The role of gypsum in bioremediation; recovery of ecosystem functions and services of polluted soils

Papel del yeso en la biorremediación; recuperación de las funciones y servicios ecosistémicos de suelos contaminados

Ph.D. Thesis / Tesis Doctoral

Helena García Robles

Programa de Doctorado Biología Fundamental y de Sistemas

Departamentos de Botánica y Edafología y Química Agrícola

Universidad de Granada 2020

UNIVERSIDAD DE GRANADA

The role of gypsum in bioremediation; recovery of ecosystem functions and services of polluted soils.

Papel del yeso en la biorremediación; recuperación de las funciones y servicios ecosistémicos de suelos contaminados.

Ph.D. Thesis

Tesis Doctoral

Programa de Doctorado Biología Fundamental y de Sistemas

Helena García Robles

Dirigida por:

Dr. Juan Lorite Moreno / Dr. Francisco José Martín Peinado

Departamentos de Botánica y Edafología y Química Agrícola

Universidad de Granada

2020

UNIVERSIDAD DE GRANADA

Editor: Universidad de Granada. Tesis Doctorales
Autor: Helena García Robles
ISBN: 978-84-1306-549-6
URI: http://hdl.handle.net/10481/63067

FUNDACIÓN
TATIANA PÉREZ DE GUZMÁN EL BUENO

Durante el tiempo de realización de esta Tesis Doctoral he disfrutado de una Beca-Contrato del Programa de Becas en Medioambiente 2016 de la Fundación Tatiana Pérez de Guzmán el Bueno.

Este trabajo ha sido financiado por los proyectos RTI 2018-094327-B-I00 del Ministerio de Ciencia, Innovación y Universidades; por el proyecto de Excelencia P11-RNM-7061 de la Junta de Andalucía (Consejería de Economía, Innovación, Ciencia y Empleo); y por KNAUF-GmbH Branch Spain a través del proyecto 3092 de la Fundación General Universidad de Granada.

La investigación presentada en esta Tesis Doctoral se ha realizado en los Departamentos de Botánica, y de Edafología y Química Agrícola de la Universidad de Granada

A mi familia, la que elegí y la que por fortuna me fue regalada

"Nuestra tarea debe ser vivir libres, ampliando nuestro círculo de compasión para abarcar a todas las criaturas vivientes y la totalidad de la naturaleza en su belleza"

Albert Einstein

AGRADECIMIENTOS

ACKNOWLEDGEMENTS

Agradecimientos / Acknowledgements

Hace ya casi 4 años que empezó esta aventura, una nueva etapa en mi vida a la que, en breve, pondré un punto y seguido. Hoy miro hacia atrás y tengo la sensación de haber llegado a la cima del Everest. Ha sido un camino largo y agridulce que termina con la felicidad propia de haber conseguido un logro personal. En este camino, muchas habéis sido las personas que, de una u otra manera, habéis puesto vuestro granito de arena para, en su conjunto, crear esta montaña.

Me gustaría empezar agradeciendo a mis directores de tesis, Juan Lorite Moreno y Francisco José Martín Peinado el gran apoyo que siempre me han brindado, así como su dedicación y enseñanzas a lo largo de todos estos años. Juan, aún recuerdo cuando llegué a tu despacho en 2014 para decirte que quería hacer el trabajo de fin de máster contigo; gracias por haber creído siempre en mí, por todas las oportunidades que me has dado, por haberme guiado en este camino y por regalarme tu amistad. A Paco, gracias por tu ayuda infinita, por ser tan humano y cercano, por estar ahí siempre para todos y para todo, por regalar tu tiempo con tanta alegría y bondad, por haberme dado tantos ánimos cuando me faltaban, y un largo etcétera de otras virtudes que se resumen en que tu gentileza no tiene límites. En resumidas cuentas, gracias a los dos por ser grandes profesionales, grandes personas, y por haber aceptado con gusto ser mis directores de tesis. Sin duda soy consciente de lo afortunada que soy.

