a; Álvarez-Mateos et al. 2019). The marble and vermicompost amendments showed an alkaline and slightly basic pH (9.1 and 7.7), respectively, while the DOR showed a distinctive slightly acidic pH after mycoremediation with *C. rigida* (6.3) (Hovorka et al. 2016), and a more acidic pH after mycoremediation with *C. radians* (5.0). The organic amendments showed elevated ECs in all cases (> 4 dS m^{-1}), although the EC was significantly lower in the DOR mycoremediated with *C. rigida*. Very low contents of (<1%) and N were detected in PS, whereas these were negligible in the marble amendment. In contrast, high OC and N contents were found in the vermicompost and DOR amendments, especially in the second amendment, in which the OC content was three-to-four-fold higher than that in the vermicompost. The organic amendments were also characterized by high available P levels (>200 mg kg^{-1} P), with that in the DOR mycoremediated with *C. rigida* being approximately two-fold higher (>400 mg kg^{-1} P) than those with *C. radians* and the vermicompost. In addition, high K levels were detected in DOR amendments, which were approximately twice as high as those measured in vermicompost. This is an important finding, considering the important role of this element in the stress tolerance of plants (Siles et al. 2015; Johnson et al. 2022).

5.3.1.2. Changes in soil chemical properties

The application of the inorganic and organic amendments and AMF inoculation strongly changed the soil chemical properties (Table 5.1). All treatments induced a sharp increase in the soil pH compared to the control polluted soil (PS), from strongly acidic (pH 3.4) to neutral (pH 7-7.5), mainly because of the great potential of the marble amendment to neutralize soil acidity (Tozsin et al. 2014; Fernández-Caliani et al. 2022). However, the organic and AMF treatments reached a slightly higher pH than the addition of marble alone (M). The treatments also induced a change in EC, which was significantly reduced in all cases compared with that of PS, even though the EC of the organic amendment materials was higher than that of PS (Table S5.1). This general decrease in EC could be attributed to the marble addition, for which a potential in decreasing EC values in soil has been previously reported (Jain et al. 2020a). This is possibly related to a reduction, induced by this fine material, in the porosity of the soil, which then increases its bulk density, thus inversely correlating with the soil EC (Jain et al. 2020b). Among the treatments, the AMF treatments (*R.irr* and *R.cus*), especially D2-*R.cus*, exhibited the largest EC decrease, displaying the capability of AMF to lower the EC in the mycorrhizosphere when high levels of salinity are present (Giri et al. 2003). In relation to the OC content in the soil, the DOR and DOR + AMF treatments, especially those including the AMF *R. custos*, significantly increased the OC content in relation to PS and M, mainly because of the elevated OC content of the DOR amendment. In contrast, treatments consisting of only AMF addition (*R.irr* and *R.cus*), as well as the vermicompost treatments, were not capable of increasing the OC content in the soil. A decrease in N content was observed in most of the treatments compared to that in PS, with the most remarkable decrease being observed under treatments with AMFs alone, as no input of N from organic amendment was added, which also indicated that overall N consumption by biological activity occurred in the treated soils. AMFs may promote the decomposition and subsequent uptake of organic N from organic sources and transfer the N to plants (Leigh et al. 2009; Whiteside et al. 2009). They also enhance the plant uptake of inorganic N from soil and increase N demand owing to the increased amounts of bacteria in the soil (Hawkins et al. 2000; Saia et al. 2014).

Table 5.1. Soil chemical properties after the different amendments and AMF treatments application. PS (control polluted soil); M (marble addition); D1 (dry olive residue mycoremediated by *C. rigida*); D2 (dry olive residue mycoremediated by *C. radians*); VC (vermicompost); *R.irr* (*R. irregularis*); *R.cus* (*R. custos*). EC (electrical conductivity); OC (organic carbon); N (total nitrogen); Pav (available phosphorus). Data are presented as the mean ± standard deviation, n=5.

Treatment	pH	EC (dS m^{-1})	OC (%)	N (%)	Pav (mg kg^{-1})	K (mg kg^{-1})
PS	3.40 ± 0.01 *a*	3.48 ± 0.07 *g*	0.72 ± 0.11 *a*	0.102 ± 0.006 *de*	8.2 ± 2.0 *a*	12.13 ± 0.14 *b*
M	7.06 ± 0.09 *b*	3.25 ± 0.05 *f*	0.72 ± 0.10 *ab*	0.074 ± 0.010 *ab*	19.6 ± 10.3 *abc*	11.13 ± 0.38 *a*
R.irr	7.50 ± 0.04 *d*	3.00 ± 0.03 *b*	0.67 ± 0.17 *a*	0.067 ± 0.007 *a*	34.0 ± 15.7 *bc*	13.00 ± 0.29 *bcd*
R.cus	7.51 ± 0.04 *d*	3.03 ± 0.03 *bc*	0.71 ± 0.13 *a*	0.063 ± 0.003 *a*	25.6 ± 6.2 *abc*	12.56 ± 0.18 *cd*
D1	7.10 ± 0.01 *b*	3.20 ± 0.11 *ef*	1.11 ± 0.23 *cd*	0.092 ± 0.007 *cd*	29.6 ± 13.7 *bc*	12.19 ± 0.62 *b*
D2	7.35 ± 0.05 *c*	3.08 ± 0.06 *bcd*	0.97 ± 0.04 *bc*	0.084 ± 0.011 *bc*	30.0 ± 14.0 *bc*	12.90 ± 0.07 *cd*
VC	7.55 ± 0.03 *d*	3.15 ± 0.02 *def*	0.81 ± 0.11 *ab*	0.074 ± 0.005 *ab*	29.2 ± 10.5 *bc*	12.88 ± 0.13 *d*
D1-*R.irr*	7.29 ± 0.04 *c*	3.14 ± 0.07 *de*	1.00 ± 0.10 *bcd*	0.085 ± 0.006 *bc*	35.9 ± 20.7 *bc*	12.28 ± 0.32 *b*
D1-*R.cus*	7.28 ± 0.12 *c*	3.11 ± 0.05 *cde*	1.10 ± 0.14 *cd*	0.078 ± 0.011 *abc*	38.9 ± 16.6 *c*	12.80 ± 0.14 *cd*
D2-*R.irr*	7.14 ± 0.10 *b*	3.17 ± 0.01 *def*	1.00 ± 0.16 *bcd*	0.077 ± 0.010 *abc*	23.9 ± 17.5 *abc*	12.28 ± 0.15 *b*
D2-*R.cus*	7.28 ± 0.10 *c*	2.76 ± 0.19 *a*	1.19 ± 0.15 *d*	0.084 ± 0.010 *bc*	28.2 ± 8.8 *bc*	12.75 ± 0.58 *cd*
VC-*R.irr*	7.52 ± 0.04 *d*	3.20 ± 0.06 *ef*	0.70 ± 0.11 *a*	0.088 ± 0.018 *bc*	21.7 ± 5.6 *abc*	12.73 ± 0.23 *cd*
VC-*R.cus*	7.46 ± 0.08 *d*	3.18 ± 0.02 *def*	0.70 ± 0.11 *a*	0.106 ± 0.014 *e*	17.0 ± 4.1 *ab*	12.51 ± 0.32 *bc*

Lowercase letters indicate significant differences among treatments according to Duncan's post hoc test (p<0.05).

Meanwhile, significant increases in available P and, to a lesser extent, the K content under the treatments compared to those in PS was also observed. For available P, treatments with DOR application, and especially the D1 combined with both AMFs, induced a greater increase. This could be directly related to the high contents of these elements in the selected organic amendments, especially in the case of the P in DOR1.

5.3.1.3. Soil enzymatic activity

Soil enzymatic activity measured at the end of experiments have been used as a bioindicator of soil quality change and biological activity in soils under the different treatments (Paz-Ferreiro et al. 2014; Silva-Castro et al. 2022). Enzymatic activity plays a key role in soil biochemical processes, controls the decomposition rate of organic matter and nutrient cycling (Kaschuk et al. 2010; Singh et al. 2019), and is highly sensitive to PTE pollution stress (Hu et al. 2014). For this purpose, soil dehydrogenase activity was measured since it reflects the oxidative activity of soil microflora and is a good indicator of overall soil microbiological activity (Burgos et al. 2002; López-Piñeiro et al. 2011), while β-glucosidase activity is involved in the C cycle, playing an important role in the degradation of cellulose to glucose, and thus in the recycling of soil organic matter and the bioavailability of soil C (Cañizares et al. 2011). Moreover, phosphatase is an enzyme involved in the mineralization of organic P and plays a key role in increasing the concentrations of inorganic phosphates in soil, while urease is an important enzyme in soil N transformation (Cui et al. 2013; Yang et al. 2016).

In this regard, the greatest enzymatic activities in all cases were found in treatments with DOR application (either alone or when combined with AMFs especially), which sharply increased all activities measured (Figure 5.2). This general enhancement in enzymatic activity after the application of this amendment has also been reported in other studies, which might be related to the high nutrient content and low amount of toxic compounds in this material after biotransformation with saprobic fungi (Siles et al. 2014b; García-Sánchez et al. 2019). In contrast, AMFs alone and vermicompost treatments (both alone and in combination with AMF) did not induce an increase in the soil enzymatic activities in general when compared to M; vermicompost only produced a significant increase in dehydrogenase activity, but to a lesser extent than the DOR treatments. This contrasts with what the findings of other studies that reported an increase in soil enzymatic activity following the application of vermicompost amendments (Tejada and Benítez

2011; Przemieniecki et al. 2021). The type of vermicompost used, its humic substances, and OC rates are significantly correlated with its influence on soil enzyme activity (Pramanik et al. 2010; García et al. 2012; Aranda et al. 2015). In our case, vermicompost showed a significantly lower OC rate than the DOR amendments, which could be related to the slight effect of vermicompost treatments on the studied enzymatic activities. With regard to the increases observed in the enzymatic (both dehydrogenase and urease) activities under the DOR treatments, all three treatments consisting of D1 highlighted as the ones that most significantly increased these activities, as the treatment with D1 alone in the case of glucosidase. Furthermore, the addition of *R. custos* to DOR enhanced the activity of dehydrogenase to a greater extent than the addition of *R. irregularis*, which is consistent with results obtained by Silva-Castro et al. (2022) in similar highly PTE-polluted soil. The general enhancement in β-glucosidase activity under the DOR treatments (both with and without AMF inoculation), might have been derived from the added amendment of stabilized organic matter via the biotransformed DOR (García-Sánchez et al. 2019), while the generally higher values obtained for phosphatase under the treatments combining DOR + AMF might have been related to the role of AMFs in the mobilization of soil phosphorus (Smith and Read 2008).

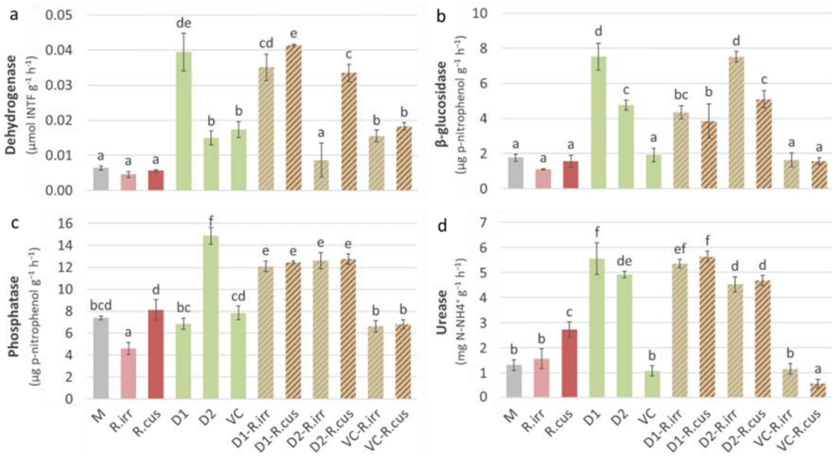


Figure 5.2. Soil enzymatic activities [dehydrogenase (a), β-glucosidase (b), phosphatase (c), and urease (d)] measured in polluted soil after the different amendment and AMF treatment applications. M (marble addition); D1 (dry olive residue mycoremediated by *C. rigida*); D2 (dry olive residue mycoremediated by *C. radians*); VC (vermicompost); *R.irr* (*R. irregularis*); *R.cus* (*R. custos*). Error bars represent the standard deviation from the mean (n=5). Lowercase letters indicate significant differences among treatments according to Duncan's post hoc test (p<0.05).

5.3.2. Effect of amendments and AMF treatments on PTE mobility

5.3.2.1. Total concentrations of PTEs

The total PTE concentration decreased in all treatments in PS (Figure 5.3; Table S5.2). However, when comparing the measured values with the current guidelines set by the Regional Government of Andalusia (BOJA, 2015), the concentrations of Pb and As were still very high in all treatments, exceeding the established regulatory thresholds for these elements for natural and urban soils (275 mg kg^{-1} for Pb, 36 mg kg^{-1} for As). Among all the treatments, D1-*R.cus* produced a greater decrease in the total concentrations of all PTEs in soil, whereas the PTE concentrations in VC-*R.cus* showed the slightest decreases, reflecting the contrasting responses of both organic amendments under the influence of the same AMF. Marble addition alone (M) also produced significant reductions in the PTE concentration compared to PS, although generally less than those caused by the addition of organic amendments and AMF inoculation.

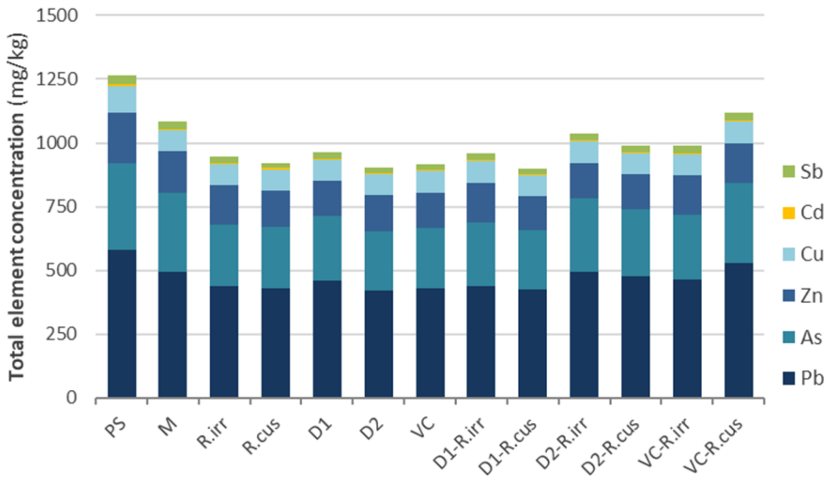


Figure 5.3. Total PTE concentrations measured in polluted soil after different amendment and AMF treatment applications (see Table S5.2 for detailed concentration values by individual element and treatment). PS (control polluted soil); M (marble addition); D1 (dry olive residue mycoremediated by *C. rigida*); D2 (dry olive residue mycoremediated by *C. radians*); VC (vermicompost); *R.irr* (*R. irregularis*); *R.cus* (*R. custos*).

These reductions might have been partly caused by a dilution effect induced by the mixture of amendments with the polluted soil, even though their application was not expected to significantly affect the total concentration of these elements (Madejón et al. 2021; Paniagua-López et al. 2023). Moreover, it might have been caused by an enhancement in the metal-accumulating ability of the plants and the increased biomass caused by the AMF and organic amendments (Jing et al. 2007; Nadeem et al. 2014).

5.3.2.2. Bioavailable concentrations of PTEs

Bioavailability is a key indicator of the potential risk that pollutants pose to the environment and living organisms and governs the ecotoxicology of pollutants in soil (Naidu et al. 2008; Zhang et al. 2013). In this study, the EDTA-extractable fraction of PTEs was measured to assess their potential bioavailability, as this is considered an accurate method for determining the bioavailable fraction of PTEs and is suitable for acidic soils (Feng et al. 2005; Wilson et al. 2010; Alibrahim and Williams 2016). Furthermore, compared to total concentrations, this fraction more accurately represents the proportion of PTEs in soil that are potentially available for uptake by plants and other living organisms (Kidd et al. 2007; Marguí et al. 2007) and that pose the greatest risk to terrestrial ecosystems (Bagherifam et al. 2019). The treatments led to different responses in the soil PTE bioavailability according to the different elements (Table 5.2). The bioavailability of elements such as Pb and Sb increased after treatment in comparison to that in PS, where the available fraction of these elements was very low. The higher EDTA-extractable concentrations of these less mobile elements under the different treatments compared with that of PS might have corresponded to the EDTA extraction capacity of the carbonate-bound and organically bound fractions of metals developed by forming strong chelates (Nakamaru and Martín-Peinado 2017). Consequently, high carbonate and OC contents in the treatments could lead to an increase in the bioavailable fraction measured for these specific elements, as previously observed in similar scenarios where carbonated and organic amendments were used (Nakamaru and Martín-Peinado 2017; García-Robles et al. 2022). However, compared to M, all organic and AMF treatments, both independently and in combination, led to significant decreases in Pb availability, especially in treatments consisting of a DOR amendment combined with AMFs, which might have been related to the higher increase in pH than by M alone. Moreover, As bioavailability remained similar to that of PS in M and the treatments with organic amendment (D1, D2, and VC), although a significant decrease was observed in most (with the exception of VC-*R.irr*) treatments where AMFs were present, leading to an increase in As availability. The general decrease in As and Pb bioavailability in the presence of AMFs might have been related to the influence of AMFs in reducing their mobility through mechanisms that include the secretion of chelating substances into soil or the superficial immobilization of these elements in their tissues by physical and chemical binding processes (Meier et al. 2012). In addition, all treatments applied to PS induced sharp reductions in the bioavailable forms of elements such as Zn, Cu, and Cd. These are highly mobile elements under acidic conditions; therefore, the large increases in pH and OC induced by all treatments strongly reduced their mobility, highlighting the role of these soil properties in controlling the mobility of these elements (Sierra-Aragón et al. 2019). It has been observed that AMFs can directly influence the mobility of these elements by changing their speciation from bioavailable to non-bioavailable forms in the rhizosphere (Jing et al. 2007). In summary, a combination of factors, including changes in the soil properties (increased pH and OC) and AMF immobilization mechanisms, were the main effects induced by the treatments, leading to the observed changes in PTE bioavailability under their influence.

Table 5.2. Bioavailable PTE concentrations (B) measured in polluted soil after different amendment and AMF treatment applications. PS (control polluted soil); M (marble addition); D1 (dry olive residue mycoremediated by *C. rigida*); D2 (dry olive residue mycoremediated by *C. radians*); VC (vermicompost); *R.irr* (*R. irregularis*); *R.cus* (*R. custos*). Data are presented as the mean ± standard deviation, n=5.

Treatment	Pb_B	As_B	Zn_B	Cu_B	Cd_B	Sb_B
PS	0.04 ± 0.04 *a*	0.81 ± 0.05 *bc*	80.62 ± 1.25 *g*	38.24 ± 1.10 *d*	0.41 ± 0.01 *g*	0.11 ± 0.05 *a*
M	0.49 ± 0.07 *g*	0.74 ± 0.15 *abc*	42.43 ± 1.76 *f*	27.12 ± 1.12 *bc*	0.32 ± 0.02 *f*	0.26 ± 0.11 *bcd*
R.irr	0.19 ± 0.11 *bcd*	0.47 ± 0.10 *a*	24.43 ± 2.16 *bc*	24.34 ± 1.64 *a*	0.26 ± 0.01 *bc*	0.24 ± 0.07 *bcd*
R.cus	0.31 ± 0.09 *def*	0.56 ± 0.14 *ab*	23.48 ± 2.31 *ab*	28.33 ± 2.31 *c*	0.24 ± 0.02 *ab*	0.20 ± 0.05 *b*
D1	0.22 ± 0.07 *bcde*	0.94 ± 0.09 *cd*	28.10 ± 1.83 *cd*	27.45 ± 1.92 *bc*	0.26 ± 0.01 *bc*	0.26 ± 0.01 *bcd*
D2	0.23 ± 0.12 *cde*	0.70 ± 0.11 *abc*	22.10 ± 1.36 *ab*	26.26 ± 1.46 *abc*	0.23 ± 0.01 *a*	0.27 ± 0.05 *bcd*
VC	0.35 ± 0.07 *ef*	0.94 ± 0.37 *cd*	25.04 ± 1.90 *bc*	28.44 ± 1.82 *c*	0.26 ± 0.01 *bc*	0.23 ± 0.06 *bc*
D1-*R.irr*	0.20 ± 0.06 *bcd*	0.59 ± 0.08 *ab*	27.28 ± 0.99 *c*	25.33 ± 0.98 *ab*	0.27 ± 0.01 *cd*	0.29 ± 0.04 *cd*
D1-*R.cus*	0.13 ± 0.09 *abc*	0.48 ± 0.14 *a*	19.95 ± 1.92 *a*	25.62 ± 2.07 *ab*	0.22 ± 0.01 *a*	0.32 ± 0.06 *d*
D2-*R.irr*	0.10 ± 0.05 *ab*	0.68 ± 0.13 *abc*	33.70 ± 1.73 *e*	26.21 ± 1.17 *abc*	0.29 ± 0.01 *de*	0.23 ± 0.05 *bc*
D2-*R.cus*	0.11 ± 0.09 *abc*	0.64 ± 0.23 *bc*	28.17 ± 6.36 *cd*	26.38 ± 0.26 *abc*	0.25 ± 0.01 *bc*	0.20 ± 0.03 *b*
VC-*R.irr*	0.37 ± 0.11 *f*	1.11 ± 0.18 *d*	32.00 ± 2.20 *e*	27.18 ± 1.56 *bc*	0.30 ± 0.02 *ef*	0.24 ± 0.04 *bcd*
VC-*R.cus*	0.24 ± 0.08 *cde*	0.67 ± 0.19 *ab*	31.56 ± 3.41 *de*	26.78 ± 2.26 *abc*	0.29 ± 0.01 *de*	0.26 ± 0.04 *bcd*

Lowercase letters indicate significant differences among treatments according to Duncan's post hoc test (p<0.05).

5.3.3. Effect of amendments and AMF treatments on wheat plants

5.3.3.1. Plant growth and mycorrhizal colonization

The application of treatments led to a significant general increase in wheat plant biomass compared to that in PS, where plant growth and survival were greatly restricted owing to the acidic pH (3.4) and high EC (3.5) present in this soil (Figure 5.4). A significant increase in soil pH after the application of the marble treatment (M) enabled plant survival and growth to a certain extent. Treatments consisting of AMF inoculation (*R.irr* and *R.cus*) barely induced a further increase in plant growth compared with those with the addition of marble alone (M). No significant increase in plant biomass after inoculation with *R. irregularis* has been observed previously (Bissonnette et al. 2010). Multiple experiments have indicated that a plant's reaction to AMF is not uniformly favorable, contingent upon both the plant and fungal genotypes, as well as environmental factors (Berger and Gutjarhr 2021). However, treatments incorporating organic amendments, with or without AMF inoculation, demonstrated superior promotion of plant biomass, particularly shoot growth, compared to treatments focused solely on mineral amendments. In contrast to M, which contributed mainly to pH neutralization, the organic amendments provided significant amounts of OC and nutrients (e.g., N, P, and K) that are naturally present in these amendments, as shown in Table S5.1, and are crucial for promoting plant growth to a greater extent. Moreover, the addition of AMFs to these organic amendments augmented this promotion in some cases, particularly in root development, as corroborated by previous studies (Alguacil et al. 2008; Curaqueo et al. 2014). The presence of AMFs can promote plant growth in polluted environments by improving plant tolerance to PTEs and their nutrition status through an increased absorption of nutrients (Nadeem et al. 2014; Bhantana et al. 2021). AMFs also provide protection to plants against PTEs through different physiological mechanisms, such as the accumulation of these elements in their structures, including spores and vesicles. In addition, the extraradical mycelia of AMFs can contribute to the immobilization of PTEs in soil through mechanisms such as the production of glomalin, a glycoprotein that acts as a chelating agent and has the potential to sequester large amounts of these elements (González-Chávez et al. 2004; Lenoir et al. 2016), and increases in the synthesis of plant phytochelatins, which also enable the chelation of PTEs (Garg and Chandel 2012; Riaz et al. 2021). Both these mechanisms promote the binding capacity of pollutants in soil, reducing their potential uptake by plants and the toxicity stress induced to them (Garg et al. 2017). Among the different treatments, D1 + *R.cus* most significantly promoted both root and shoot growth. Furthermore, the vermicompost treatments led to higher shoot growth compared with the other treatments, which might have corresponded to the higher P levels detected in the shoots of plants grown under these treatments (data not shown). This might have been because vermicompost greatly increases the available P by inhibiting the fixation of labile P by soil particles, leading to an increase in shoot P concentrations (Sáinz et al. 1998; F. Zhang et al. 2020). Therefore, the combination of AMF inoculation with organic amendments may be beneficial for increasing the potential of these fungi to enhance plant growth, as a synergistic effect is produced by the positive effect of organic matter on the growth of the external mycelia of AMFs (F.Y. Wang et al. 2013; Kohler et al. 2015). In addition, their combined application contributes to an increased diversity of soil microbial populations, which may improve the performance of these bioremediation-based treatments by promoting collaborative interactions between the different microbial communities present (Sepehri and Sarrafzadeh 2018, 2019).

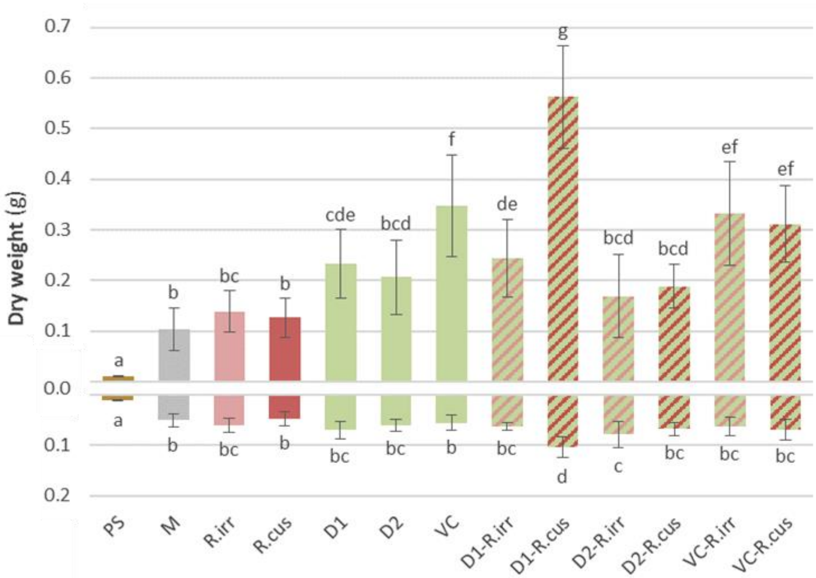


Figure 5.4. Total biomass (dry weight) of wheat plant roots (down) and shoots (up) in polluted soil after different amendment and AMF treatment applications. PS (control polluted soil); M (marble addition); D1 (dry olive residue mycoremediated by *C. rigida*); D2 (dry olive residue mycoremediated by *C. radians*); VC (vermicompost); *R.irr* (*R. irregularis*); *R.cus* (*R. custos*). Error bars represent the standard deviation from the mean (n=5). Lowercase letters indicate significant differences among treatments according to Duncan's post hoc test (p<0.05).

Moreover, mycorrhization in wheat roots, accounted as the degree to which plant roots were colonized by AMF, was measured in the treatments in which AMF were inoculated (Figure S5.2). No significant differences were observed between AMF treatments, consistent with the findings reported by Silva-Castro et al. (2022), who studied various AMF strains in soil polluted with PTEs. In the organic amendment treatments, the percentage of mycorrhization decreased, particularly under vermicompost treatments, an effect also detected in other studies (Beck 2012; Pierart et al. 2019). The DOR amendments with AMFs did not induce greater mycorrhization of the roots than AMF inoculation alone, as previously observed by García-Sánchez et al. (2019). In all cases, root colonization ranged from 5% to 10%. However, *R. custos* showed slightly higher mycorrhization rates than *R. irregularis* when combined with the different organic amendments, particularly D2. This might have been due to the better mycorrhization capacity of *R. custos* under pollution stress conditions, as this AMF was isolated from a naturally PTE-polluted environment (Cano et al. 2009).

5.3.3.2. Concentration of PTEs in plants

The very limited plant growth in PS soil did not allow for the analysis of the PTE content in plants growing in this soil, as the plant material harvested from it was too scarce. However, the analysis of PTE content in plants grown under the different treatments showed significantly higher concentrations in the roots than in the shoots across all treatments (Figure S5.3). This contrast was particularly notable for Pb, with concentrations in the roots being in the range of approximately 50-to-200-fold higher than those in the shoots, followed by As (30-to-100-fold) and Cd (up to 90-fold under specific treatments). Conversely, elements such as Cu and Sb exhibited concentrations in roots that were 5-to-20-fold higher than those in shoots. Finally, Zn exhibited more equilibrated concentrations between the roots and shoots, although they were still slightly higher in the roots. The significantly higher accumulation of PTEs in the roots than in the shoots, enhanced by the influence of treatments, might have interesting implications for phytoremediation programs intended for the remediation of PTE-polluted sites. The promotion of PTE immobilization at the root level contributes to the phytostabilization of pollutants, thus reducing the potential toxicity risk to the ecosystem (Yang et al. 2014).

The PTE concentrations measured in the plants also highlighted the overall effectiveness of all treatments tested in decreasing the concentration of most of the PTEs studied in plant tissues compared with sole marble application (M), with the exception of Cu in the roots and Cd in the shoots (Figure 5.5). This is consistent with the higher concentrations measured in M for both the total and, especially, bioavailable PTE fractions compared to the other treatments, which directly modulate plant uptake. The most significant reductions in PTEs concentrations driven by the treatments were observed for Pb, As, and Sb in both roots and shoots, as well as for Cu and Zn, especially in shoots, showing the greatest deviations from most of the treatments in the PCA analysis. Meanwhile, M showed a strong direct correlation with these elements in the PCA, as the highest plant uptake for all of them occurred under this treatment. In contrast, treatment with D1 combined with AMFs (D1-*R.irr* and D1-*R.cus*) generally displayed a lower uptake of most of the PTEs in the plants, both in the roots and shoots, as evidenced by the PCA results, where these treatments were positioned furthest from most elements and the M treatment. This was particularly significant for Pb in both plant parts, and for As, Cd, and Sb in the roots (Figure S5.3). However, the general influence observed for all treatments tested, leading to a reduced uptake of PTEs by plants to different extents compared to that in M, highlights that the combined application of organic amendments with AMFs was effective in promoting survival and conferring resistance to plants in polluted soils by different mechanisms.

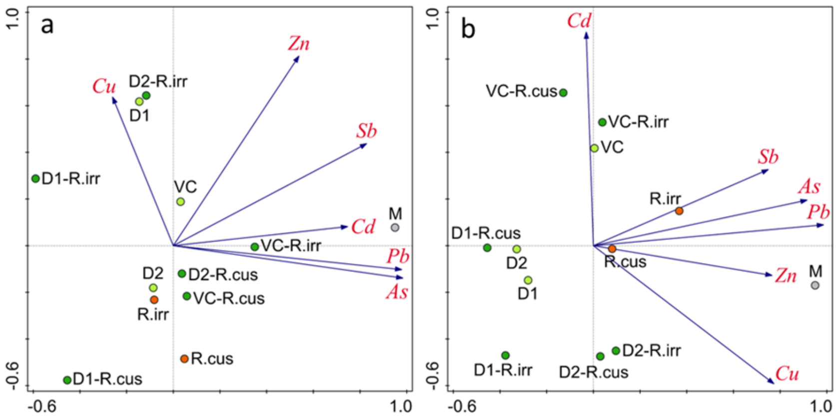


Figure 5.5. Principal component analysis results for different treatments and their correlations with PTEs concentrations in plants roots (a) and shoots (b). The first two principal components explained over 90% of the variance in both cases. M (marble addition); D1 (dry olive residue mycoremediated by *C. rigida*); D2 (dry olive residue mycoremediated by *C. radians*); VC (vermicompost); *R.irr* (*R. irregularis*); *R.cus* (*R. custos*).

As previously discussed, the treatments improved PTE immobilization in the soil through different mechanisms, which reduced the PTE availability. Treatments that showed greater decreases in plant uptake compared to that of M (DOR combined with AMF treatments) coincided with those for which lower bioavailable fractions of PTEs were measured in soil (Table 5.2). Therefore, the influence of treatments on the immobilization of pollutants in the soil was evidenced as a primary process for their protective effect on the plants growing in polluted soils. AMFs play an important role in reducing PTE transfer to plants, as they can enhance the phytostabilization of these elements by increasing their retention in the hyphae and roots, thus reducing their translocation to the shoots (Soares and Siqueira 2008; Meier et al. 2012; Cabral et al. 2015). This is consistent with the highest PTEs reduction rates observed in the shoots of the plants, especially in treatments with AMFs in combination with DOR amendments (Figure S5.3). García-Sánchez et al. (2021) documented an enhancement of mycorrhizostabilization mechanisms through the application of organic amendments, primarily attributed to the AMF promotion of glomalin and phytochelatin production, consequently stimulating these mechanisms. In the present study, this was evidenced by the fact that AMF treatments without organic amendments (i.e., *R.irr* and *R.cus*), especially *R.irr*, were less effective in reducing the concentrations of Pb and As in the shoots. Moreover, increases in PTE concentrations in plants under the treatments were also found in some cases, with the most remarkable being the increase in Cd concentration in the shoots under the vermicompost treatments, which showed a sharp increase compared with that of M and the other treatments. This was indicated by the strong positive correlation shown in the PCA results (Figure 5.5b). A higher accumulation of Cd in the leaves of plants growing in soil amended with vermicompost was previously identified by Sebastian and Prasad (2013), although in our study, the exceptional Cd accumulation in shoots under the vermicompost treatments was not related to a negative effect on their growth.

5.3.3.3 Transfer of PTEs from soil to plant

Concentrations extracted by EDTA were selected for BAF and BCF calculation, since this fraction better represents the bioavailable forms of PTEs for plants (Marguí et al. 2007; Parra et al. 2014). In general, the applied treatments were effective in significantly reducing the shoot uptake of PTEs compared to M, especially for Pb, As, Cu, and Sb, leading to significantly lower BAFs for these elements under most treatments (Table 5.3). Nevertheless, a slight accumulation of Pb, As, Zn, and Sb in wheat shoots was still observed (BAF>1), although to a significantly lesser extent than in M. Moreover, treatments with only AMF inoculation showed lower BAF reductions for these elements, especially in the case of Pb and As with *R. irregularis* inoculation, which remained at levels similar to those measured in M. Conversely, the DOR amendments, especially the combination of D1 with both AMFs, showed the lowest accumulations of certain elements (i.e. Pb and Sb) in shoots. No accumulation in the shoots (BAF<1) was found for Cu and Cd, although for the latter, BAF>1 was observed for the vermicompost treatments.

Regarding PTE accumulation in roots, concentrations measured in this part of the plant for all studied elements were significantly higher than their bioavailable fraction in soils (BCF>1) in all treatments, being especially high for Pb and As, but also for Cd and Sb (Table 5.3). In contrast, the accumulation of Zn and Cu in the roots was much lower, and low accumulation was also observed in the shoots for both elements. These elements are considered to have low accumulation capacity in different grass plants (Bhatti et al. 2016; Andrejić et at. 2018). However, only for Zn did shoot concentrations approximate the values measured in the roots, exhibiting a TF closer to one. In any case, no translocation from the roots to shoots was estimated for this element under any treatment (TF<1) (Table S5.3). For all other elements, the minimal uptake in shoots compared to the substantial accumulation in roots led to a very low TF under all treatments, especially for Pb and As (TF<0.01). This highlighted the effectiveness of the treatments in preventing the transfer of pollutants to the aboveground parts of the plants. In fact, the significantly higher accumulation of PTEs in the root tissues was attributed to AMF mechanisms, which immobilized them in the mycorrhizosphere and fungal structures, thereby restricting their translocation to shoots and consequently promoting the phytostabilization process (Singh et al. 2019). This process can be further improved by the addition of organic amendments (García-Sánchez et al. 2017), as observed in our results, particularly for DOR-based amendments. Among other factors promoting phytostabilization that can be attributed to organic amendments is their direct effect in promoting plant growth and, more specifically, root development. This results in increased soil colonization by roots and greater biomass in this part of plants, leading to a higher capacity of plants to accumulate and immobilize greater amounts of PTEs.

Table 5.3. Transfer of PTEs from soil to plant (BAF: bioaccumulation factor; BCF: bioconcentration factor) in each of the different studied treatments. M (marble addition); D1 (dry olive residue mycoremediated by *C. rigida*); D2 (dry olive residue mycoremediated by *C. radians*); VC (vermicompost); *R.irr* (*R. irregularis*); *R.cus* (*R. custos*). Data are presented as the mean ± standard deviation, n=5.