Gracias a los Departamento de Botánica, y Edafología y Química Agrícola por haber sido mi segunda casa todos estos años. Gracias a Paqui por ser la alegría personificada, por siempre estar ahí para sacarme una sonrisa y aconsejarme. Gracias a Julio Peñas, Domingo, Siham, Eva y Noelia, por vuestro gran apoyo, vuestros consejos y vuestra amistad. Gracias a Ana Teresa, por todas tus enseñanzas y tu cariño, por esa relación tan cercana que hemos establecido desde el principio. Gracias a Pedro por rebosar bondad y jovialidad por los poros. Gracias a Julio de la Rosa, Ingrid y Juan Francisco, por todas nuestras charlas y risas, y por todo el ánimo que me habéis dado. Gracias a Antonio Mendoza, porque no se puede ser más buena gente que tú; solo siento que no hayamos coincidido más. Gracias a Katy y Sorboni, por compartir conmigo sus culturas y simpatía. Gracias a Consuelo, Gabriel, Paco Valle, Víctor y Samira por ser grandes compañeros/as y por todos los buenos momentos que hemos compartido. Gracias a Pepe, por tu simpatía, tu cariño y por todo lo que me has ayudado. Gracias a Luz por todas las gestiones y tu simpatía. Gracias a Belén, por toda tu ayuda y por nuestras conversaciones; te he echado mucho de menos y te deseo lo mejor. Gracias a todos los compañeros del Departamento de Botánica en Farmacia pues, aunque hemos coincidido poco, os tengo en gran estima. Gracias también a las compañeras del herbario Mariate, Carmen y Laura, y a David Nesbitt. Gracias a

Marta Nieto, por compartir toda su sabiduría predoctoral conmigo y estar tan pendiente. Quiero reservar el último gracias de este Departamento a mis compañeros de batalla: Mode, Isma, Deivid y Antonio González; sin vosotros, este camino hubiera sido bastante más arduo. Gracias por las risas, los lloros, el apoyo, en definitiva, por vuestra amistad. Solo quiero recordaros que hay luz al final del túnel. Os paso el relevo y os cedo toda mi energía.

…Habiendo terminado con los agradecimientos a la sexta planta (Botánica), cojo el ascensor (como tantas veces he hecho) para continuar por la planta baja (Edafología)…

Dentro del Departamento de Edafología y Química Agrícola, quiero dedicar un gracias muy especial a Emilia, Irene, Manolo Sierra y Javier, por tantas risas, tanto cariño y tanto apoyo. Manolo, aún me da la risa cuando veo el quadrat de muestreo, o como tú lo llamas, la rejilla del lavaplatos. Un millón de gracias a Manoli y Yolanda, por haber tenido tanta paciencia conmigo y por haberme ayudado tanto. A mis compis Ana Romero, Layla, Marino, Mario Gutiérrez, los Antonios, Sofía, Azman, Mikel, Dani, Salsabil, Obid y Rocio, quiero agradeceros todos los buenos momentos que hemos pasado juntos, os deseo lo mejor. A mi gran amiga Eliane, simplemente gracias por ser quién eres; te echo de menos. Por último, quisiera agradecer a Mariano Simón que nos ayudara a montar las parcelas experimentales y, en especial, por todo lo que me ha enseñado sin él saberlo; descansa en paz.

Gracias a Pipo, por tu empeño, ayuda, apoyo, comprensión y simpatía… ¡Parece que he llegado a la meta! Aprovecho para extender mi gratitud a los compañeros del IFAPA que tanto me ayudaron durante el experimento del invernadero, especialmente a Pepe Vílchez.

El próximo agradecimiento es para los miembros de mi Tribunal de Tesis: Eva Cañadas Sánchez, Javier Martínez Garzón, Ana Romero Freire, José Álvarez Rogel, Susana Loureiro, Irene Ortíz Bernad y Elma Lahive, por acompañarme en un día tan señalado y ofrecerme sus valiosos consejos. Thank you! I would also like to thank Yasuo and Nazaret for being my international referees and taking the time to read this thesis and give me their advice. I hope you will all enjoy it!