Treatment	Pb_BAF	As_BAF	Zn_BAF	Cu_BAF	Cd_BAF	Sb_BAF
M	10.12 ± 2.30 *c*	5.09 ± 3.44 *b*	2.14 ± 0.23 *cde*	0.58 ± 0.32 *b*	0.22 ± 0.03 *a*	8.95 ± 4.66 *e*
R.irr	12.31 ± 2.93 *c*	5.23 ± 1.52 *b*	3.05 ± 0.40 *f*	0.33 ± 0.05 *a*	1.09 ± 0.15 *c*	2.82 ± 0.95 *abc*
R.cus	4.34 ± 1.64 *ab*	2.15 ± 0.72 *a*	2.60 ± 0.41 *e*	0.36 ± 0.19 *a*	0.46 ± 0.08 *ab*	6.31 ± 3.03 *d*
D1	2.98 ± 1.62 *ab*	0.71 ± 0.24 *a*	2.09 ± 0.24 *cd*	0.23 ± 0.03 *a*	0.38 ± 0.13 *ab*	2.26 ± 0.28 *ab*
D2	2.91 ± 1.46 *ab*	1.14 ± 0.67 *a*	2.33 ± 0.26 *cde*	0.21 ± 0.02 *a*	0.39 ± 0.21 *ab*	1.93 ± 1.06 *ab*
VC	3.73 ± 0.77 *ab*	1.12 ± 0.14 *a*	2.36 ± 0.30 *cde*	*0.23* ± 0.04 *a*	2.07 ± 0.98 *d*	5.89 ± 1.99 *d*
D1-*R.irr*	1.32 ± 0.65 *a*	1.26 ± 0.55 *a*	2.22 ± 0.34 *cde*	*0.31* ± 0.16 *a*	0.30 ± 0.15 *a*	1.05 ± 0.79 *a*
D1-*R.cus*	2.46 ± 2.01 *ab*	1.73 ± 0.62 *a*	2.54 ± 0.40 *de*	0.20 ± 0.02 *a*	0.76 ± 0.20 *bc*	1.17 ± 0.09 *a*
D2-*R.irr*	9.27 ± 4.30 *c*	2.09 ± 0.36 *a*	1.41 ± 0.24 *a*	0.23 ± 0.04 *a*	0.16 ± 0.05 *a*	2.79 ± 0.71 *abc*
D2-*R.cus*	5.68 ± 2.36 *b*	1.54 ± 1.36 *a*	2.51 ± 0.49 *de*	0.38 ± 0.17 *a*	0.38 ± 0.15 *ab*	5.48 ± 1.52 *cd*
VC-*R.irr*	3.84 ± 2.14 *ab*	1.23 ± 0.44 *a*	1.93 ± 0.16 *bc*	0.24 ± 0.02 *a*	2.32 ± 0.22 *d*	5.12 ± 1.40 *cd*
VC-*R.cus*	3.42 ± 2.41 *ab*	2.41 ± 0.63 *a*	1.64 ± 0.15 *ab*	0.19 ± 0.02 *a*	2.14 ± 0.19 *d*	3.98 ± 0.85 *bcd*
	Pb_BCF	As_BCF	Zn_BCF	Cu_BCF	Cd_BCF	Sb_BCF
M	615.9 ± 97.1 *bc*	189.9 ± 29.2 *d*	2.57 ± 0.47 *a*	2.99 ± 0.39 *ab*	15.16 ± 3.99 *ab*	61.68 ± 29.38 *d*
R.irr	589.0 ± 253.8 *bc*	150.1 ± 35.2 *cd*	3.30 ± 0.49 *abc*	3.28 ± 0.96 *ab*	15.14 ± 1.80 *ab*	34.29 ± 12.24 *abc*
R.cus	369.0 ± 110.1 *ab*	148.7 ± 45.4 *cd*	3.08 ± 0.52 *ab*	2.34 ± 0.25 *a*	19.96 ± 2.60 *c*	47.45 ± 17.38 *cd*
D1	433.8 ± 174.3 *abc*	66.0 ± 18.1 *a*	3.32 ± 0.62 *bc*	4.45 ± 0.68 *c*	12.94 ± 1.34 *a*	31.76 ± 8.53 *abc*
D2	426.5 ± 78.7 *abc*	103.4 ± 18.3 *abc*	3.44 ± 0.66 *bc*	3.43 ± 0.58 *b*	15.76 ± 1.97 *ab*	29.84 ± 7.02 *abc*
VC	322.3 ± 88.1 *a*	95.0 ± 52.3 *abc*	3.85 ± 0.44 *c*	2.92 ± 0.91 *ab*	16.81 ± 3.22 *b*	42.17 ± 13.77 *bc*
D1-*R.irr*	268.8 ± 68.2 *a*	76.2 ± 21.9 *ab*	3.07 ± 0.38 *ab*	3.42 ± 0.33 *b*	12.36 ± 1.22 *a*	26.66 ± 5.46 ab
D1-*R.cus*	517.9 ± 185.6 *abc*	132.0 ± 59.4 *bc*	3.05 ± 0.23 *ab*	3.59 ± 0.79 *bc*	12.67 ± 1.11 *a*	18.74 ± 8.45 *a*
D2-*R.irr*	596.8 ± 297.3 *bc*	100.6 ± 32.9 *abc*	3.14 ± 0.34 *ab*	3.59 ± 1.14 *bc*	14.31 ± 3.87 *ab*	46.47 ± 14.28 *bcd*
D2-*R.cus*	670.8 ± 134.8 *c*	133.8 ± 39.9 *c*	3.19 ± 0.47 *abc*	3.11 ± 0.49 *ab*	14.92 ± 1.08 *ab*	41.81 ± 9.27 *bc*
VC-*R.irr*	439.4 ± 130.1 *abc*	94.0 ± 28.9 *abc*	2.94 ± 0.58 *ab*	3.20 ± 0.23 *ab*	14.74 ± 1.98 *ab*	41.54 ± 10.06 *bc*
VC-*R.cus*	495.1 ± 126.4 *abc*	127.0 ± 35.8 *bc*	2.85 ± 0.41 *ab*	2.80 ± 0.59 *ab*	13.26 ± 2.70 *ab*	30.32 ± 5.36 *abc*

Lowercase letters indicate significant differences among treatments according to Duncan's post hoc test ($p<0.05$).

5.3. Conclusions

The application of amendments to polluted soil led to an overall improvement in the main soil properties and reduced soil toxicity by decreasing the concentrations of some PTEs. The treatments tested showed different degrees of effectiveness in recovering soil function and reducing pollution levels. The treatment based only on an inorganic liming amendment (marble sludge) was effective in neutralizing the soil pH, thereby reducing the availability of highly mobile elements such as Zn, Cu, and Cd and allowing plants to survive in polluted soil. In contrast, it was less effective in promoting plant growth and soil enzymatic activities and in providing protection to plants against PTE uptake. However, the combined application of inorganic and organic amendments increased their effectiveness in promoting plant growth and survival, and improving soil biological status. Vermicompost-based treatments led to significant increases in the plant shoots, but their performance in reducing PTE toxicity and providing plants with protection mechanisms against pollutant uptake was less effective than that of treatments based on DOR biotransformed by saprobic fungi. Treatments incorporating this amendment led to the greatest increase in the wheat plant biomass and soil enzymatic activity, especially when combined with the inoculation of AMFs. In this regard, the treatment consisting of a combination of marble with DOR biotransformed by *C. rigida* (DOR1) and inoculated with AMFs showed the highest potential for reducing PTE concentrations in plants and inducing growth, thus playing an important bioprotective role in the plants. Therefore, the joint implementation of DOR and AMFs further enhanced their efficacy through the synergistic effects produced between them, promoting the immobilization of PTEs in soil and stimulating the phytostabilization mechanisms induced by AMFs. Among the AMFs tested, *R. custos* showed slightly better performance than *R. irregularis*, suggesting the potential benefits of using indigenous, locally adapted fungi from polluted sites, as they may perform better under pollution conditions and consequently show higher remediation potential.

Our results demonstrate that mycoremediated DOR may be an efficient product for use as a soil organic amendment for remediating metal(loid)-polluted soils. Furthermore, our findings suggest that the application of biotransformed DOR in combination with AMFs may enhance the remediation potential of soils polluted with PTEs, representing a promising remediation strategy for the recovery of soil function in polluted areas.

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Chapter 6

General discussion

6.1. Soil pollution by PTEs and need for action

Potentially toxic elements (PTEs) are elements normally present in nature, but human industrialization and activities have led to the presence of concentrations of these elements higher than permissible all over the world. They may be toxic even at very low concentrations and are non-biodegradable, so they can persist for exceptionally long time in the ecosystem and be accumulated and biomagnified to concentrations potentially toxic to organisms of every level in the food chain, including humans, being most of these elements carcinogenic. It is therefore crucial to identify efficient tools and approaches to assess the potential risks of exposure to these PTEs, to measure the toxicity levels in scenarios where soil pollution by these elements is present, and to develop sustainable and efficient strategies for the remediation of these sites.

The Guadiamar Green Corridor (GGC) (SW Spain) represents an exemplary scenario of the degree to which a natural ecosystem can be deeply damaged and degraded over the long term by anthropogenic activities, and more specifically by PTEs pollution, leading to high levels of persistent soil pollution. Even though great efforts were taken after the Aznalcóllar mining spill to clean the affected area, the magnitude of this accident made these tasks insufficient. This scenario is also an example of a long-term soil monitoring program, which has been developed since the accident occurred and that continues nowadays. In this sense, soil pollution by the Aznalcóllar mining spill has been monitored over time by analyzing a total of 100 sampling points distributed along the entire affected area at three depths (0-10, 10-30, and 30-50 cm), assessing the evolution of the soil properties and potential changes in the mobility and availability of pollutants in the long term. This was firstly done in 1998 only several months after the accident took place (after the tailings were removed), and was repeated in 1999 (after the cleaning of the highly polluted areas), in 2004 (after the tilling of the upper 20-25 cm), and in 2013 (15 years after the accident).

The comparative study of the first three sampling campaigns (up to 2004) revealed that the removal of the tailings left a heterogeneous distribution pattern of the pollutants, with highly polluted soil patches alternating with less polluted areas, and that the cleanup did not substantially lower the concentration in the highly polluted soils (Simón et al. 2008a). Six years after the spill and at the end of all remediation measures, the intervention levels defined by the Regional Government of Andalusia were exceeded in the uppermost 10 cm in 35% of the soils. In contrast, after the monitoring of 2013, Martín-Peinado et al. (2015) observed that 15 years after the accident the soil pH tended to rise and concentration of pollutants tended to diminish over time, being the total concentration of PTEs measured in the affected soils within the levels allowed by the regional legislation in most of the GGC. However, Martín-Peinado et al. (2015) also pointed out that the effects of the deficient removal of tailings after the spill were still perceptible, since in many places the tailings remained mixed with the soil, giving rise to soil patches with very acidic pH and high pollutants concentrations, easily detectable by the null growth of vegetation on them. These highly polluted bare soil patches are still present throughout the GGC more than two decades after the spill, and represent a major source of residual pollution from which pollutants can spread through the solid and liquid phases to the surrounding areas. For this reason, the implementation of actions and development of strategies for the remediation of these polluted soils based on effective, low-cost, and environmentally friendly soil remediation techniques is still required.

For all the above mentioned, the GGC is considered as an ideal scenario that can be used as a real natural laboratory for developing and evaluating not only different soil pollution remediation strategies but also additional research and studies in a great variety of fields linked to the heterogeneous conditions that may be found within this area, ranging from unpolluted areas to highly PTEs polluted areas to naturally recovered areas. Processes such as spontaneous vegetation colonization and development in soils polluted at different degrees, microbiological community adaptation to different pollution levels, or the response of a wide variety of model organisms to unfavorable soil conditions and toxicity by PTEs under real environmental conditions and mixture effects of the pollutants could be evaluated in this area. Furthermore, the influence of soil remediation and bioremediation treatments and techniques on all these parameters could be evaluated in-situ and monitored over time, to more accurately assess their real impact in environmental risk assessments. This would assist in developing comprehensive protocols and strategies for the remediation of PTEs polluted soils based on the generated knowledge, and that could be implemented and extrapolated to similar scenarios worldwide.

6.2. Evaluation of soil pollution and toxicity in the Guadiamar Green Corridor (GGC)

An evaluation of the status of soils of the GGC and pollution levels present in them was firstly assessed in **chapters 2**, **3**, **4**, and **5**. This was done to obtain an approximation of the conditions existing in the soils used as references for the different status and pollution levels existing within the studied area in the GGC, which were the unaffected natural soils (US), the naturally recovered soils (RS), and the highly polluted soils not subjected to remediation treatments used as residual pollution control (PS). This first evaluation was used afterwards as a reference for evaluating to which extent the remediation actions were effective in recovering conditions in the treated polluted soils and in approximating them to the recovered and natural soils.

In this regard, the US within the GGC were found to be slightly acidic, present low electrical conductivity (EC) and $CaCO_3$ content, and a high organic carbon content (OC). On the other hand, the PS were found to be strongly acidic, with high EC, and very low $CaCO_3$ and OC contents. Meanwhile, the RS sampled in the area of study within the GGC showed a certain variability in their properties related to the wide range of vegetation development and recovery levels that occur in these soils. In any case, RS were characterized by being less acidic than the PS, with a pH ranging from acidic in areas closer to the polluted soil patches to a pH even more elevated than the US of the GGC in more recovered areas. The spatial pH variations in RS are associated to the $CaCO_3$ content in these soils, which also showed a high variability. This could be related to the large-scale remediation actions taken in the area after the accident, which involved the use of heavy machinery and resulted in a certain heterogeneity in the application of the liming amendments. This, in combination with a subsequent divergent spatial evolution of soil conditions over time, led to the varied conditions present nowadays. Furthermore, the OC in RS also showed spatial variability, reaching values at certain areas higher than those found in US, as a result of developing a higher density of vegetation cover on them.

In relation to the soil pollution levels in the reference soils, the total PTEs concentrations measured showed that Pb and As in both PS and RS exceeded the regulatory thresholds established by the regional administration (BOJA 2015). Although in RS the concentrations of Pb and As exceeded these guideline values, and were significantly higher than the background levels in the area, considered the ones measured in the US, they were significantly lower than those measured in the PS, where the low mobility of these elements encourages their accumulation in the uppermost part of the soil (García-Carmona et al. 2019a). On the contrary, total concentrations of Cu, Zn, and Cd measured in PS and RS did not approximate to the regional intervention values. However, the concentrations of Cu and Zn in RS were twice higher than in PS. These are highly mobile elements under acidic conditions, so that under PS conditions the spread of these elements by the runoff waters and their infiltration in depth are intensified (Martín-Peinado et al. 2015), thus reducing their total concentrations in the uppermost centimeters of this soil. Meanwhile, in RS the precipitation of these elements as insoluble forms due to the slightly acidic soil pH (Rieuwerts et al. 1998), together with their association with OC by specific binding to it by adsorption and complexation (Kumpiene et al. 2008), led to the higher total concentrations of Cu and Zn to be detected in RS. In contrast, this was not observed for Cd, possibly related to the high heterogeneity in the spatial distribution of the pollution in the area.

The opposite to what was observed for total PTEs concentrations was found for their water-soluble fractions, considered as a good indicator of their short-term dynamics and mobility (Beesley et al. 2010). This fraction showed that the soluble concentrations of the low mobile elements, Pb and As, were higher in RS than in PS, where lowest mobility of these elements was found. These elements are usually related to the labile organic matter and phosphates forms, which have been identified as key factors in the desorption of Pb and As by competing effects for surface sites in the soil, thus increasing their availability by increasing their soluble and labile forms (Egli et al. 2010; Sierra-Aragón et al. 2019). In addition, the rise in pH can promote the desorption of As from iron oxides and organic matter, also increasing its solubility in RS (Martín-Peinado et al. 2011). Conversely, Cu, Zn, and Cd soluble concentrations were found to be significantly lower in RS than in PS, since the water solubility of these elements is strongly negatively correlated with soil pH, reaching their highest mobility under acidic conditions (Laurent et al. 2020; Kicińska et al. 2022). Moreover, the affinity of Cu, Zn, and Cd for organic matter has been widely reported, exhibiting a high influence in the specific adsorption of these elements by metal oxides and hydroxides that are considered less reversible reactions (Bradl 2004; Kabata-Pendias 2010).

The EDTA-extractable fraction of the PTEs, considered as their bioavailable fraction, and a more reliable prediction of long-term availability of these elements in soils (Hurdebise et al. 2015), showed a similar tendency to the water-soluble fraction for Pb, As, and Zn, being both fractions for Pb and As higher in RS than in PS and the opposite for Zn. However, a different tendency in the EDTA-extractable fraction for Cu and Cd was observed compared to their water-soluble fraction, showing for these elements higher bioavailable concentrations in RS than in PS, similar to what was observed for Pb and As. This can be related to the high EDTA capacity to effectively extract the organically-bound fractions of PTEs present in soil by forming strong chelates, thus indicating the maximum potential PTEs extractability (Labanowski et al. 2008; Nakamaru and Martín-Peinado 2017). This occurred for all elements except for Zn, which can be explained by the different lability of complexed and bound elements in soil and differences in their kinetics of desorption/dissolution that lead to different binding strengths (Sun et al. 2001; Finžgar and Lestan 2006). In fact, the lower removal of Zn by EDTA compared to other PTEs was previously observed by other authors (Udovic et al. 2007; Udovic and Lestan 2009), who found that after Zn fractionation, the majority of it was predominantly present in the residual fraction over that bound to organic matter, which can explain why EDTA was not particularly effective in Zn removal.

6.3. Influence of in-situ remediation actions on soil pollution

A set of different remediation treatments and techniques were applied in the field to the PS to evaluate their effectiveness in improving soil conditions and reducing the pollution and toxicity levels, and therefore to assess their potential for the remediation of PTE-polluted soils. The treatments were proposed based on the conditions present in these highly degraded soils and related to their pollution. In this sense, and according to the highly acidic pH of the PS, two different liming treatments, based on gypsum mining spoil and marble sludge, were selected aimed at correcting the soil high acidity. Furthermore, two treatments based on physical techniques, landfarming and biopiles, the latter considered as the mixture at equal volumes of PS and RS, were selected to correct the limiting structure of the topsoil and favor water retention and the settlement of plant seeds that could subsequently establish in the treated soil, as well as the incorporation of organic matter in the case of the biopile-based treatment. Finally, all these treatments were applied in duplicate in combination with an organic amendment, vermicompost, to contribute to all of them with the extra addition of organic matter and counteract the lack of organic content in the polluted soil.

6.3.1. Improvement of soil conditions

After the exposure over time to the treatments applied, a characterization of the main soil properties and analysis of PTEs fractions in the treated polluted soils were carried out in **chapters 2**, **3**, and **4**, to measure the magnitude of the changes induced by the actions implemented on them when compared to untreated polluted soils (PS).

The remediation treatments and techniques evaluated led to differing results in terms of effectiveness in improving soil conditions and reducing the toxicity levels in them. Due to the high acidity of the polluted soils, liming treatments, based on gypsum and marble materials, were highly effective in significantly increasing the soil pH, especially marble-based treatments, which in the doses applied led to a complete soil pH neutralization. These results are in agreement with previous studies in the area using other carbonated materials and residues, ranging from pure lime to others such as sugar-refinery scum and sugar beet lime, for the neutralization of soil acidity due to their elevated $CaCO_3$ content (Clemente et al. 2003; Madejón et al. 2006; Aguilar et al. 2007). On the other hand, landfarming treatments (**chapter 2**) and biopile treatments (**chapters 2**, **3**, and **4**) were less effective in rising the soil pH, although they also significantly reduced the strong acidity on the treated soils compared to PS.

Moreover, treatments including the organic amendment (vermicompost) increased the OC in soils although to levels still far from those found in RS and US. Treatments without vermicompost amendment also contributed to a slight increase in soil OC, although not significantly, as a result of the soil crust breakage, allowing the deposition of plant seeds and their eventual development over time. Associated with the breakage of the soil crust by the treatments and the addition of the inorganic and organic components, which altogether improved the soil structure, all treatments were effective in significantly reducing the elevated EC of the PS at similar extent, reducing it even by half, although EC in treated soils was still considerably higher compared to the RS, where EC was about half to that measured in the treated soils. The improved soil structure and the presence of organic matter in the treated soils prevented surface crusting and favored infiltration, promoting the soluble salts leaching and washing from the soil (Pastor-Jáuregui et al. 2020b). In addition, the persistent high EC in PS over time is also derives from the presence of residual sludge aggregates that are frequently found in this soil, and which remain in constant oxidation causing the progressive acidification of the soil by the continuous release of sulfates and leaching of PTEs (López-Pamo et al. 1999).

6.3.2. PTEs availability and toxicity

The water-soluble and the EDTA-extractable fractions of the PTEs present under all treatments tested were measured to assess the soil toxicity levels and the changes produced in them after the application of the treatments to the polluted soil. This was repeated for every individual sampling due to the high variability and heterogeneity of soil pollution even within a same plot and treatment. These fractions were used to this end since they are considered as a more accurate indicator of the actual bioavailability of the PTEs, being this a better indicator of the potential risk that the pollutants pose to the environment and to living organisms, and determining the ecotoxicity in the soil (Bolan et al. 2008; Zhang et al. 2013). In contrast, PTEs total concentrations are less relevant in terms of their toxicity and behavior in the soil since this fraction does not reflect soil chemistry, and the amount of an element that is actually available to the living organisms is different from that assessed by strong acid digestions, which can dissolve PTEs fractions that are not available for organisms, such as those bound to silicates (Buscaroli 2017).