Un gracias infinito a mi super equipo particular de muestreo: Minerva, Mario Paniagua, Cristian, Sandra, Olivia, Regina y Miguelillo. Chicos/as, estoy en deuda con vosotros/as, sin vuestra ayuda esta tesis no hubiera sido posible, LITERALMENTE. Mine, ¡cuánto me hubiera gustado hacer esta tesis contigo! A lo mejor hubiéramos encontrado el pájaro “ambulancia” juntas… Mario, gracias por siempre estar dispuesto a ayudarme, espero poder hacer lo mismo por ti en el futuro. Gracias a Sandra y a Olivia por haberle puesto tantas ganas, porque me habéis dado oxígeno y tranquilidad, sois muy grandes chicas. Por cierto, si tuvierais nostalgia, aún tengo guardadas unas cuantas plantitas ricas en arsénico y un molinillo de café; solo decidme día y hora. A Miguelillo… ¡ese compi magnífico! Gracias por haberme dado siempre lo mejor de ti, por tu ayuda y apoyo infinitos, por los conciertos de guitarra; gracias por siempre compartir alegría, comprensión, paciencia y bondad a raudales. A todos, gracias por vuestra amistad, y por todas las risas e inolvidables momentos en Aznalcóllar (¡y sobre todo en Granada!).

Un gracias enorme a María, Carolina y Manu Pacheco. ¡Cuánto dinero nos hemos ahorrado en psicólogos con nuestras terapias particulares arreglando el mundo! Llegaremos donde nos propongamos; ¡que se echen a temblar! ¡Ánimo en la recta final!

A Moha. Gracias, gracias y más gracias por tantas risas, tanto apoyo, tantos consejos, tanto ánimo, tanto cariño; por guiarme cuando me pierdo; en definitiva, por ser el gran amigo que eres.

A Jesús Muñoz y Carliños, por conseguir que R al fin fuera mi aliado. Carliños, gracias por tantas horas de ayuda y tantos buenos momentos. ¡Ánimo, que eres el siguiente!

To Erik Smolders and his team, especially to Gina, Daniela and Mieke. Thank you very much for your warm welcome into your lab and for all your help.

Mi más sincero agradecimiento a Sonia Mediavilla. Por ser la mejor profesora que jamás he tenido y tendré, además de una grandísima amiga y mentora. Si no fuera porque creíste en mí, probablemente esta tesis no existiría. La ciencia cobra sentido con gente como tú al frente, nunca lo olvides.

Un inmenso y afectuoso gracias a mis amigos/as. A los/las de Salamanca, en especial a Taniusqui (que incansablemente me escucha, apoya y aconseja como la mejor de las amigas), Alexito, Lore, Chemi, Conchi, Pabli, Fran, Nando, Ele, Merce, María, Ro y David González Juanes, mi más sincero agradecimiento por quererme como lo hacéis y por apoyarme en la distancia. A Vero, por darme tanta cordura y comprensión. También quiero agradecer a mis biologuines del alma e hijo adoptivo por ser tan buenos amigos y una inmensa fuente de energía para mí. A mis amigos/as de Granada, en especial a Merce Cazorla, Omar, Biomer, As, Antonio y Tania, por vuestro cariño, paciencia infinita y apoyo, un millón de gracias. A mis amigos/as del mundo, en especial a José, Esther, Ani y Leo, porque aun estando lejos, me habéis acompañado y apoyado en este proceso. En adelante, siempre y cuando una pandemia no nos frene, quiero recuperar todo el tiempo perdido y disfrutar de vosotros/as. A todos/as, sois un tesoro, ¡os quiero!

Agradecimientos / Acknowledgements

A Julia, mi hermana americana. Sencillamente gracias por regalarme tu amistad, tu bondad, tu energía y tu cariño. Gracias por siempre estar ahí, por cargar con mi mochila cuando la tesis me pesaba. Me has apoyado y comprendido cada instante. Ojalá la gente como tú abundara en el mundo, pues éste sería un lugar increíble. Estoy orgullosa de ti. Te quiero.