In this regard, in water-soluble and EDTA extractable fractions significant reductions were detected in all treatments both after 12 and 18 months compared to those measured in the PS, although these reductions differed among the treatments. Marble-based treatments showed the highest effectiveness in reducing to a greater extent both water-soluble and bioavailable PTEs concentrations, as well as gypsum-based treatments in the case of water-soluble fraction (**chapter 2**), in most cases these treatments reducing water-soluble concentrations to levels similar to or even below those found in the US (**chapters 3** and **4**). The strong reductions in these PTEs fractions were associated to the significant increase in soil pH after the application of these treatments resulting in the effective immobilization of the highly mobile elements such as Cu, Zn, and Cd, in accordance with previous studies that highlighted the effectiveness of soil amendments rich in $CaCO_3$ in retaining these elements (Aguilar et al. 2004a). However, Simón et al. (2005b) reported that the mobilization of As could increase at soil pH>6.5, and that an excessively high pH could limit the fixation of As increasing its bioavailability. Consequently, while gypsum-based treatments showed an optimal pH for the retention of this element, soil pH under marble-based amendments, which approximated to the neutrality, exceeded in this sense the optimal values, aligning with occasional detections of slight increases in the As mobile fractions under these treatments. The increases in the bioavailable forms of elements such as As and Pb could also be related to their resolubilization in the presence of organic matter by competing mechanisms for sorption sites in the soil (Angin et al. 2008; Aftabtalab et al. 2022). As such, when planning soil remediation strategies including liming and organic amendments, it is crucial to accurately estimate the doses to be applied in order to effectively control the mobility of PTEs in polluted soils (Simón et al. 2010).

On the contrary to the liming treatments, biopile and landfarming techniques showed lower general reductions in the PTEs water-soluble and bioavailable fractions, especially in the bioavailable fraction under biopile treatments (chapters 2 and 4), which was strongly associated with the more acidic soil pH measured under these treatments, that were less effective in rising the pH in the treated soils. In addition, Cu, Zn, and Cd water-soluble fractions under biopile and landfarming techniques stood out for showing notably higher levels compared to the liming-based treatments and even exceeding the maximum pollutant levels considered for these elements (Rajendran et al. 2022). This implies that these treatments were less feasible in reducing the solubility of the most mobile elements as they had lower influence in rising the soil pH, and were therefore less effective in controlling PTEs pollution.

6.4. Biological assessment of soil toxicity

The ecotoxicological approach using toxicity bioassays on target organisms at different trophic levels and with different exposure routes and sensitivities to soil properties and PTEs toxicity has been recommended to complement chemical analysis for a correct ecotoxicological risk assessment (González et al. 2011). These ecotoxicological tests aim to provide a realistic prediction of the behavior of pollutants in the environment, and they represent effective tools to assess the actual level of pollution of soils considering the bioavailability of the PTEs, thus more accurately establishing the toxicity risks in polluted soil ecosystems (Gruiz et al. 2016).

Most commonly, soil toxicity bioassays are focused on microorganisms, plants, and invertebrates, and can be divided into two different groups: those using a liquid phase (soil extract, pore water, leachate, etc.), and those using the soil solid phase (García-Carmona et al. 2017). The effectiveness of the remediation treatments applied to the PS in the reduction of PTEs bioavailability and soil toxicity was assessed by using ecotoxicological tests in both soil phases and with different bioindicator organisms on each one (**chapter 2**). In the toxicity bioassays performed, it was expected that each organism would respond differently according to their own sensitivity and to the different pollution levels present in each of the treated soils.

The bioassays, indeed, showed different responses of the organisms according to the treatments applied. The amendments applied to the PS strongly modified pollutants concentrations, with significant influence in water-soluble and bioavailable (EDTA-extractable) concentrations at different extents. The response of soil microbiological communities to the treatments was measured through the soil heterotrophic respiration rate using the soil solid phase. This bioassay showed that the treatments led to significant increases in soil microbiological activity, although not reaching the same levels as in the RS. Nevertheless, the treatments showed different degrees of recovery of the microbiological activity due to the different extents to which they decreased the PTEs availability in the treated soils and improved the main soil properties. In this regard, treatments with a higher content of $CaCO_3$ and a pH closer to neutrality showed higher soil respiration rates, being marble-based treatments those showing rates more similar to that in the RS. This is consistent with the findings of Ramsey et al. (2005), who indicated that the increased acidity and PTEs concentrations are primary limiting factors of soil respiration in mine waste-polluted soils. Moreover, treatments with vermicompost amendment showed higher rates compared to the same treatment without the organic amendment, highlighting the positive effect of the soil organic carbon content in the microbiological activity in the soil (Bastida et al. 2006). Regarding the suitability of using soil basal respiration test as an indicator of microbial stress in relation to PTEs pollution in soils, Romero-Freire et al. (2016b) questioned its use for showing a low sensitivity to soil pollution. However, according to the results obtained in this work it can be concluded that this test was a useful tool to estimate the inhibitory effects on microbial activity caused by PTEs toxicity in the soils evaluated, showing high sensitivity to the different conditions and correlating accurately with the responses obtained in other bioassays.

Moreover, toxicity bioassays were also applied to terrestrial plants (*Lactuca sativa* and *Hordeum vulgare*) in both the liquid and the solid phase, to measure the response of this group of organisms to the soil pollution levels under every treatment. The responses of these plants to the treatments indicated that the bioassay performed in the liquid phase, using *L. sativa*, was more sensitive than the one in the solid phase with *H. vulgare*, which showed a slight and uniform response of all the treatments compared to the response obtained for RS. This indicates that bioassays using the liquid phase may be more accurate in reflecting the behavior of mobile phases, and more sensitive to the risks of dispersion, solubilization and bioavailability of pollutants in the environment, thus being a better estimator of soil phytotoxicity than bioassays using the solid phase. Regardless of their different sensitivities, these bioassays showed a similar response, with all treatments significantly reducing the toxicity of PS. However, in both the liquid and solid phase of the soil, the gypsum and marble-based treatments highlighted as those approximating the most to the results obtained in RS.

This was supported by the responses obtained for other bioassays performed using organisms living in the liquid phase, such as the algae growth inhibition and daphnia mortality toxicity tests. These tests, based on the liquid phase, also showed more contrasting and conclusive differences between treatments than the bioassays using the solid phase. In this sense, the responses to the treatments observed through these bioassays were in agreement, highlighting liming treatments, particularly GV, and M and MV, as those exhibiting the lowest toxic effects to the test organisms and the most similar responses to those obtained in RS. According to the PTEs fractions, these treatments were the ones that showed higher reductions in PTEs concentrations for all fractions compared to those measured in PS, especially in the water-soluble concentrations, suggesting that the reduction of soluble concentrations leads to a decrease in the toxicity risk to the ecosystem. In this regard, Rocha et al. (2011) pointed out that the ecotoxicological responses of these aquatic organisms to soil toxicity, as well as the adverse effects of the soil extracts on them, was related to the solubility of PTEs together with the soil properties controlling this fraction.

These findings emphasize that PTEs water-soluble fraction is a critical factor controlling the soil toxicity levels for organisms, with a greater influence than other PTEs fractions on them. Therefore, the distinct responses and sensitivities that organisms exhibit to PTEs may be dependent on the fraction to which they are primarily exposed. For these reasons, when evaluating soil toxicity risks and the changes induced by remediation actions on polluted soils, the combined assessment of PTEs fractionation and the ecotoxicological response of different target organisms is encouraged for a more accurate evaluation of the effects induced by the actions implemented and, hence, of their actual effectiveness on reducing soil pollution levels and the associated risks to the ecosystem.

6.5. Evolution of ecological succession in polluted soils after remediation

Ecological succession is the process by which the structure and composition of a biological community evolves over time following a disturbance (Walker et al 2010), including the interactions that occur among the main groups of soil organisms. In the GGC ecosystem, where the existing biological community was severely damaged and partly eliminated after the mining spill, the ecological succession occurred assisted by the remediation actions implemented in the years following the accident. However, this process occurred to varying degrees spatially, with some areas remaining where succession did not occur due to excessive pollution levels.

The remediation treatments evaluated aimed to facilitate and accelerate the natural ecological succession in areas where it was not naturally occurring by the extremely limiting conditions. The success of these treatments in triggering succession and facilitating the recovery of biological communities in the highly degraded soil was assessed by analyzing the status of these communities at various levels.

6.5.1. Vegetation development and adaptation on remediated soils

In ecological succession following a major disturbance, such as a mining spill, pioneer species recolonizing the degraded area during the early stages are predominantly annual plants, grasses and opportunistic species benefiting from the absence of competitors (Párraga-Aguado et al. 2013). The emergence of this primary vegetation is of great importance, as this species can act as "nurse plants", facilitating the subsequent colonization by other plant species less tolerant to stress conditions (Navarro-Cano et al. 2018). Moreover, the establishment of an initial vegetation cover is vital as it provides additional physical protection to the soil and enhances its physicochemical conditions, such as increasing organic carbon content and improving soil structure and stabilization, thus reducing the potential for pollutant particles to be redistributed by wind or water (Pérez-de-Mora et al. 2006b).

Therefore, when assessing the effectiveness of soil remediation treatments in polluted areas, passive restoration, defined as the natural recolonization of the polluted area by native vegetation that occurs along with the improvement in soil conditions, serves as a crucial indicator of soil pollution levels and the extent to which they are reduced by the treatments applied (Álvarez-Rogel et al. 2021). In consequence, the occurrence of this process in both the reference and the treated soils was measured in chapter 3 as an indicator of the effectiveness of different soil remediation treatments in facilitating the vegetation recovery in polluted soils.

In this context, PS showed a complete absence of vegetation growth due to its high levels of pollution, strong acidic pH, high EC, and very low OC, being these conditions extremely limiting for the natural establishment of vegetation. The application of the treatments to PS, which resulted in a general improvement of soil properties and a reduction in soil toxicity, was therefore essential to enable vegetation to colonize the soil, with varying degrees of success depending on the treatment. Among the treatments, those based on vermicompost showed the greatest vegetation cover and species richness, approaching the conditions present in RS. These treatments significantly increased the OC in the soil, addressing one of the most critical factors limiting plant growth in mine tailings sites (Pardo et al. 2017). Also, the addition of OC into the soil increased soil water retention capacity (chapter 2), which enhances plant productivity (Lal 2020). Overall, water-soluble and EDTA extractable PTEs fractions in all treatments were reduced to concentrations similar to RS and, consequently, were not limiting factors for vegetation growth. Thus, soil OC, which was associated with the extent of vegetation cover present in each treatment, emerged as the primary factor driving vegetation recovery in the treated soils. Vermicompost treatments were therefore the most effective in promoting plant recolonization and development on polluted soils, consistent with the findings of Sierra-Aragón et al. (2019) who observed a positive development of vegetation in PTEs polluted soils in the same area following the improvement of soil fertility produced by the addition of a vermicompost-based amendment. This underscores the importance of adding organic amendments to barren polluted soils to facilitate the reestablishment and recolonization by pioneer vegetation (Wong 2003).

In the studied soils, two native plant species, *Lamarckia aurea* and *Spergularia rubra,* identified as highly tolerant to PTEs pollution in the area, acted as pioneer species colonizing the treated soils. These species were strongly associated with soils exhibiting the highest levels of pollution, and were almost exclusively present in them, with *L. aurea* predominantly found in the first vegetation belt surrounding the PS, in agreement with García-Carmona et al. (2019a) who previously observed this vegetation distribution within the same study area. However, their dominance decreased as the soil conditions improved, allowing less pollution-tolerant species to grow, indicating a reduction in the toxicity levels. In relation to the vegetation status in the area, Pérez-de-Mora et al. (2011) carried out a detailed vegetation survey in an experimental area (20 x 50 m) within the GGC, detecting during three consecutive spring campaigns (2006-2008) a total of 53 vascular plant species, most of which were annual (37), although biennials (12) and perennials (4) were also present. In the present study, a mean of 12 different plant species growing in US subplots (4 m^2) were found. In RS subplots (4 m^2) the mean species richness was slightly reduced to 10 species, in line with the findings of García-Carmona et al. (2019a), who observed a similar plant diversity in these soils. Meanwhile, intermediate values for this parameter in the treated polluted soils were measured (proximate to 5 in treated soils without vermicompost addition, and about 7 in those with vermicompost addition).

Furthermore, the assessment of the PTEs accumulation capacity of *L. aurea and S. rubra* revealed that both species showed remarkably high accumulation capacity of PTEs, especially Pb and As, in both their roots and shoots, in accordance with previous observations (García-Carmona et al. 2019a). Notably, *L. aurea* demonstrated exceptional accumulation of Pb and As in its roots, which can explain its predominance in the soils with the most limited vegetation development and where no other plant species can thrive. The extraordinary PTEs accumulation capacity shown by these species indicates that they have developed adaptive physiological mechanisms enabling them to accumulate large amounts of PTEs without exhibiting toxicity symptoms (Mehes-Smith et al. 2013), and thus tolerate and thrive in severely polluted soil conditions. In consequence, *L. aurea* and *S. rubra* can be considered key species in the area, as their early presence in polluted soils can promote soil improvements and facilitate the subsequent recolonization of these soils by species less tolerant to stress conditions. Additionally, these species can serve as bioindicators of PTEs pollution in similar polluted areas.

The establishment of these pioneer species in treated soils is important not only as an indicator of soil pollution levels and as a mechanism for further soil improvement, but also because their presence may enhance microbial activity. They do this by providing soil microbial communities with suitable habitat and essential OC and nutrient contents, which are crucial for performing key soil processes (Álvarez-Rogel et al. 2021). Likewise, the development of the aboveground communities is often closely linked to the belowground communities in the soil, and interactions between vegetation and soil microorganisms, primarily mediated by soil conditions, have important implications for community structure and ecosystem functioning at the local scale (van der Putten et al. 2009). For this reason, when evaluating the rate of succession driven by treatments, it is important to take into account not only the aboveground processes, as reflected in vegetation response, but also those occurring belowground.

6.5.2. Soil microbiological status

Soil microbiology, to a great extent, governs the functioning of the ecosystems. Changes in the biomass, diversity, and metabolic features of soil microbial community are believed to reflect soil quality status of ecosystems (Zhu et al. 2012). Therefore, to develop effective bioremediation strategies and to study the impact of remediation actions comprehensively, it is worth including a thorough analysis of the soil's microbiological status. This was achieved by analyzing the composition, diversity, structure and functionality of microbial communities in some of the studied soils (chapter 4). Specifically, this was assessed in the reference soils, PS and RS, and in two selected treated soils using different remediation approaches: one with only an inorganic component, the marble amended soils, which stood out for being the most effective treatment in the preceding analyses by most significantly reducing PTEs concentrations and buffering the strongly acidic conditions of PS, leading to a higher reduction of soil toxicity according to the bioassays performed (chapter 2); and another one with only an organic component, the biopile technique, for being a treatment that assists in the incorporation into the polluted soil of the native microbial communities, present in the adjacent recovered soil, and for being a treatment that showed promising results in previous studies in the area for the reduction of soil toxicity (García-Carmona et al. 2017). In both cases, the microbiological assessment of these treatments was carried out when they were applied alone, so that no external source of microorganisms was added (i.e., without vermicompost addition).

The excess of PTEs concentrations in soils disrupt soil microbial activity, as observed through a toxicity bioassay used as a general approximation of the soil microbiological status. In this sense, a drastic reduction in soil heterotrophic respiration rate was detected in PS, aligning with the observed decreases in total abundances, community structure, and diversity of microbial communities in this soil. This confirms that microbial biomass and taxonomic diversity of soil communities were significantly affected by the presence of PTEs concentrations. Under such conditions, the microbial biomass may be reduced, as microorganisms may divert energy towards growth and cell maintenance to withstand pollutant stress (Minnikova et al. 2017). Furthermore, it is expected that the taxonomic diversity and abundance of microbial community structure will decrease under long-term PTEs pollution. The most sensitive microorganisms typically experience a sharp decrease in species and abundance, while resistant organisms increase their prevalence as they better adapt to the limiting conditions, thus causing shifts in community structure (Zhao et al. 2020).

In addition to PTEs concentrations, the very low OC and available water contents, along with a strongly acidic pH in PS, also altered the soil microbiota, leading to a decline in the total abundance, structure, and diversity of soil microorganisms. With regard to soil OC, the toxic effects of PTEs on soil microorganisms are greater in soils with low OC (Moreno et al. 2009), while a higher OC content in soil permits microbial populations cope with the stress induced by PTEs pollution. In addition, pH is considered an essential factor affecting the metabolic activities, diversity, and community composition of soil microorganism, with these parameters being particularly susceptible to acidic pH (Naz et al. 2022). Similarly, available water content has also been identified as a key factor in maintaining soil microbial communities, affecting their composition and activity, with low values leading to reduced microbial activity and abundance (Wu et al. 2017; Zhao et al. 2020). For all of the above, the soil microbiological quality of PS was low, negatively impacting a variety of soil biochemical processes and disrupting the nutrient cycling and capacity to perform key ecological functions (Giller et al. 1998), thereby hindering the establishment of vegetation in these soils.

On the other hand, the soil treatments for which their microbiological status was evaluated (marble and biopile) showed effectiveness in restoring soil quality, as the abundance and structure of microbial populations under both treatments were restored to levels proximate to those found in RS. Compared to PS, the marble and biopile-based treatments produced significant increases in pH and OC values, respectively, which were associated with significant reductions in the PTEs soluble fraction (chapter 4). Moreover, the available water content in soils treated with these techniques was expected to be significantly higher than in PS, according to the results in chapter 2, and especially in the case of marble, which is attributed with a high capacity for retaining bioavailable water by increasing water storage with a low matric potential (Simón et al. 2014). Thus, the improvements in these soil properties induced by the treatments were crucial for restoring the soil's microbiological status to conditions closer to those in RS, confirming their effectiveness in facilitating the successional change in the microbial community structure of the treated soils.

6.6. Potential of bioremediation for enhanced soil pollution remediation

Based on the results in chapters 2, 3, and 4, the highly polluted soils showed a severely restricted plant growth and a low soil microbiological quality, since these soils were characterized by a strongly acidic pH, high EC, poor soil structure, low water-holding capacity, lack of organic matter, and nutrient deficiency. However, the application of remediation treatments to the PS led to significant improvements in soil quality, reducing the mobility and toxicity of PTEs, and allowing vegetation to develop. Despite these improvements, the conditions and quality of the treated soils after remediation were still less favorable compared to the RS. Furthermore, there is a potential risk of PTEs remobilization in the treated soils related to the influence of the treatments, which should be monitored over time. This highlights that physicochemical techniques for the remediation of polluted soils are in some cases not enough to permanently alleviate pollution risks. Consequently, soil bioremediation processes may need to be implemented in parallel to promote the remedial effects of other applied techniques.

Microorganisms can provide effective and economically feasible solutions for enhancing soil cleanup and remediation, by improving soil quality and facilitating the establishment of vegetation under stress conditions. Arbuscular mycorrhizal fungi (AMFs), one of the most important groups of soil microorganisms and present in most terrestrial ecosystems forming symbiotic associations with the roots of most plants, are widely recognized for the potential benefit they provide to the soil-plant system, mainly through nutritional, functional, and structural improvements (Smith and Read 2008). In addition, their ability to immobilize, extract, and concentrate PTEs both in their structures and in the soil helps plants tolerate stress in polluted soils (Garg et al. 2017; Riaz et al. 2021).

Therefore, the potential of bioremediation treatments based on AMFs for enhancing the performance of the physicochemical remediation treatments applied to PTEs polluted soils was assessed in **chapter 5**. The remediation treatment that showed the highest overall effectiveness in the field-based in-situ remediation approach, the marble-based amendment, along with the organic amendment, the vermicompost, were selected to assess their potential for soil remediation combined with AMFs as a bioremediation element. Additionally, a second organic amendment consisting of dry olive reside (DOR) was included and evaluated in this bioremediation-based approach. This amendment was biotransformed by selected saprobic fungi prior to its application to eliminate the phytotoxic properties naturally present in this residue. The combined application of these selected remediation treatments with the inoculation of AMFs to the PS was evaluated under greenhouse conditions. The assessment focused on improvements in soil physicochemical and biological status, as well as on the survival and performance of a model plant (wheat) growing in the treated soils, to estimate soil toxicity levels and the bioprotective role induced by the AMFs.