Por último, quiero regalar mi más profundo y sincero agradecimiento a mi familia. Me van a faltar palabras. A mi hermano Javito, por ser tan puro y maravilloso, por protegerme y apoyarme como lo haces, por abrazarme y darme fuerzas siempre que lo necesito; eres el mejor hermano del mundo y estoy muy orgullosa de ti. Gracias a mi primuco del alma, Julino, sencillamente no sé qué haría sin ti (si tú lloras, yo lloro, ya sabes), gracias por siempre llevarme de la mano y protegerme; por favor, nunca me sueltes, porque saber que estás conmigo me alegra la vida. Gracias a mis queridas primas y primos: Cris, Azu, David, Antonius y Pedri, que más allá de primos, sois grandes amigos. Gracias a mis tíos y tías por siempre darme ánimos y su cariño. A mi abuela Gabi, mi abuelo Tomás y mi tío Pedro, por haberme querido tanto y por acompañarme allí donde estéis; os echo muchísimo en falta. Gracias a mis padres adoptivos, o suegros como otra gente los llama, por cuidar de mí, por mantenerme bien alimentada y por hacerme sentir una más de la familia. A mis cuñados Julia y Alex, por ser tan maravillosos. Finalmente, quiero dedicar mis últimas palabras y el mayor de todos mis agradecimientos a mi compañero de vida, mis padres y mi abuela Mati; a vosotros os dedico esta tesis. A Javi, por darme luz y oxígeno cada día, por buscarme siempre las sonrisas, por ayudarme a levantar cuando me caigo, por recordarme quien soy cuando lo olvido, por quererme con tanta fuerza y sinceridad, por comprenderme, por ser mi confidente, por sacar lo mejor de mí; nunca creí poder tener tanta suerte y espero saber regalarte lo mismo. A MIS PADRES, Julio y Amalia, simplemente deciros que, si hubiera podido elegir padres, sin duda hubierais sido vosotros. Gracias por vuestras innumerables y valiosísimas enseñanzas, por vuestro amor y apoyo incondicionales, por confiar en mi a ciegas, por ser mi pilar, por darme fuerza y alegría, por haber hecho de mi la persona que soy. Mis logros son el resultado de vuestro esfuerzo. SOIS LOS MEJORES, todas las palabras son pocas. A mi abue Matildina quiero agradecerle que me llene el corazón de ternura y alegría cada día, que a sus 91 añitos se siga comiendo el mundo a bocados (por favor, ¡no pares nunca!), que crea en mi por encima de todo como yo creo en ella, que me dé tanta fuerza y cariño, que haya dedicado gran parte de su vida a cuidarme con tanta dedicación y a enseñarme tantísimas cosas valiosas porque, aunque ella no lo crea, entiende de todo; eres mi debilidad y te estaré eternamente agradecida. A todos/as, os quiero infinitamente y os llevo conmigo.

Por unas razones o por otras, todos/as sois coautores/as de esta tesis. GRACIAS

CONTENTS

SUMMARY

SUMMARY

Soil is one of the main providers of environmental functions and services, its productivity being one of the most essential services for human survival and development. Nevertheless, anthropogenic activities such as mining, may seriously degrade it, causing relevant health and ecological hazards. In this sense, soil acidification, salinization and pollution are a great concern worldwide, for which many degraded soil ecosystems would require assisted natural remediation. The aim of this thesis is to assess the role of gypsum mining spoil as an amendment of soils affected by acidification, salinization and Potentially Harmful Elements (PHEs), in order to revalue this mining waste material while assisting the natural remediation of degraded soils and their associated plant communities. The study area is located in the Guadiamar Green Corridor (GGC, Southern Spain), home to one of the worst European ecological disasters that has ever occurred. In 1998, the Aznalcóllar pyrite mine pond breached and released acidic waters and tailings highly polluted with PHEs. Thereupon, multiple cleaning and rehabilitation activities were undertaken in the area to guarantee the population's safety and its complete recovery over time. However, many years following the disaster, there are still numerous remnant spots of polluted soils where vegetation cannot even germinate.

In **Chapter 1**, we assessed the residual pollution in the uppermost sector of the GGC, in terms of PHE mobility and plant performance. For this purpose, we focused on analysing the selective extractions of soil PHEs and their relation to the main soil properties, vegetation distribution and PHE uptake pattern. We observed that PHE pollution in the GGC is still very heterogeneous and that plant species distribution, richness and abundance are conditioned by a gradient of soil pollution, passing from total inhibition of vegetation in the most polluted areas to a rich pool of herbaceous and shrub species in the most recovered ones. These findings suggest that further remediation measures are needed. Before using a new soil amendment in the field, it is advisable to previously test its effects under controlled conditions. For this end, in **Chapter 2**, we tested in a greenhouse experiment the role of gypsum mining spoil in improving soil properties, reducing PHE availability and promoting seed germination and survival, and plant growth. For this experiment we used the most polluted soils in the GGC, which appeared as unvegetated soil patches. We concluded that effective doses of gypsum mining spoil can be used to remediate soils polluted with PHEs and to significantly enhance plant performance. In the light of the positive results obtained for gypsum mining spoil in the greenhouse experiment, we aimed at assessing its bioremediation potential under field conditions. Hence, in **Chapter 3**, we described a field experiment undertaken in the sector of the GGC most affected by residual pollution, where we tested the effect of gypsum mining spoil on the remediation of soil properties and the reduction of PHE mobility and toxicity, through the analysis of PHE fractions and a toxicity bioassay with *Lactuca sativa* L. To compare and evaluate this effect, we also used other waste materials and remediation treatments previously used in the area or in similar scenarios. Our study showed that gypsum mining spoil presents promising results, although marble sludge, with or without vermicompost, was the treatment that most effectively reduced PHE toxicity risk. Since residual pollution has been limiting the recovery of vegetation, in **Chapter 4**, we focused on evaluating the effect of gypsum mining spoil on the spontaneous recovery of native vegetation and PHE uptake pattern. Further, we compared this effect with those obtained by the other soil amendments and treatments tested in Chapter 3. In this case, the treatment that showed the highest herbaceous species cover and diversity was the mixture of vegetated and unvegetated affected soils. This treatment is not feasible at large scale, although in medium to small extensions (consistent with the scale of the unvegetated soil spots) where only annual vegetation grows, it would represent a suitable treatment to consider. However, unless PHEs are retained within the soil matrix, the potential mobilization of some residual pollutants would be present in this treatment. In this sense, marble sludge (with and without vermicompost), followed by gypsum mining spoil with vermicompost, were the treatments that most effectively reduced PHE availability and increased plant recovery altogether. These findings suggest that the use of organo-mineral amendments will play a key role in the remediation of areas affected by residual pollution of PHEs.

Due to the promising results obtained for marble sludge, gypsum mining spoil, and vermicompost in soil remediation and vegetation recovery, further studies should be conducted to assess their positive effects in the long-term and to prevent future acidification processes that could remobilize PHEs in soil. All the lessons learnt in this study provide valuable information about new remediation strategies that could be transposed to similar scenarios over the world.