Overall, the bioremediation approach based on AMFs showed promising results as a complement to physicochemical methods for enhancing their effectiveness in soil remediation processes. The application of the inorganic liming amendment alone (marble sludge) was effective in neutralizing the soil pH, thereby reducing the availability of highly mobile elements and allowing plants to survive in the PS, consistent with the results obtained in the field-based experiments (**chapters 2**, **3**, and **4**). However, it was less effective in promoting plant growth, improving soil biological status, and protecting plants against PTE uptake. In contrast, the combined application of inorganic and organic amendments together with AMF inoculation resulted in greater reductions of PTEs mobility, improved soil biological status by enhancing soil enzymatic activities, and promoted plant growth.

The greater reductions in PTEs bioavailability in the presence of AMFs were related to the higher phytostabilization enhanced by the fungi, increasing the retention of these elements in the hyphae and roots. The combined application of organic amendments with AMF inoculation further enhanced the mycorrhizostabilization of PTEs attributed to AMFs mechanisms, due to the synergistic effect produced by the positive impact of the organic matter on the growth of the AMF external mycelia (F.Y. Wang et al. 2013; Kohler et al. 2015). This promoted the production of chelating substances into the soil, such as glomalin and phytochelatins, which immobilize these elements in the mycorrhizosphere (García-Sánchez et al. 2021), as well as the immobilization of the PTEs in the fungal structures by physical and chemical processes, leading in both cases to the reduction in PTEs mobility (Meier et al. 2012; Faizan et al. 2024).

In addition, the application of organic amendments in combination with AMFs significantly enhanced the potential of these fungi to boost plant growth. These amendments provided significant amounts of OC and nutrients, crucial for promoting plant growth, and AMF inoculation improved plant tolerance to PTEs as well as the nutrition status through an increased absorption of nutrients (Arriagada et al. 2014; Bhantana et al. 2021). Furthermore, their combined application contributes to an increased diversity of soil microbial populations, promoting collaborative interactions between them and thus improving the performance of the bioremediation-based treatments (Sepehri and Sarrafzadeh 2018).

Altogether, the combination of the inorganic and organic amendments with AMFs inoculation led to the greatest plant performance in the treated polluted soil. This approach was more effective with DOR-based organic amendments compared to vermicompost, which exhibited a lower potential, and which may be mainly driven by an overall better quality of the biotransformed DOR. Compared to vermicompost, DOR showed lower EC and significantly higher levels of OC and nutrients, which are crucial for plant growth and stress tolerance (Siles et al. 2015; Johnson et al. 2022). Moreover, regarding soil biological status, the significantly higher OC and nutrients content in DOR, combined with its reduced amount of toxic compounds after biotransformation with saprobic fungi (García-Sánchez et al. 2019), exerted great influence in enhancing soil enzymatic activities. In contrast, vermicompost had a minimal effect on these activities, probably related to its significantly lower OC.

Regarding PTEs uptake, treatments combining organic amendments and AMF inoculation were more effective in significantly reducing PTEs in plant shoots compared to the liming treatment alone, especially when using DOR as organic amendment. Conversely, all treatments showed high PTEs accumulation in plant roots, highlighting their effectiveness in preventing the transfer of pollutants to the aboveground part of the plants, as previously observed by García-Sánchez et al. (2017) using similar treatments. This significantly higher PTES accumulation in root tissues was attributed to AMF mechanisms that restrict their translocation to shoots and consequently promote the phytoextraction process in plant roots (Singh et al. 2019). Moreover, the organic amendments contributed to improve plant nutrition, which promoted roots growth and increased soil colonization, thereby enhancing the plant's ability to accumulate and immobilize greater amounts of PTEs.

Overall, the bioremediation treatments tested were effective in increasing the potential of physicochemical treatments for remediating the PTE-polluted soils. Particularly, the joint implementation of biotransformed DOR and AMF inoculation further enhanced their efficacy through synergistic effects, promoting both the immobilization of PTEs in soil and stimulating the phytoextraction mechanisms induced by AMFs. This combined application increased plant tolerance to PTEs and resulted in a better growth of wheat plants under adverse soil conditions. Therefore, the bioremediation approach evaluated could represent a reliable soil remediation strategy, supporting the successful recovery of the microbial populations and the establishment of the plant communities in the treated soils, thus facilitating the restoration of soil functions in polluted areas.

General conclusions

1. Soils residually polluted in the Guadiamar Green Corridor (GGC) have limited recovery potential due to the elevated potentially toxic elements (PTEs) concentrations, low organic matter content, high acidity, and salinity in these soils. Liming amendments from industrial wastes applied to polluted soils in situ, together with vermicompost, were the most effective treatments in improving soil properties and controlling PTEs mobility. Neutralization of soil acidity was key in controlling the solubility and bioavailability of PTEs. Consequently, the use of gypsum mining spoil and, above all, marble sludge as amendment represents an efficient and low-cost solution for the remediation of soils polluted by PTEs.

2. The assessment of ecotoxicological response in multiple organisms is crucial to accurately evaluate the effect of the remediation techniques under natural, multi-stress conditions. According to both liquid- and solid-phase ecotoxicity tests applied, marble-based treatments were the most effective in reducing soil toxicity due to their strong pH neutralization, which reduced PTEs solubility and minimized toxicity risks. Liquid-phase tests showed a higher sensitivity to soil toxicity, so their use should be preferred in ecotoxicity studies, without excluding the application of solid-phase tests.

3. The treatments applied to the PTE-polluted soils in the GGC were highly effective in promoting spontaneous vegetation growth by improving soil properties and reducing PTEs availability. In this regard, vermicompost was particularly effective due to the improved fertility resulting from the increase in soil organic carbon, leading to greater plant diversity and increased vegetation cover.

4. Two native plant species, *Spergularia rubra* and *Lamarckia aurea*, exhibited remarkable ability to accumulate Pb and As in their roots. They can be considered key species in the studied area, as they not only serve as pioneers in recolonizing the degraded soils but also facilitate the subsequent recolonization by other species less tolerant to high levels of pollution. The success of the remediation strategy is therefore promoted by the early recolonization of the soil by these highly pollution-tolerant species, which enhance further evolution of the soil physical, biological, and chemical conditions.

5. Biopile and marble-based treatments significantly influenced soil microbial communities, especially at the bacterial genus level, which is effective for detecting disturbances caused by PTEs in soil. These treatments significantly increased the abundances of total bacterial and archaeal populations, as well as key functional groups like ammonium-oxidizing bacteria and denitrifiers, demonstrating their effectiveness in restoring soil microbial community.

6. The combined study of soil toxicity and the structure of vegetation and bacterial communities following remediation actions highlights the close relationships between soil properties, vegetation status, microbial activity, and toxicity bioassays. These factors can be applied as tools for soil quality assessment and planning remediation strategies for soils polluted by PTEs.

7. The remediation process can be improved by the application of biotransformed dry olive residue (DOR) by saprobic fungi, which combined with marble-based amendment represents a highly effective organic treatment for remediating PTE-polluted soils. This combination showed high effectiveness in improving soil physicochemical and biological status, promoting plant growth and survival, and reducing PTEs toxicity and plant uptake.

8. A bioremediation approach combining marble, biotransformed DOR, and arbuscular mycorrhizal fungi (AMFs) enhances PTEs immobilization in polluted soils by stimulating the phytostabilization process induced by AMFs. This synergy improves plant protection and increases the overall effectiveness of the remediation process, representing a promising sustainable bioremediation strategy for restoring soil functions and reducing toxicity in areas polluted by PTEs.

Conclusiones generales

1. Los suelos residualmente contaminados del Corredor Verde del Guadiamar (GGC) tienen un potencial de recuperación limitado debido a las elevadas concentraciones de elementos potencialmente tóxicos (PTEs), el bajo contenido en materia orgánica, y la elevada acidez y salinidad de estos suelos. Las enmiendas encalantes procedentes de residuos industriales aplicadas a los suelos contaminados in situ, junto con vermicompost, fueron los tratamientos más eficaces para mejorar las propiedades del suelo y controlar la movilidad de los PTEs. En consecuencia, el uso de residuos de yeso y, especialmente, de lodos del corte y pulido de mármol como enmiendas representa una solución eficaz y de bajo coste para la remediación de suelos contaminados por PTEs.

2. La evaluación de la respuesta ecotoxicológica en un grupo diverso de organismos es crucial para evaluar con precisión el efecto de las técnicas de remediación bajo condiciones naturales con factores de estrés múltiples. De acuerdo con las pruebas de ecotoxicidad aplicadas tanto en fase líquida como sólida, los tratamientos basados en mármol fueron los más eficaces para reducir la toxicidad del suelo debido a su fuerte efecto neutralizador del pH. Esto redujo la solubilidad de los PTEs, minimizando así los riesgos de toxicidad. Las pruebas en fase líquida mostraron una mayor sensibilidad a la toxicidad del suelo, por lo que su uso en estudios de ecotoxicidad debería ser preferente, sin excluir la aplicación de pruebas en fase sólida.

3. Los tratamientos de remediación aplicados a los suelos del GGC contaminados por PTEs mostraron una elevada eficacia para promover el crecimiento espontáneo de la vegetación al mejorar las propiedades del suelo y reducir la disponibilidad de los PTEs. El vermicompost resultó ser especialmente eficaz debido a la mejora en la fertilidad del suelo por el aumento en el contenido de carbono orgánico, lo que propició una mayor diversidad y cubierta vegetal en los suelos tratados con esta enmienda.

4. Dos especies nativas de plantas, *Spergularia rubra* y *Lamarckia aurea*, mostraron una notable capacidad para acumular Pb y As en sus raíces. Estas pueden considerarse especies clave en la zona de estudio, dado que no solo actúan como especies pioneras en la recolonización de los suelos degradados, sino que facilitan la posterior recolonización por parte de otras especies menos tolerantes a los altos niveles de contaminación. Por tanto, el éxito de la estrategia de remediación se ve favorecido por la

pronta recolonización del suelo por estas especies altamente tolerantes a la contaminación, cuya presencia potencia la evolución y mejora de las condiciones físicas, biológicas y químicas del suelo.

5. Los tratamientos basados en biopilas y en mármol influyeron significativamente en las comunidades microbianas del suelo, especialmente a nivel de género bacteriano, el cual puede ser útil para detectar alteraciones causadas por los PTEs en el suelo. Estos tratamientos aumentaron significativamente la abundancia de las poblaciones bacterianas y arqueas totales, así como grupos funcionales clave como las bacterias oxidantes del amonio y las desnitrificantes, lo que demuestra la eficacia de estos tratamientos en la restauración de la comunidad microbiana del suelo.

6. El estudio combinado de la toxicidad del suelo y la estructura de las comunidades vegetales y bacterianas tras las medidas de remediación pone de manifiesto las estrechas relaciones existentes entre las propiedades del suelo, el estado de la vegetación, la actividad microbiana y los bioensayos de toxicidad. Estos factores pueden ser utilizados como herramientas para la evaluación de la calidad del suelo y la planificación de estrategias de remediación de suelos contaminados por PTEs.

7. La aplicación de alperujo (DOR) biotransformado por hongos saprobios puede mejorar el proceso de remediación de suelos contaminados por PTEs, el cual combinado con mármol destacó como un tratamiento orgánico altamente eficaz para dicho fin. Esta combinación demostró una elevada eficacia en la mejora de las estado fisicoquímico y biológico del suelo, promoviendo la supervivencia y el crecimiento de las plantas, así como reduciendo la toxicidad de los PTEs y su acumulación en las plantas.

8. Un enfoque de biorremediación basado en la combinación de mármol, DOR biotransformado y hongos micorrícicos arbusculares (AMFs) potencia la inmovilización de los PTEs en el suelo contaminado al estimular el proceso de fitoestabilización inducido por los AMFs. Esta sinergia mejora la protección de las plantas y aumenta la eficacia global del proceso de remediación, mostrando así un elevado potencial como estrategia de biorremediación sostenible para restaurar las funciones del suelo y reducir la toxicidad en zonas contaminadas por PTEs.

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Supplementary materials

Chapter 2. Supplementary materials

Table S2.1. Guideline values (mg kg^{-1}) to declare a soil as potentially polluted by legislations of different countries.

Element	Spain		China [3]	Canada [4]	Portugal [5]
	Andalusia [1]	Galicia [2]			
As	36	50	15	12	11
Cd	25	2 (1)	0.2	1.4	1
Cu	595	50	35	63	62
Pb	275	100 (80)	35	70	45
Zn	10,000	300 (200)	100	200	290

The value presented is the most restrictive by land use: Andalusia - other uses (generally, agricultural), Galicia the same (in brackets ecosystem protection value), Canada - agricultural and/or residential/parkland use, Portugal - agricultural use, and China - Level I soils.

[1] Generic Reference Value (NGR) for trace elements in the Region of Andalusia. BOJA (Boletín Oficial de la Junta de Andalucía). (2015). *Decreto 18/2015, por el que se aprueba el reglamento que regula el régimen aplicable a los suelos contaminados*. BOJA, 38, 28-64.http://www.juntadeandalucia.es/medioambiente/web/2012_provisional/2015/reglamento_suelos_contaminados.pdf

[2] Generic Reference Value (NXR) for contaminants in the Region of Galicia. DOG (Diario Oficial de Galicia). (2009). *Decreto 60/2009, do 26 de febreiro, sobre solos potencialmente contaminados e procedemento para a declaración de solos contaminados*. DOG, 57, 5920-5936. https://www.lex.gal/galilex/4384

[3] Environmental quality standards for soils (HM) in China. MEEPRC (Ministry of Ecology and Environment of the People's Republic of China). (2018). *Soil Environmental Quality Risk. Control Standard for Soil Contamination of Agricultural Land (GB 15618–2018)*. https://www.fao.org/faolex/results/details/es/c/LEX-FAOC136767/

[4] Canadian Soil Quality Guidelines. CCME (Canada Council of Ministers of the Environment). (2007). *Canadian Soil Quality Guidelines for the Protection of Environmental and Human Health: Summary Tables* (Updated September, 2007). https://support.esdat.net/Environmental%20Standards/canada/soil/rev_soil_summary_tbl_7.0_e.pdf

[5] Reference values for main soil pollutants in Portugal. APA (Agência Portuguesa do Ambiente). (2019). *Solos Contaminados - Guia Técnico - Valores de referência para solo. Revisão 3 - Setembro de 2022*. Agência Portuguesa do Ambiente. https://sniambgeoviewer.apambiente.pt/GeoDocs/geoportaldocs/AtQualSolos/Guia_Tecnico_Valores%20de%20Referencia_2019_01.pdf

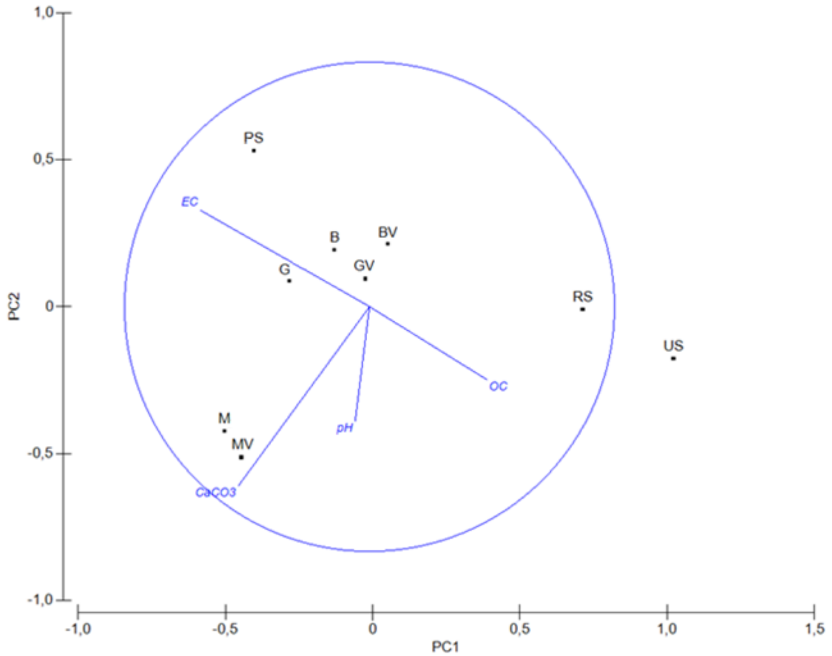

Figure S3.1. PCA analysis of main soil properties related to soils and treatments.

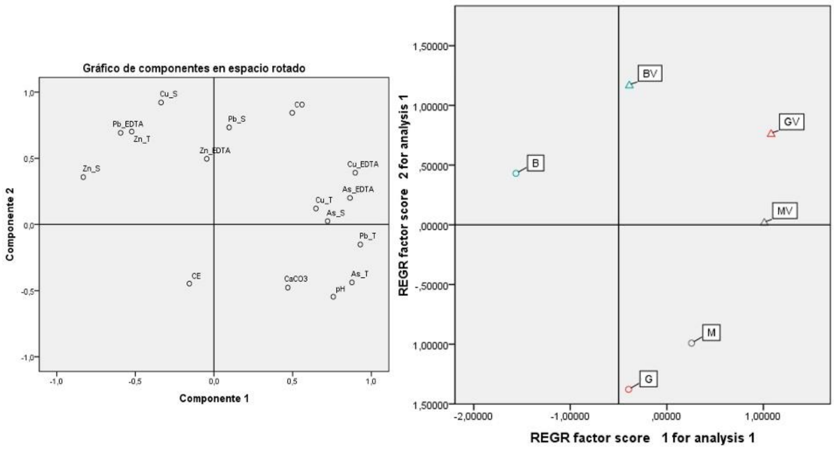

Figure S3.2. PCA analysis of main soil properties and metal concentrations related to treatments.

Table S3.1. Chemical properties and element content in organic and inorganic amendments. Chemical properties: pH; EC: electrical conductivity; OC: organic carbon; $CaCO_3$: calcium carbonates. Potentially Toxic Elements (PTEs): Cu, Zn, As and Pb. Letter "T" after PTEs refers to total concentrations. NA= Not Available.

Properties	Biopile (mean±SD)	Vermicompost [1] (mean±SD)	Marble sludge [2] (mean±SD)	Gypsum mining spoil [3] (mean±SD)
pH	4.28 ± 0.48	7.88 ± 0.12	8.27±0.13	7.50 ± 0.20
EC (dS m^{-1})	0.98 ± 0.61	4.18 ± 0.25	1.13 ± 0.08	2.90 ± 0.04
OC (%)	3.68 ± 1.46	23.16 ± 1.87	0.16 ± 0.11	0.04 ± 0.03
$CaCO_3$ (%)	0.52 ± 0.25	NA	99.99 ± 0.01	27.25 ± 3.52
Cu_T (mg kg^{-1})	216.8 ± 127.2	44.38 ± 3.56	4.01 ± 0.03	10.10 ± 0.84
Zn_T (mg kg^{-1})	416.7 ± 229.6	17.18 ± 2.58	5.45 ± 0.80	18.07 ± 2.06
As_T (mg kg^{-1})	279.7 ± 44.7	1.81 ± 0.27	0.77 ± 0.17	3.50 ± 0.41
Pb_T (mg kg^{-1})	501.3 ± 142.3	3.90 ± 0.72	2.44 ± 0.35	4.39 ± 0.73

[1]Nakamaru YM, Martín-Peinado FJ (2017) Effect of soil organic matter on antimony bioavailability after the remediation process. Environ Pollut 228:425-432. https://doi.org/10.1016/j.envpol.2017.05.042

[2]Aguilar-Garrido A, Paniagua-López M, Sierra-Aragón M, Martínez-Garzón FJ, Martín-Peinado FJ (2023) Remediation potential of mining, agro-industrial, and urban wastes against acid mine drainage. Sci Rep 13:12120. https://doi.org/10.1038/s41598-023-39266-4

[3]Ballesteros-Jiménez M (2018) Restoration of gypsum habitats affected by quarrying: Guidance for assisting vegetation recovery. Granada: Doctoral Thesis, Universidad de Granada, 2018. Available at: http://digibug.ugr.es/handle/10481/49583

Table S3.2. Spearman's significant correlations between PTEs concentrations in plants and their total and bioavailable concentrations in soils.