RESUMEN

RESUMEN

El suelo es uno de los principales proveedores de funciones y servicios ecosistémicos, siendo su productividad uno de los servicios más esenciales para la supervivencia y el desarrollo de la humanidad. No obstante, las actividades antrópicas como la minería pueden degradarlo gravemente, causando peligros relevantes para la salud y los ecosistemas. En este sentido, la acidificación, la salinización y la contaminación de los suelos son motivo de gran preocupación en todo el mundo, por lo que muchos suelos degradados requerirían remediación natural asistida. El objetivo de esta tesis es evaluar el papel del yeso de rechazo como enmienda de suelos afectados por acidificación, salinización y elementos potencialmente tóxicos (EPTs), con el fin de revalorizar este residuo minero al mismo tiempo que se promovería la remediación natural de los suelos degradados y la regeneración de sus comunidades vegetales asociadas. Como área de estudio, elegimos el Corredor Verde del Guadiamar (CVG, Sur de España), escenario de uno de los peores desastres ecológicos europeos de la historia. En 1998, la balsa de la mina de pirita de Aznalcóllar colapsó, liberando aguas ácidas y lodos altamente contaminados con EPTs. En consecuencia, se emprendieron múltiples actividades de limpieza y rehabilitación en la zona para garantizar la seguridad de la población y la completa recuperación de la zona a lo largo del tiempo. Sin embargo, muchos años después de la catástrofe, todavía quedan numerosas manchas de suelos contaminados donde la vegetación ni siquiera puede emerger.

En el **Capítulo 1**, evaluamos la contaminación residual en el sector más alto del CVG, en términos de movilidad de los EPTs y de recuperación vegetal. Para ello, nos centramos en el análisis de extracciones selectivas de los EPTs del suelo y su relación con las principales propiedades del suelo, la distribución de la vegetación y el patrón de absorción de EPTs. Observamos que la contaminación por EPTs en el CVG es todavía muy heterogénea y que la distribución, riqueza y abundancia de especies vegetales están condicionadas por un gradiente de contaminación del suelo, que pasa de la inhibición total de la vegetación en las zonas más contaminadas a una diversa comunidad de especies herbáceas y arbustivas en las más recuperadas. Una de las conclusiones principales es la necesidad de aplicar medidas de remediación en la zona. Antes de utilizar una nueva enmienda del suelo en campo, es aconsejable testar previamente sus efectos en condiciones controladas. Por consiguiente, en el

Capítulo 2, mediante un experimento de invernadero, probamos el papel del yeso de rechazo en la mejora de las propiedades del suelo, la reducción de la disponibilidad de EPTs, la potenciación de la emergencia y supervivencia de semillas, y el crecimiento de las plantas. Para este experimento, utilizamos los suelos más contaminados del CVG, que aparecían como manchas de suelo sin vegetación. Llegamos a la conclusión de que, con dosis efectivas, el yeso de rechazo se podría emplear para remediar los suelos contaminados con EPTs y para mejorar significativamente el rendimiento de las plantas. A la luz de los resultados positivos obtenidos para el yeso de rechazo en el experimento de invernadero, nos propusimos evaluar su potencial de biorremediación en condiciones de campo. Así pues, en el **Capítulo 3** describimos un experimento de campo realizado en el sector del CGV más afectado por contaminación residual, en el que probamos el efecto del yeso de rechazo en la restauración de las propiedades del suelo y la reducción de la movilidad y la toxicidad de EPTs, mediante el análisis de fracciones de EPTs y un bioensayo de toxicidad con *Lactuca sativa* L. Para comparar y evaluar este efecto, utilizamos también otros materiales de desecho y tratamientos aplicados con anterioridad en la zona o en escenarios similares. Nuestro estudio demostró que el yeso de rechazo presenta resultados prometedores, aunque el lodo de mármol, con o sin vermicompost, fue el tratamiento que más eficazmente redujo el riesgo de toxicidad de los EPTs. Dado que la contaminación residual ha estado limitando la recuperación de la vegetación, en el **Capítulo 4**, nos centramos en evaluar el efecto del yeso de rechazo en la recuperación espontánea de la vegetación autóctona y el patrón de absorción de EPTs, comparando también este efecto con los obtenidos por las otras enmiendas del suelo y tratamientos empleados en el Capítulo 3. En este caso, el tratamiento que obtuvo la mayor cobertura y diversidad de especies herbáceas fue la mezcla de suelo contaminado vegetado con suelo contaminado aún sin vegetación. Este tratamiento no es factible en grandes extensiones, sin embargo, en extensiones medianas o pequeñas (en consonancia con la escala de nuestras manchas de suelo sin vegetación) donde sólo crece vegetación anual, sí podría representar un tratamiento a considerar. No obstante, pese a que los EPTs están mayoritariamente retenidos dentro de la matriz del suelo, algunos contaminantes podrían presentar una cierta movilización potencial en este tratamiento. En este sentido, el lodo de mármol (con y sin vermicompost), seguido del yeso de rechazo con vermicompost, fueron los tratamientos que más redujeron la disponibilidad de EPTs a la par que aumentaron la recuperación de la vegetación, lo que sugiere que el uso de enmiendas órgano-minerales podría