	Spergularia rubra		*Lamarckia aurea*	
	Pb_Shoot	Pb_Root	Pb_Shoot	Pb_Root
Pb_T	0.637**	0.734**	0.484*	0.536**
	As_Shoot	As_Root	As_Shoot	As_Root
As_T	0.610**	0.489*	0.825**	0.619**
	Zn_Shoot	Zn_Root	Zn_Shoot	Zn_Root
Zn_T	0.837**	0.888**	0.592**	0.616**
	Cu_Shoot	Cu_Root	Cu_Shoot	Cu_Root
Cu_T	0.463**	0.610**	- -	0.530**
	Zn_Shoot	Zn_Root	Zn_Shoot	Zn_Root
Zn_W	0.645**	0.576**	0.576**	0.424*
	Pb_Shoot	Pb_Root	Pb_Shoot	Pb_Root
Pb_EDTA	-0.554**	-0.460*	-0.537**	-0.576**
	As_Shoot	As_Root	As_Shoot	As_Root
As_EDTA	0.487*	0.471*	0.553**	0.749**
	Zn_Shoot	Zn_Root	Zn_Shoot	Zn_Root
Zn_EDTA	0.543**	0.669**	- -	- -
	Cu_Shoot	Cu_Root	Cu_Shoot	Cu_Root
Cu_EDTA	0.462*	0.700**	- -	0.450*

Chapter 4. Supplementary materials

Table S4.1. Primers used for the quantification of the abundance of selected microorganism in soil samples.

Group	Gene marker	Size (bp)	Primer		References
			Name	Sequence	
Total *Bacteria*	V3-16S rRNA	194	P1 (341F) P2 (534R)	CCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	Muyzer et al. (1993)
AOB	*amo*A	490	AmoA1F AmoA1R	GGGGTTTCTACTGGTGGT CCCCTCKGSAAAGCCTTCTTC	Rotthauwe et al. (1997)
Denitrifiers	*nosZ*	276	nosZ1840F nosZ2090R	CGCRACGGCAASAAGGTSMSSGT CAKRTGCAKSGCRTGGCAGAA	Henry et al. (2006)
Total *Archaea*	16S rRNA	456	UNI-b-rev ARCH915	GA GGGCGGTGTGTRCAA AGGAATTGGCGGGGGAGCAC	Yu et al. (2008)

Table S4.2. qPCR cycling conditions.

		Total *Bacteria*	AOB	Denitrifiers	Total *Archaea*
Amplification (40 cycles)	Initial denaturalization	95ºC, 3 minutes	95ºC, 7 minutes	95ºC, 10 minutes	72ºC, 7 minutes
	Denaturalization	95ºC, 30 seconds	95ºC, 30 seconds	95ºC, 15 seconds	95ºC, 30 seconds
	Primers annealing	60ºC, 40 seconds	52ºC, 30 seconds	65ºC, 30 seconds	60ºC, 30 seconds
	Elongation	72ºC, 30 seconds	72ºC, 30 seconds	72ºC, 30 seconds	72ºC, 30 seconds
	Final extension		72ºC, 5 minutes		95ºC, 7 minutes

Table S4.3. Number of sequences and OTUs and alpha diversity indices (n=3) of the Illumina high-throughput sequencing for the different soil fractions in limed soil (M), biopile soil (B), control soil (CT) and recovered soil (RS). According to the Kruskal-Wallis and Conover-Iman tests (p<0.05), different letters indicates significant differences among samples.

	Number of sequences	Number of OTUs	Simpson	Shannon
M	22861 ± 1960	2775 ± 90 B	0.01 ± 0.001 A	6.36 ± 0.11 A
B	24433 ± 1630	2967 ± 651 A	0.01 ± 0.021 A	6.20 ± 0.44 A
CT	23641 ± 711	741 ± 295 AB	0.14 ± 0.094 A	3.61 ± 1.02 A
RS	6146 ± 1207	2008 ± 322 B	0.01 ± 0.003 A	6.54 ± 0.19 A

Table S4.4. Average abundance (n=3) ± standard deviation of phyla belonging to the *Archaea* domain by Illumina high-throughput sequencing for the different soil fractions in limed soil (M), biopile soil (B), control soil (CT) and recovered soil (RS). According to the Kruskal-Wallis and Conover-Iman tests (p<0.05), different letters indicate significant differences among samples. n.d.: non detected.

	M	B	CT	RS
Archaea_unclassified	0.07 ± 0.07 A	n.d. A	n.d. A	n.d. A
Euryarchaeota	3.26 ± 3.26 A	0.04 ± 0.04 A	n.d. A	1.98 ± 0.17 A
Thaumarchaeota	96.67 ± 3.33 B	99.96 ± 0.04 B	100 ± 0.00 A	98.02 ± 0.17 AB

Table S4.5. Average abundance (n=3) ± standard deviation of genera classified within the *Archaea* domain by Illumina high-throughput sequencing for the different soil fractions in limed soil (M), biopile soil (B), control soil (CT) and recovered soil (RS). According to the Kruskal-Wallis and Conover-Iman tests (p<0.05), different letters indicate significant differences among samples. n.d.: non detected and *: not classified into the genus level.

	M	B	CT	RS
Archaea_unclassified*	0.07 ± 0.07 A	n.d. A	n.d. A	n.d. A
Euryarchaeota_unclassified*	n.d. B	0.02 ± 0.02 B	n.d. B	1.45 ± 0.08 A
Methanomicrobiales_unclassified*	0.70 ± 0.70 A	n.d. A	n.d. A	n.d. A
Methanoregula	0.32 ± 0.32 A	n.d. A	n.d. A	n.d. A
Methanosaeta	2.24 ± 2.24 A	n.d. A	n.d. A	n.d. A
Thermoplasmata_unclassified*	n.d. B	0.02 ± 0.02 B	n.d. B	0.55 ± 0.09 A
Candidatus_Nitrososphaera	0.14 ± 0.07 B	0.06 ± 0.02 B	n.d. B	0.61 ± 0.35 A
Nitrososphaeraceae_unclassified*	96.53 ± 3.37 A	99.91 ± 0.05 A	100 ± 0.00 A	97.40 ± 0.19 A

Table S4.6. Average abundance (n=3) ± standard deviation of phyla within the *Bacteria* domain by Illumina high-throughput sequencing for the different soil fractions in limed soil (M), biopile soil (B), control soil (CT) and recovered soil (RS). According to the Kruskal-Wallis and Conover-Iman tests (p<0.05), different letters indicate significant differences among samples. n.d.: non detected.

	M	B	CT	RS
Acidobacteria	3.56 ± 0.20 A	3.12 ± 1.03 A	4.32 ± 2.29 A	5.61 ± 0.12 A
Actinobacteria	25.71 ± 1.34 A	29.90 ± 3.40 A	26.80 ± 0.04 A	32.66 ± 0.71 A
Armatimonadetes	0.16 ± 0.03 A	0.07 ± 0.04 A	0.04 ± 0.04 A	0.12 ± 0.03 A
Bacteroidetes	10.80 ± 0.32 A	4.81 ± 1.88 A	4.18 ± 2.35 B	7.04 ± 0.52 AB
BRC1	0.05 ± 0.02 A	n.d. B	n.d. B	0.02 ± 0.1 AB
Chlamydiae	0.04 ± 0.01 A	0.01 ± 0.01 B	n.d. B	0.03 ± 0.01 A
Chloroflexi	3.43 ± 0.09 A	2.32 ± 1.09 A	0.84 ± 0.61 A	2.15 ± 0.10 A
Cloacimonetes	0.01 ± 0.01 A	n.d. A	n.d. A	n.d. A
Cyanobacteria	1.03 ± 0.35 A	0.56 ± 0.41 A	0.03 ± 0.03 B	0.18 ± 0.01 A
Deinococcus-Thermus	0.43 ± 0.12 A	0.07 ± 0.05 B	n.d. B	0.01 ± 0.01 B
FBP	0.01 ± 0.01 A	0.01 ± 0.01 A	n.d. A	0.03 ± 0.02 A
Fibrobacteres	0.10 ± 0.01 A	0.01 ± 0.01 B	n.d. B	0.01 ± 0.01 B
Firmicutes	1.31 ± 0.15 A	7.08 ± 2.28 A	5.22 ± 4.93 A	1.33 ± 0.08 A
Gemmatimonadetes	1.57 ± 0.18 A	1.68 ± 0.76 A	0.27 ± 0.26 A	1.14 ± 0.13 A
Nitrospirae	0.35 ± 0.03 A	0.19 ± 0.11 AB	n.d. B	0.06 ± 0.03 B
Patescibacteria	5.19 ± 0.78 A	3.88 ± 1.27 A	1.66 ± 1.51 A	2.45 ± 0.51 A
Planctomycetes	2.02 ± 0.97 A	1.59 ± 0.56 A	0.32 ± 0.31 A	1.04 ± 0.11 A
Proteobacteria	29.39 ± 0.97 A	20.53 ± 5.81 A	45.03 ± 25.33 A	32.02 ± 0.67 A
Spirochaetes	0.02 ± 0.01 A	n.d. A	n.d. A	n.d. A
Tenericutes	n.d. A	n.d. A	n.d. A	0.03 ± 0.01 A
Verrucomicrobia	1.20 ± 0.12 C	0.51 ± 0.27 C	0.10 ± 0.08 B	0.71 ± 0.10 A
Bacteria_unclassified	136.63 ± 0.90 A	23.66 ± 13.28 BC	11.19 ± 6.08 C	13.35 ± 021 B

Table S4.7. Average abundance (n=3) ± standard deviation of genera classified within the *Bacteria* domain by Illumina high-throughput sequencing for the different soil fractions in limed soil (M), biopile soil (B), control soil (CT) and recovered soil (RS). According to the Kruskal-Wallis and Conover-Iman tests (p<0.05), different letters indicate significant differences among samples. n.d.: non detected and *: not classified into the genus level.

	M	B	CT	RS
Pyrinomonadaceae_RB41*	n.d. A	0.32 ± 0.23 A	2.90 ± 2.47 A	0.92 ± 0.48 A
Subgroup_6_unclassified*	2.12 ± 0.19 A	1.24 ± 0.69 A	0.80 ± 0.79 A	1.86 ± 0.24 A
Acidimicrobiia_unclassified*	0.87 ± 0.03 A	1.61 ± 0.91 A	0.27 ± 0.25 A	0.55 ± 0.13 A
Microtrichales_unclassified*	0.39 ± 0.04 A	0.69 ± 0.20 A	0.73 ± 0.73 A	0.66 ± 0.02 A
Actinobacteria_unclassified*	1.40 ± 0.18 A	1.68 ± 0.31 A	7.62 ± 6.83 A	4.08 ± 0.48 A
Blastococcus	1.01 ± 0.07 A	0.67 ± 0.23 AB	0.15 ± 0.14 B	0.55 ± 0.09 B
Geodermatophilus	0.17 ± 0.04 A	0.68 ± 0.34 A	0.62 ± 0.44 A	0.58 ± 0.09 A
Intrasporangiaceae_unclassified*	0.69 ± 0.11 A	1.59 ± 0.54 A	0.20 ± 0.12 B	0.18 ± 0.03 B
Microbacteriaceae_unclassified*	0.18 ± 0.02 A	1.79 ± 0.37 A	0.87 ± 0.86 A	1.21 ± 0.15 A
Arthrobacter	2.94 ± 0.59 A	2.33 ± 1.14 A	0.19 ± 0.18 A	0.60 ± 0.19 A
Micrococcaceae_unclassified*	0.56 ± 0.07 A	1.47 ± 0.82 A	0.14 ± 0.13 A	0.24 ± 0.07 A
Pseudarthrobacter	4.74 ± 0.59 A	3.34 ± 1.49 A	1.44 ± 1.42 A	0.87 ± 0.33 A
Micrococcales_unclassified*	0.70 ± 0.13 A	0.76 ± 0.34 A	2.41 ± 2.09 B	0.84 ± 0.07 B
Actinoplanes	0.81 ± 0.18 A	0.21 ± 0.11 A	0.39 ± 0.24 A	0.87 ± 0.17 A
Nocardioidaceae_unclassified*	1.31 ± 0.50 A	0.08 ± 0.03 A	0.41 ± 0.41 A	0.05 ± 0.01 A
Nocardioides	3.72 ± 0.30 A	0.84 ± 0.33 A	1.32 ± 1.32 A	2.05 ± 0.44 A
Cutibacterium	0.01 ± 0.01 B	0.01 ± 0.01 B	1.70 ± 0.78 A	0.01 ± 0.01 B
Pseudonocardia	0.03 ± 0.01 A	0.42 ± 0.21 A	3.33 ± 2.63 A	1.39 ± 0.05 A
Actinobacteria_unclassified*	1.39 ± 0.17 B	2.80 ± 0.31 B	0.17 ± 0.17 C	2.60 ± 0.22 A
Solirubrobacter	1.04 ± 0.08 B	1.76 ± 0.33 AB	0.64 ± 0.64 B	3.59 ± 0.30 A
Bacteroidia_unclassified*	2.15 ± 0.04 A	1.01 ± 0.45 A	0.93 ± 0.43 A	1.24 ± 0.17 A
Chitinophagaceae_unclassified*	2.95 ± 0.17 A	1.55 ± 0.73 A	1.41 ± 1.34 A	3.37 ± 0.46 A
Microscillaceae_unclassified*	2.33 ± 0.11 A	0.93 ± 0.44 B	0.20 ± 0.09 B	1.07 ± 0.11 B
Chloroflexi_unclassified*	1.46 ± 0.15 A	0.68 ± 0.33 B	0.30 ± 0.15 B	0.64 ± 0.06 B
Bacillaceae_unclassified*	0.13 ± 0.02 B	1.51 ± 0.13 A	0.22 ± 0.22 B	0.68 ± 0.08 A
Bacillales_unclassified*	0.11 ± 0.01 A	0.96 ± 0.47 A	0.47 ± 0.37 A	0.15 ± 0.03 A
Sulfobacillus	0.02 ± 0.01 A	0.04 ± 0.03 A	1.95 ± 1.95 A	n.d. A
Gemmatimonadaceae_unclassified*	1.13 ± 0.11 A	1.17 ± 0.50 A	0.10 ± 0.08 A	0.90 ± 0.03 A
Saccharimonadales_unclassified*	4.68 ± 0.76 A	3.51 ± 1.25 A	1.62 ± 1.51 A	2.10 ± 0.37 A
Acetobacteraceae_unclassified*	0.35 ± 0.01 A	1.79 ± 1.38 A	0.06 ± 0.06 A	0.27 ± 0.12 A
Alphaproteobacteria_unclassified*	1.07 ± 0.09 A	1.40 ± 0.68 A	2.74 ± 0.46 A	3.41 ± 0.20 A

Beijerinckiaceae_unclassified*	0.93 ± 0.17 A	0.41 ± 0.20 A	0.25 ± 0.25 A	0.87 ± 0.08 A
Rhizobiales_unclassified*	1.38 ± 0.24 A	2.52 ± 1.23 A	18.57 ± 17.25 A	6.95 ± 0.39 A
Rhodobacteraceae_unclassified*	1.47 ± 0.15 A	0.20 ± 0.03 AB	0.15 ± 0.03 B	0.12 ± 0.04 B
Sphingomonadaceae_unclassified*	1.35 ± 0.15 A	0.78 ± 0.37 A	0.10 ± 0.10 B	0.67 ± 0.13 AB
Sphingomonas	3.14 ± 0.45 A	2.41 ± 1.15 A	0.66 ± 0.64 A	1.29 ± 0.07 A
Burkholderiaceae_unclassified*	7.35 ± 0.36 A	3.47 ± 0.80 A	4.40 ± 1.73 A	5.11 ± 0.23 A
Nitrosospira	0.74 ± 0.03 A	0.17 ± 0.08 A	0.75 ± 0.39 A	0.12 ± 0.01 A
Proteobacteria_unclassified*	0.53 ± 0.09 A	0.33 ± 0.04 A	10.21 ± 9.83 A	0.68 ± 0.13 A
Bacteria_unclassified*	13.69 ± 0.90 A	23.72 ± 13.28 A	11.19 ± 6.08 A	13.36 ± 0.21 A

Table S4.8. Pearson product-moment correlation coefficients (r) between NMS vectors in Figure 4.5, which represent the physicochemical properties and the gene copy number per gram of soil of total *Bacteria* and *Archaea*, ammonium oxidizing bacteria (AOB) and nosZ-bearing bacteria (denityfying) in soils. (pH; EC: electric conductivity (dS m^{-1}); SOC: soil organic carbon (g kg^{-1}); $CaCO_3$ (%); Silt, Clay and Sand (%); CIC: cation exchange capacity ($cmol_{(+)}$ kg^{-1}); Cu_T, Zn_T, As_T and Pb_T: total trace elements concentrations (mg kg^{-1} dry soil); Cu_W, Zn_W, As_W and Pb_W: water soluble trace elements concentrations ($cmol_{(+)}$ kg^{-1}); Cu_EDTA, Zn_EDTA, As_EDTA and Pb_EDTA: bioavailable fraction of trace elements extracted with EDTA ($cmol_{(+)}$ kg^{-1}). Correlations ≥ 0.7 are black boldfaced and correlations ≤ -0.7 are red boldfaced.