desempeñar un papel fundamental en la recuperación de zonas afectadas por contaminación residual de EPTs.

Debido a los prometedores resultados obtenidos para el lodo de mármol, el yeso de rechazo, y el vermicompost en la remediación del suelo y la recuperación de la vegetación, deberían realizarse ulteriores estudios para evaluar sus efectos positivos a largo plazo y evitar futuros procesos de acidificación que podrían volver a movilizar los EPTs en el suelo. Todas las lecciones aprendidas en este estudio proporcionan información valiosa sobre nuevas estrategias de remediación que podrían transponerse a escenarios similares en todo el mundo.

INTRODUCTION

INTRODUCTION

1. Soil pollution

Soil is one of the main providers of environmental functions and services, its productivity being the most essential service for human survival and development (Kabata-Pendias and Pendias, 2000). Even though, an intense and unsustainable use of soil by human beings is leading to the intense degradation of many terrestrial ecosystems. The soil contains one of the most diverse associations of living organisms (Campbell, 2008), and thus, it is considered an ecosystem in itself (Ponge, 2015), whose most visible tenants are plants. Soil acts not only as support for vegetation, but also as resource supplier and an environmental filter, diminishing pollution affection towards plants. In return, plants have the capacity to enhance the already high soil resilience (Seybold et al., 1999), decreasing the negative impacts of anthropogenic activities on soils (Gondard et al., 2003). Hence, vegetation curbs soil erosion and positively modify soil physicochemical and biological properties by, for instance, improving soil structure and dealing with pollutants (Ehrenfeld et al., 2005; Pilon-Smits, 2005). As well, plant richness conditions the diversity of soil microbial communities and the ecosystem processes they mediate (i.e. nutrient cycling) (Zak et al., 2003), which are essential for the proper functioning of soil ecosystems. Consequently, plant-soil interdependence is evident and strong, and the interactions between them are the key to understand their mutual ecology (Ehrenfeld et al., 2005). The soil-plant system (Jeffrey, 1987) has a direct impact on the optimization of basic ecosystem functions such as the water cycle, and plays a key role on carbon fixation and climate change control (De-Deyn et al., 2008; Ponge, 2015).

Soil pollution caused by harmful anthropogenic activities has become a major concern worldwide (Liu, et al., 2018). Centuries of anthropogenic activities have resulted in accumulation of pollutants in soils that, in many cases, may generate a risk of environmental toxicity and, thus, a threat for living organisms (Rodríguez-Eugenio et al., 2018). The main anthropogenic sources of soil pollution involve those related to industrial (i.e. mining), urban and agricultural activities, with most polluted soils in the world containing complex mixtures of both organic and inorganic pollutants. The Intergovernmental Technical Panel on Soils (ITPS) identified soil pollution as the third threat to soil functions in Europe (FAO and ITPS, 2015), where it has been estimated that approximately 2.8 million sites are polluted, and that only around 2.3% of those have been remediated (Payá-Pérez et al., 2018). In Spain, the I National

Plan for the Recovery of Polluted Soils (1995-2005) (BOE, 1995) identified 4,532 potentially polluted sites, but only 270 have been recovered; according to the II National Plan for the Recovery of Polluted Soils (2008-2015), at least 170 M € from public funds were invested to this end (MARM, 2009, - PNIR, Appendix 13-).