	Total *Bacteria*	Total *Archaea*	AOB	Denitrifying
pH	**0.99**	**0.72**	0.59	**0.88**
EC	**-0.99**	**-0.89**	**-0.80**	**-0.71**
$CaCO_3$	0.49	**0.90**	**0.96**	-0.14
SOC	**0.98**	**0.90**	**0.82**	0.68
Silt	-0.60	-0.03	0.14	**-0.96**
Clay	**0.73**	0.20	0.03	**0.99**
Sand	0.53	-0.06	-0.22	**0.94**
CIC	**0.99**	**0.70**	0.57	**0.89**
Cu_T	**0.86**	**1.00**	**0.97**	0.38
Zn_T	**0.94**	**0.96**	**0.90**	0.55
As_T	**-0.75**	-0.23	-0.06	**-1.00**
Pb_T	**-0.98**	-0.69	-0.56	**-0.90**
Cu_W	**-0.93**	-0.55	-0.40	**-0.96**
Zn_W	**-0.99**	**-0.72**	-0.59	**-0.88**
As_W	-0.56	0.02	0.19	**-0.95**
Pb_W	**-1.00**	**-0.76**	-0.64	**-0.85**
Cu_E	**0.98**	**0.92**	**0.84**	0.66
Zn_E	**-1.00**	**-0.81**	-0.69	**-0.81**
As_E	0.66	0.10	-0.07	**0.98**
Pb_E	**0.77**	0.25	0.09	**1.00**

Table S4.9. Pearson product-moment correlation coefficients (r) between the NMS vectors in Figure 4.6, which represent the physicochemical properties and the dominant bacterial genera (RA>0.5%) in soils. pH; EC: electric conductivity (dS m^{-1}); SOC: soil organic carbon (g kg^{-1}); $CaCO_3$ (%); Silt, Clay and Sand (%); CIC: cation exchange capacity (cmol(+) kg^{-1}); Cu_T, Zn_T, As_T and Pb_T: total trace elements concentrations (mg kg^{-1} dry soil); Cu_W, Zn_W, As_W and Pb_W: water soluble trace elements concentrations (cmol(+) kg^{-1}); Cu_EDTA, Zn_EDTA, As_EDTA and Pb_EDTA: bioavailable fraction of trace elements extracted with EDTA (cmol(+) kg^{-1}). Correlations ≥ 0.7 are black boldfaced and correlations ≤ -0.7 are red boldfaced.

	pH	EC	$CaCO_3$	SOC	Silt	Clay	Sand	CIC	Cu_T	Zn_T	As_T	Pb_T	Cu_S	Zn_S	As_S	Pb_S	Cu_E	Zn_E	As_E	Pb_E
Pyrinomonadaceae_RB41	0,62	-0,37	-0,51	0,34	**-0,99**	**0,96**	**1,00**	0,64	-0,01	0,18	**-0,95**	-0,65	**-0,78**	-0,63	**-1,00**	-0,57	0,31	-0,51	**0,98**	**0,94**
Subgroup_6_unclassified	-0,29	0,01	**0,79**	0,03	**0,88**	**-0,78**	**-0,91**	-0,31	0,37	0,19	**0,76**	0,33	0,50	0,30	**0,90**	0,23	0,06	0,16	**-0,84**	**-0,75**
Acidimicrobiia_unclassified	-0,51	0,24	0,62	-0,21	**0,97**	**-0,91**	**-0,98**	-0,53	0,14	-0,05	**0,89**	0,55	0,69	0,52	**0,98**	0,46	-0,18	0,39	**-0,95**	**-0,88**
Microtrichales_unclassified	0,68	**-0,86**	**0,92**	**0,88**	0,03	0,15	-0,11	0,66	**0,99**	**0,94**	-0,18	-0,65	-0,50	-0,68	0,07	**-0,72**	**0,89**	**-0,77**	0,05	0,20
Actinobacteria_unclassified	0,57	-0,31	-0,57	0,27	**-0,98**	**0,93**	**0,99**	0,58	-0,08	0,11	**-0,92**	-0,60	**-0,73**	-0,57	**-0,99**	-0,51	0,24	-0,45	**0,96**	**0,91**
Blastococcus	**-0,88**	**0,70**	0,14	-0,68	**0,96**	**-0,99**	**-0,94**	**-0,89**	-0,38	-0,55	**1,00**	**0,90**	**0,96**	**0,88**	**0,95**	**0,85**	-0,65	**0,81**	**-0,98**	**-1,00**
Geodermatophilus	0,63	-0,38	-0,51	0,34	**-0,99**	**0,96**	**1,00**	0,64	0,00	0,19	**-0,95**	-0,66	**-0,78**	-0,63	**-1,00**	-0,58	0,31	-0,52	**0,98**	**0,94**
Intrasporangiaceae_unclassified	**-0,97**	**1,00**	-0,56	**-1,00**	0,53	-0,67	-0,46	**-0,97**	**-0,90**	**-0,97**	0,69	**0,96**	**0,90**	**0,97**	0,49	**0,98**	**-0,99**	**0,99**	-0,59	**-0,71**
Microbacteriaceae_unclassified	0,63	**-0,82**	**0,95**	**0,84**	0,10	0,08	-0,18	0,61	**0,98**	**0,92**	-0,11	-0,60	-0,44	-0,62	0,14	-0,67	**0,86**	**-0,73**	-0,02	0,13
Arthrobacter	**-0,97**	**0,87**	-0,12	**-0,85**	**0,86**	**-0,93**	**-0,81**	**-0,98**	-0,61	**-0,75**	**0,94**	**0,98**	**1,00**	**0,97**	**0,83**	**0,96**	**-0,83**	**0,93**	**-0,89**	**-0,95**
Micrococcaceae_unclassified	**-0,95**	**1,00**	-0,62	**-1,00**	0,46	-0,61	-0,39	**-0,94**	**-0,93**	**-0,99**	0,63	**0,94**	**0,86**	**0,95**	0,42	**0,97**	**-1,00**	**0,98**	-0,53	-0,65
Pseudarthrobacter	**-0,94**	**1,00**	-0,65	**-1,00**	0,43	-0,58	-0,35	**-0,93**	**-0,95**	**-0,99**	0,60	**0,92**	**0,84**	**0,94**	0,39	**0,96**	**-1,00**	**0,98**	-0,49	-0,62
Micrococcales_unclassified	0,50	-0,23	-0,64	0,19	**-0,96**	**0,90**	**0,98**	0,52	-0,16	0,03	**-0,89**	-0,53	-0,68	-0,50	**-0,97**	-0,45	0,16	-0,38	**0,94**	**0,88**
Actinoplanes	0,41	-0,65	**1,00**	0,68	0,35	-0,18	-0,42	0,39	**0,89**	**0,79**	0,14	-0,37	-0,20	-0,41	0,39	-0,47	**0,70**	-0,53	-0,27	-0,12
Nocardioidaceae_unclassified	-0,01	-0,28	**0,93**	0,32	**0,71**	-0,57	**-0,76**	-0,03	0,62	0,46	0,54	0,05	0,23	0,01	**0,74**	-0,05	0,34	-0,13	-0,65	-0,52
Nocardioides	-0,03	-0,26	**0,93**	0,30	**0,72**	-0,59	**-0,77**	-0,05	0,61	0,45	0,56	0,07	0,25	0,03	**0,75**	-0,03	0,32	-0,11	-0,66	-0,54
Cutibacterium	0,64	-0,40	-0,49	0,36	**-1,00**	**0,96**	**1,00**	0,66	0,02	0,21	**-0,95**	-0,67	**-0,80**	-0,65	**-1,00**	-0,59	0,33	-0,53	**0,99**	**0,95**
Pseudonocardia	0,66	-0,41	-0,48	0,38	**-1,00**	**0,97**	**1,00**	0,67	0,03	0,22	**-0,96**	-0,68	**-0,80**	-0,66	**-1,00**	-0,61	0,35	-0,55	**0,99**	**0,95**
Actinobacteria_unclassified	**-0,99**	**0,90**	-0,21	**-0,89**	**0,81**	**-0,90**	**-0,76**	**-0,99**	-0,67	**-0,80**	**0,91**	**0,99**	**1,00**	**0,99**	**0,78**	**0,98**	**-0,87**	**0,96**	**-0,85**	**-0,92**
Solirubrobacter	0,14	-0,42	**0,98**	0,45	0,59	-0,44	-0,66	0,12	**0,73**	0,59	0,41	-0,10	0,08	-0,14	0,63	-0,20	0,48	-0,27	-0,53	-0,39

Bacteria_unclassified	0,75	-0,52	-0,36	0,49	-1,00	0,99	0,99	0,76	0,16	0,35	-0,99	-0,77	-0,87	-0,75	-1,00	-0,70	0,47	-0,65	1,00	0,98
Bacteroidia_unclassified	-0,78	0,56	0,32	-0,53	1,00	-1,00	-0,98	-0,79	-0,21	-0,39	0,99	0,80	0,90	0,78	0,99	0,74	-0,51	0,68	-1,00	-0,99
Chitinophagaceae_unclassified	-0,02	-0,27	0,93	0,30	0,72	-0,58	-0,77	-0,04	0,61	0,45	0,56	0,06	0,24	0,03	0,75	-0,04	0,33	-0,11	-0,66	-0,54
Microscillaceae_unclassified	-0,87	0,70	0,15	-0,67	0,96	-1,00	-0,94	-0,88	-0,37	-0,54	1,00	0,89	0,96	0,88	0,95	0,84	-0,65	0,80	-0,98	-1,00
Chloroflexi_unclassified	-0,92	0,76	0,06	-0,74	0,94	-0,98	-0,90	-0,92	-0,46	-0,62	0,99	0,93	0,98	0,92	0,92	0,89	-0,72	0,85	-0,96	-0,99
Bacillaceae_unclassified	-1,00	0,96	-0,35	-0,95	0,71	-0,82	-0,65	-1,00	-0,78	-0,88	0,84	1,00	0,98	1,00	0,68	1,00	-0,94	0,99	-0,76	-0,85
Bacillales_unclassified	0,84	-0,96	0,81	0,97	-0,21	0,38	0,13	0,82	0,99	1,00	-0,41	-0,81	-0,70	-0,83	-0,17	-0,87	0,98	-0,90	0,29	0,43
Sulfobacillus	0,82	-0,95	0,82	0,96	-0,19	0,35	0,10	0,81	1,00	0,99	-0,38	-0,80	-0,67	-0,82	-0,14	-0,85	0,97	-0,89	0,26	0,41
Gemmatimonadaceae_unclassified	-0,98	0,89	-0,17	-0,87	0,83	-0,92	-0,78	-0,99	-0,65	-0,78	0,93	0,99	1,00	0,98	0,81	0,97	-0,86	0,95	-0,87	-0,94
Saccharimonadales_unclassified	-0,69	0,45	0,44	-0,42	1,00	-0,98	-1,00	-0,71	-0,08	-0,27	0,97	0,72	0,83	0,69	1,00	0,65	-0,39	0,59	-0,99	-0,97
Acetobacteraceae_unclassified	-0,95	0,82	-0,03	-0,79	0,90	-0,96	-0,86	-0,95	-0,53	-0,69	0,97	0,96	0,99	0,95	0,88	0,93	-0,78	0,90	-0,93	-0,98
Alphaproteobacteria_unclassified	0,62	-0,37	-0,52	0,34	-0,99	0,96	1,00	0,64	-0,01	0,18	-0,95	-0,65	-0,78	-0,63	-1,00	-0,57	0,31	-0,51	0,98	0,94
Beijerinckiaceae_unclassified	-0,44	0,17	0,69	-0,13	0,94	-0,87	-0,97	-0,46	0,22	0,03	0,86	0,48	0,63	0,45	0,96	0,39	-0,10	0,32	-0,92	-0,84
Rhizobiales_unclassified	-0,21	-0,08	0,84	0,12	0,83	-0,72	-0,87	-0,23	0,46	0,28	0,70	0,24	0,42	0,21	0,86	0,15	0,15	0,07	-0,79	-0,68
Rhodobacteraceae_unclassified	-0,77	0,56	0,33	-0,52	1,00	-1,00	-0,99	-0,79	-0,20	-0,38	0,99	0,80	0,89	0,77	0,99	0,73	-0,50	0,68	-1,00	-0,99
Sphingomonadaceae_unclassified	-0,93	0,78	0,03	-0,76	0,92	-0,98	-0,89	-0,93	-0,48	-0,64	0,98	0,94	0,99	0,93	0,91	0,90	-0,74	0,87	-0,95	-0,99
Sphingomonas	-0,90	0,73	0,10	-0,71	0,95	-0,99	-0,92	-0,91	-0,42	-0,59	0,99	0,91	0,97	0,90	0,93	0,87	-0,69	0,83	-0,97	-1,00
Burkholderiaceae_unclassified	-0,88	0,98	-0,76	-0,99	0,29	-0,45	-0,21	-0,87	-0,98	-1,00	0,48	0,86	0,75	0,88	0,25	0,91	-0,99	0,93	-0,36	-0,50
Nitrosospira	0,23	-0,50	0,99	0,53	0,52	-0,36	-0,58	0,21	0,79	0,66	0,33	-0,19	-0,01	-0,23	0,55	-0,29	0,56	-0,36	-0,45	-0,31
Proteobacteria_unclassified	0,10	-0,38	0,97	0,42	0,62	-0,48	-0,69	0,08	0,70	0,56	0,45	-0,06	0,12	-0,10	0,66	-0,16	0,44	-0,23	-0,56	-0,43

Table S4.10. Spearman's rank correlation among basal respiration, root elongation and germination rate of soil and the remaining different abiotic and biotic properties determined in this work. Basal respiration (g CO_2 h^{-1} g^{-1}); root elongation and germination rate (%), pH; EC: electric conductivity (dS m^{-1}); SOC: soil organic carbon (g kg^{-1}); $CaCO_3$ (%); Silt, Clay and Sand (%); CIC: cation exchange capacity (cmol(+) kg^{-1}); Cu_T, As_T and Pb_T: total trace elements concentrations (mg kg^{-1} dry soil); Cu_W, Zn_W and Pb_W: water soluble trace elements concentrations (cmol(+) kg^{-1}); Zn_EDTA, and Pb_EDTA: bioavailable fraction of trace elements extracted with EDTA (cmol(+) kg^{-1}), OTUs Number; Simpson and Shannon indices values; Total *Bacteria*, Total *Archaea*, AOB and Denitrifiers populations (copy number gene g^{-1}); and relative abundance at the genus level (%).

Variables	Basal respiration	Root elongation	Germination rate
Basal respiration	1.00	0.78	0.73
Root elongation	0.78	1.00	0.75
Germination rate	0.73	0.75	1.00
pH	0.66	0.29	0.73
EC	-0.95	-0.70	-0.73
$CaCO_3$	0.27	0.05	0.60
SOC	0.55	0.78	0.54
Silt	-0.26	-0.39	-0.68
Clay	0.17	0.39	0.65
Sand	0.21	0.24	0.24
CIC	0.68	0.80	0.40
Cu_T	0.55	0.62	0.28
Zn_T	0.34	0.56	0.10
As_T	-0.63	-0.57	-0.67
Pb_T	-0.80	-0.76	-0.73
Cu_W	-0.48	-0.29	-0.73
Zn_W	-0.46	-0.31	-0.76
As_W	0.00	0.01	-0.40
Pb_W	-0.49	-0.34	-0.73
Cu_EDTA	0.49	0.58	0.09
Zn_EDTA	-0.44	-0.34	-0.76
As_EDTA	0.40	0.52	0.32
Pb_EDTA	0.62	0.78	0.56
OTUs Number	0.44	0.48	0.73
Simpson index	-0.76	-0.38	-0.47
Shannon index	0.78	0.66	0.66
Total *Bacteria*	0.92	0.75	0.73
AOB	0.84	0.64	0.75
Desnitrificantes	0.83	0.73	0.73
Total *Archaea*	0.87	0.79	0.75
Pyrinomonadaceae_RB41	-0.10	-0.07	-0.45

Subgroup_6_unclassified	0.17	0.04	0.28
Acidimicrobiia_unclassified	0.16	0.21	0.53
Microtrichales_unclassified	0.26	0.38	0.15
Actinobacteria_unclassified	0.33	0.22	0.05
Blastococcus	0.41	0.20	0.60
Geodermatophilus	0.31	0.35	-0.02
Intrasporangiaceae_unclassified	0.10	0.25	0.50
Microbacteriaceae_unclassified	0.30	0.47	0.15
Arthrobacter	0.33	0.32	0.60
Micrococcaceae_unclassified	0.08	0.18	0.51
Pseudarthrobacter	0.13	0.08	0.46
Micrococcales_unclassified	0.15	0.11	0.05
Actinoplanes	**0.63**	0.10	0.25
Nocardioidaceae_unclassified	0.12	-0.10	0.22
Nocardioides	0.29	-0.11	0.22
Cutibacterium	-0.65	-0.78	-0.76
Pseudonocardia	0.13	0.03	-0.28
Actinobacteria_unclassified	0.50	**0.80**	**0.73**
Solirubrobacter	**0.62**	**0.68**	0.40
Bacteria_unclassified	0.16	-0.02	0.17
Bacteroidia_unclassified	0.15	-0.15	0.30
Chitinophagaceae_unclassified	0.52	0.24	0.22
Microscillaceae_unclassified	0.31	0.12	0.57
Chloroflexi_unclassified	0.45	0.17	**0.61**
Bacillaceae_unclassified	0.33	**0.72**	0.47
Bacillales_unclassified	-0.22	0.15	-0.12
Sulfobacillus	-0.32	-0.31	-0.21
Gemmatimonadaceae_unclassified	0.47	0.40	**0.66**
Saccharimonadales_unclassified	0.32	0.13	0.35
Acetobacteraceae_unclassified	0.51	**0.61**	**0.67**
Alphaproteobacteria_unclassified	0.20	0.12	-0.13
Beijerinckiaceae_unclassified	0.54	0.30	0.48
Rhizobiales_unclassified	0.26	0.36	0.08
Rhodobacteraceae_unclassified	0.04	-0.03	0.40
Sphingomonadaceae_unclassified	0.40	0.29	**0.67**
Sphingomonas	0.22	0.20	0.47
Burkholderiaceae_unclassified	0.15	-0.21	0.11
Nitrosospira	-0.34	-0.46	-0.26
Proteobacteria_unclassified	0.14	0.12	-0.05

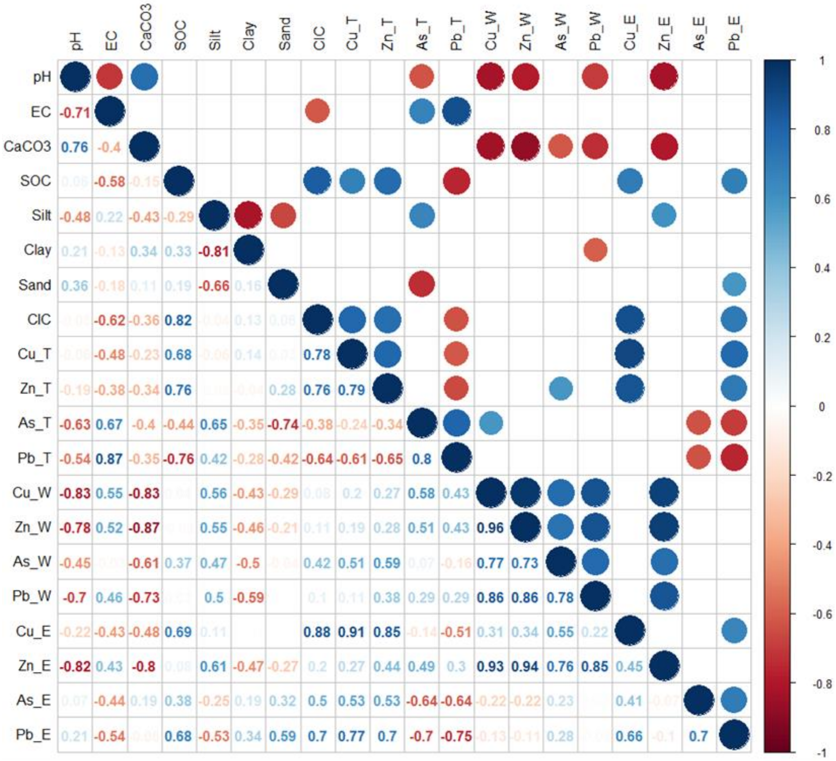

Figure S4.1. Spearman's correlations values among the soil properties. pH; EC: electric conductivity (dS m^{-1}); SOC: soil organic carbon (g kg^{-1}); $CaCO_3$ (%); Silt, Clay and Sand (%); CIC: cation exchange capacity (cmol(+) kg^{-1}); Cu_T, Zn_T, As_T and Pb_T: total extracted; Cu_W, Zn_W and As_W and Pb_T: water soluble; Cu_E, Zn_E, As_E and PB_E: bioavailable fraction extracted with EDTA.

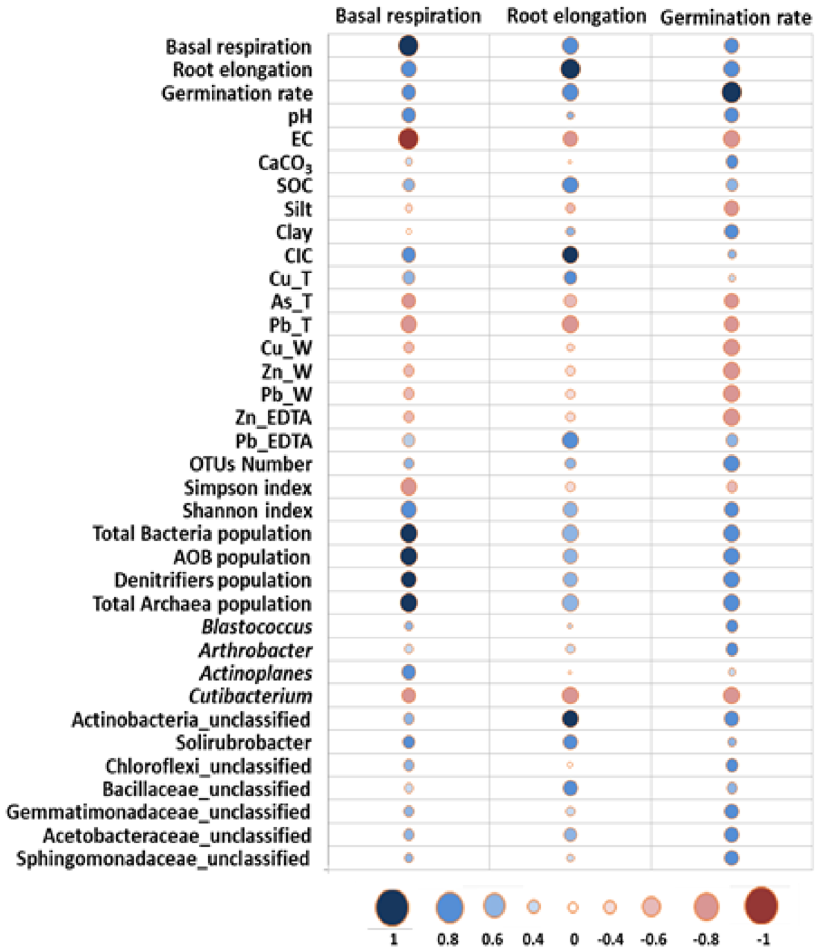

Figure S4.2. Spearman's correlations values among basal respiration, root elongation and germination rate and the remaining variables in soils determined in this work. Only the variables that were significantly correlated (r>0.7) at least to one of basal respiration, root elongation and germination rate are represented. Basal respiration (g CO_2 h^{-1} g^{-1}); root elongation and germination rate (%), pH; EC: electrical conductivity (dS m^{-1}); SOC: soil organic carbon (g kg^{-1}); $CaCO_3$ (%); Silt, Clay and Sand (%); CIC: cation exchange capacity (cmol(+) kg^{-1}); Cu_T, As_T and Pb_T: total trace elements concentrations (mg kg^{-1} dry soil); Cu_W, Zn_W and Pb_W: water soluble trace elements concentrations (cmol(+) kg^{-1}); Zn_EDTA, and Pb_EDTA: bioavailable fraction of trace elements extracted with EDTA (cmol(+) kg^{-1}), OTUs Number; Simpson and Shannon index values; Total Bacteria, Total Archaea, AOB and Denitrifiers populations (copy number gene g^{-1}); and realative abundance of *Blastococcus*, *Arthrobacter*, *Actinoplanes*, *Cutibacterium*, Actinobacteria_unclassified, *Solirubrobacter*, Chloroflexi_unclassified, Bacillaceae_unclassified, Gemmatimonadaceae_unclassified, Acetobacteraceae_ unclassified, Sphingomonadaceae_unclassified (%).

Chapter 5 . Supplementary materials

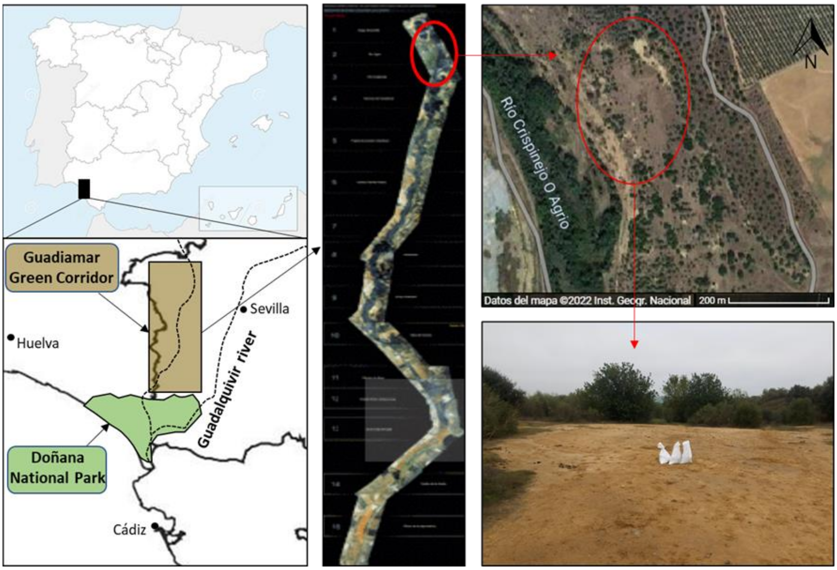

Figure S5.1. Sampling location of the residual polluted soil used in the experimental set up of this study (modified from Paniagua-López et al., 2023).

Table S5.1. The main chemical properties of the control polluted soil and the individual amendments. DOR-*C.rig* (DOR mycoremediated by *C. rigida*); DOR-*C.rad* (DOR mycoremediated by *C. radians*); EC (electrical conductivity); OC (organic carbon); N (total nitrogen); Pav (available phosphorus); n.d.: not detected. Data are presented as mean ± standard desviation, n=3.

	pH	EC (dS/m)	OC (%)	N (%)	Pav (mg kg^{-1})	K (g kg^{-1})
Polluted soil	3.40 ± 0.01 *a*	3.48 ± 0.07 *b*	0.72 ± 0.11 *a*	0.102 ± 0.006 *b*	8.2 ± 2.0 *a*	12.13 ± 0.14 *b*
Marble sludge	9.10 ± 0.12 *e*	1.20 ± 0.07 *a*	0.06 ± 0.01 *a*	0.011 ± 0.003 *a*	n.d.	1.82 ± 0.21 *a*
DOR-*C.rig*	6.32 ± 0.03 *c*	4.24 ± 0.11 *c*	38.42 ± 2.36 *c*	1.920 ± 0.018 *c*	401.2 ± 30.6 *c*	32.09 ± 1.79 *d*
DOR-*C.rad*	4.95 ± 0.02 *b*	4.82 ± 0.05 *d*	51.92 ± 0.47 *d*	2.186 ± 0.016 *d*	219.6 ± 36.7 *b*	41.45 ± 0.59 *e*
Vermicompost	7.66 ± 0.02 *d*	4.92 ± 0.18 *d*	13.59 ± 2.94 *b*	1.848 ± 0.063 *c*	231.4 ± 9.7 *b*	17.59 ± 0.25 *c*

Table S5.2. Total PTEs concentrations (T) measured in polluted soil after the different amendments and AMF fungi treatments application. PS (control polluted soil); M (marble addition); D1 (dry olive residue mycoremediated by *C. rigida*); D2 (dry olive residue mycoremediated by *C. radians*); VC (vermicompost); *R.irr* (*R. irregularis*); *R.cus* (*R. custos*).

Treatment	Pb_T	As_T	Zn_T	Cu_T	Cd_T	Sb_T
PS	580.7 ± 24.4 *e*	338.6 ± 9.0 *d*	198.6 ± 2.7 *d*	105.9 ± 4.4 *b*	6.9 ± 0.4 *d*	32.8 ± 5.4 *c*
M	492.0 ± 25.3 *cd*	312.0 ± 12.9 *bc*	162.7 ± 15.6 *c*	82.7 ± 8.5 *a*	5.7 ± 0.3 *c*	30.9 ± 2.7 *bc*
R.irr	437.0 ± 13.6 *ab*	243.8 ± 9.2 *a*	155.1 ± 2.4 *c*	81.4 ± 4.0 *a*	4.2 ± 0.2 *a*	24.3 ± 5.9 *abc*
R.cus	429.4 ± 8.4 *ab*	242.1 ± 8.0 *a*	140.3 ± 2.0 *ab*	84.6 ± 8.2 *a*	5.0 ± 0.2 *b*	21.2 ± 1.7 *a*
D1	459.9 ± 10.0 *abc*	253.9 ± 5.0 *a*	137.9 ± 12.2 *a*	81.1 ± 2.3 *a*	4.7 ± 0.3 *ab*	25.1 ± 6.9 *abc*
D2	421.9 ± 13.9 *a*	233.7 ± 4.3 *a*	139.0 ± 3.2 *a*	83.5 ± 8.7 *a*	4.2 ± 0.2 *a*	20.7 ± 3.1 *a*
VC	428.5 ± 11.9 *ab*	236.4 ± 3.4 *a*	140.5 ± 4.1 *ab*	84.1 ± 3.9 *a*	4.6 ± 0.2 *ab*	20.2 ± 4.1 *a*
D1-*R.irr*	436.2 ± 14.1 *ab*	251.2 ± 9.9 *a*	155.5 ± 5.8 *c*	86.1 ± 8.8 *a*	4.8 ± 0.3 *b*	24.2 ± 5.1 *abc*
D1-*R.cus*	424.2 ± 5.1 *a*	233.4 ± 7.6 *a*	135.8 ± 3.7 *a*	79.7 ± 2.7 *a*	4.2 ± 0.2 *a*	22.6 ± 5.9 *ab*
D2-*R.irr*	493.9 ± 44.4 *cd*	287.1 ± 13.3 *b*	141.5 ± 4.8 *ab*	84.3 ± 3.6 *a*	5.8 ± 0.1 *c*	23.5 ± 8.1 *ab*
D2-*R.cus*	477.1 ± 57.7 *bc*	260.5 ± 36.6 *a*	140.4 ± 8.5 *ab*	82.8 ± 6.1 *a*	4.8 ± 0.6 *b*	22.5 ± 1.3 *ab*
VC-*R.irr*	465.7 ± 32.8 *abc*	253.6 ± 26.5 *a*	153.4 ± 8.0 *bc*	83.1 ± 7.6 *a*	4.8 ± 0.3 *b*	29.0 ± 4.2 *abc*
VC-*R.cus*	528.7 ± 23.0 *d*	313.4 ± 7.4 *c*	153.8 ± 9.0 *bc*	87.7 ± 2.6 *a*	6.1 ± 0.3 *c*	27.2 ± 0.7 *abc*

Lowercase letters indicate significant differences among treatments according to Duncan's post hoc test (p<0.05).

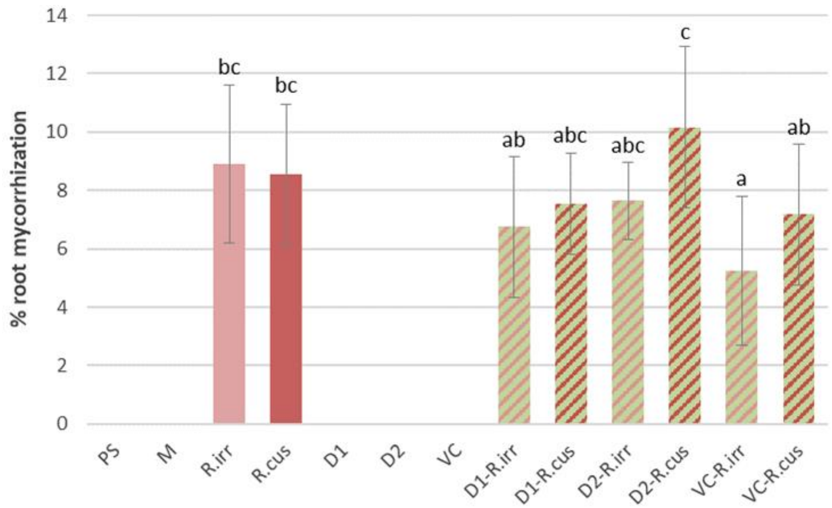

Figure S5.2. Percentages of root mycorrhization in polluted soil after the different amendments and AMF treatments application. PS (control polluted soil); M (marble addition); D1 (dry olive residue mycoremediated by *C. rigida*); D2 (dry olive residue mycoremediated by *C. radians*); VC (vermicompost); *R.irr* (*R. irregularis*); *R.cus* (*R. custos*). Error bars represent standard deviation from the mean (n=5). Lowercase letters indicate significant differences among treatments according to Duncan's post hoc test (p<0.05).

Table S5.3. Transfer of PTEs from plant roots to shoots (TF: translocation factor) in each of the different studied treatments. M (marble addition); D1 (dry olive residue mycoremediated by *C. rigida*); D2 (dry olive residue mycoremediated by *C. radians*); VC (vermicompost); *R.irr* (*R. irregularis*); *R.cus* (*R. custos*). Data are presented as mean ± standard desviation, n=5.

Treatment	Pb_TF	As_TF	Zn_TF	Cu_TF	Cd_TF	Sb_TF
M	0.017 ± 0.007 *d*	0.025 ± 0.014 *b*	0.865 ± 0.253 *de*	0.194 ± 0.103 *c*	0.015 ± 0.004 *a*	0.143 ± 0.034 *d*
R.irr	0.023 ± 0.008 *e*	0.036 ± 0.010 *c*	0.937 ± 0.171 *e*	0.105 ± 0.015 *ab*	0.072 ± 0.010 *b*	0.086 ± 0.030 *abc*
R.cus	0.012 ± 0.003 *bc*	0.015 ± 0.005 *ab*	0.854 ± 0.147 *de*	0.151 ± 0.062 *bc*	0.024 ± 0.006 *a*	0.152 ± 0.097 *d*
D1	0.007 ± 0.001 *ab*	0.010 ± 0.004 *a*	0.623 ± 0.097 *abc*	0.051 ± 0.004 *a*	0.030 ± 0.012 *a*	0.077 ± 0.027 *ab*
D2	0.007 ± 0.003 *ab*	0.011 ± 0.007 *a*	0.700 ± 0.169 *bcd*	0.062 ± 0.015 *a*	0.025 ± 0.014 *a*	0.063 ± 0.032 *a*
VC	0.012 ± 0.002 *bc*	0.015 ± 0.008 *ab*	0.616 ± 0.077 *abc*	0.084 ± 0.026 *a*	0.124 ± 0.057 *c*	0.139 ± 0.004 *cd*
D1-*R.irr*	0.005 ± 0.001 *a*	0.018 ± 0.012 *ab*	0.740 ± 0.207 *bcd*	0.092 ± 0.047 *a*	0.025 ± 0.013 *a*	0.044 ± 0.037 *a*
D1-*R.cus*	0.005 ± 0.003 *a*	0.014 ± 0.002 *a*	0.835 ± 0.136 *de*	0.060 ± 0.016 *a*	0.060 ± 0.012 *b*	0.073 ± 0.030 *ab*
D2-*R.irr*	0.016 ± 0.003 *cd*	0.022 ± 0.004 *ab*	0.450 ± 0.050 *a*	0.082 ± 0.036 *a*	0.012 ± 0.004 *a*	0.063 ± 0.022 *a*
D2-*R.cus*	0.009 ± 0.002 *ab*	0.011 ± 0.010 *a*	0.824 ± 0.131 *cde*	0.109 ± 0.029 *ab*	0.025 ± 0.009 *a*	0.125 ± 0.018 *bcd*
VC-*R.irr*	0.009 ± 0.003 *ab*	0.013 ± 0.005 *a*	0.669 ± 0.107 *bcd*	0.074 ± 0.005 *a*	0.159 ± 0.017 *d*	0.124 ± 0.021 *bcd*
VC-*R.cus*	0.008 ± 0.004 *ab*	0.019 ± 0.004 *ab*	0.583 ± 0.095 *ab*	0.069 ± 0.013 *a*	0.166 ± 0.033 *d*	0.135 ± 0.043 *cd*

Lowercase letters indicate significant differences among treatments according to Duncan's post hoc test (p<0.05).

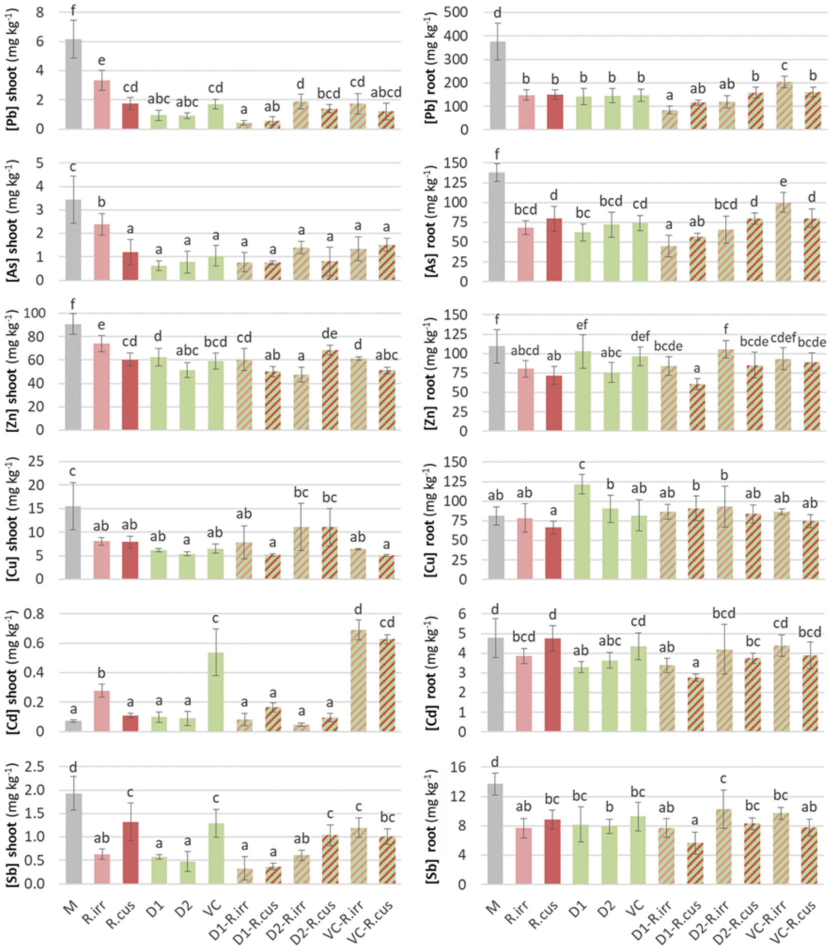

Figure S5.3. PTEs concentrations (mg kg^{-1}) in wheat plant shoots (left) and roots (right) in polluted soil after the different amendments and AMF treatments application. M (marble addition); D1 (dry olive residue mycoremediated by *C. rigida*); D2 (dry olive residue mycoremediated by *C. radians*); VC (vermicompost); *R.irr* (*R. irregularis*); *R.cus* (*R. custos*). Error bars represent standard deviation from the mean (n=5). Lowercase letters indicate significant differences among treatments according to Duncan's post hoc test (p<0.05).

List of acronyms

Acronym	Description
AMF(s)	Arbuscular mycorrhizal fungi
ANR	Assisted natural remediation
AOB	Ammonia-oxidizing bacteria
AWC	Available water content
B	Biopiles (50% w/w mixture of RS and PS)
BAF	Bioaccumulation factor
BCF	Bioconcentration factor
BV	Biopiles + vermicompost
$CaCO_3$	Calcium carbonate
CEC/CIC	Cation exchange capacity
CRM	Certified reference material
CT	Control/contaminated soil (equivalent to PS)
D1	Dry olive residue mycoremediated by *C. rigida*
D2	Dry olive residue mycoremediated by *C. radians*
DOR	Dry olive residue
EC	Electrical conductivity
EDTA	Ethylenediaminetetraacetic acid
G	Gypsum mining spoil
GGC	Guadiamar Green Corridor
GV	Gypsum mining spoil + vermicompost
ICP-MS	Inductively coupled plasma-mass spectrometry
ICP-OES	Inductively coupled plasma-optical emission spectrometry
M	Marble sludge
MV	Marble sludge + vermicompost
NMDS/NMS	Non-metric multidimensional scaling
OC	Organic carbon
OTU(s)	Operational taxonomic units
PCA	Principal component analysis
PCR	Polymerase chain reaction
PS	Polluted soil (unvegetated polluted soil within the affected area)
PTE(s)	Potentially toxic elements

Acronym	Description
RA	Relative abundance
RS	Recovered soil (soil naturally revegetated within the affected area)
SOC	Soil organic carbon
TF	Translocation factor
US	Unaffected soil (Natural soil adjacent to the affected area)
VC	Vermicompost
WFC	Water field capacity
XRF	X-ray fluorescence
Xx_B/EDTA	EDTA-extracted bioavailable PTEs fraction
Xx_T	Total PTEs fraction
Xx_W	Water-soluble PTEs fraction

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