Among all chemical pollutants, Potentially Harmful Elements (PHEs), which include metals and metalloids, are of major concern since they can persist in soils for a long period of time (Pilon-Smits, 2005) and may have a cumulative effect in organisms, becoming the main source of global environmental pollution with harmful implications also for human health (Muyessar and Linsheng, 2016). Some PHEs are essential for biological functions, such as Cu and Zn, but even though, when exceeding specific thresholds, they all become potentially toxic for live (Gall et al., 2015). Regarding food chain pollution and health risk, As, Cd and Pb are among the most dangerous PHEs (Simon, 2014), since they may become toxic at very low concentrations (Rahman and Singh, 2019).

In order to assess the potential toxicity of PHEs, knowledge of the soil properties related to PHE mobility and availability (i.e.: pH, CEC, clay fraction, organic matter content, etc.) is required. Depending on the element and the soil type and soil use considered, intervention levels are established for each region or country for regulatory purposes. Intervention levels are based on human and ecotoxicological risks and serve as soil quality standards in order to determine whether soils are polluted and require intervention (Lijzen et al., 2001). Nonetheless, intervention levels are usually focused on PHE total concentrations regardless soil properties (Romero-Freire et al., 2014; 2015), and soluble, exchangeable and bioavailable fractions should be also analyzed to evaluate the short and medium-term toxicity and potential dispersion of pollutants in the environment. Moreover, conducting toxicity bioassays would provide crucial information about the ecological risk of PHEs, and could support legislation regarding polluted soils (BOE, 2005).

2. Assisted Natural Remediation and waste materials as soil amendments

2.1. Assisted Natural Remediation

Soil pollution can be managed through *ex situ* and/or *in situ* techniques (Liu et al. 2018); the first ones are more effective in shorter time and easier to monitor, but require the excavation and removal of the affected soils, what generates a great additional impact in an already disturbed environment, as well as health risk, waste production and higher costs (Azubuike et al. 2016), what makes them a less realistic and feasible option for large extensions of polluted soils (Illera et al., 2004; Burgos et al., 2008); the second ones, although frequently considered less effective, especially in cold regions, are usually more cost-effective and, since they tend to preserve the natural structure and functions of soils, they are lately seen as a more eco-efficient solution (Liu et al., 2018).

In drier climates, the natural remediation of polluted soils would be too slow (Adriano et al., 2004). As such, Assisted Natural Remediation (ANR) is crucial for the rehabilitation of the ecosystem. The ANR of polluted areas consists, among others, in the *in-situ* application of soil amendments to immobilize pollutants within the soil matrix and restore soil properties and cycles (Kumpiene et al., 2019), and to promote vegetation recovery (Adriano et al., 2004). In the case of the remediation of soils polluted with PHEs, amendments rich in calcium, iron and/or organic matter have previously proved their effectiveness on a field scale (Adriano et al., 2004; Clemente et al., 2015; Xiong et al., 2015; Aguilar et al., 2007).

2.2. Waste materials as soil amendments

The generation of large amounts of waste materials in different human activities is a global issue (Velenturf et al., 2019). Thus, the revalorization through their use as soil amendments is an eco-friendly, cost-effective and feasible solution (Rodríguez-Jordá et al., 2012) in line with zero waste principles (Greyson, 2007). This thesis was conceived in this line and oriented to study the potential use of organic and inorganic amendments in the remediation of soils acidified, salinized, and polluted with potentially harmful elements. The search for new sustainable treatments for this purpose has made this work primarily focus on gypsum mining spoil, amendment with a high potential in the remediation of polluted soils but with scarce available information so far. Moreover, the comparison with other inorganic (marble sludge) and organic (vermicompost) amendments has also been studied.

2.2.1. Gypsum mining spoil

Gypsum mining spoil is a waste material generated at initial stages of gypsum mining, as a result of the bulk mineral processing. At global scale, gypsum world production in 2018 reached 160 million tons (U.S. Geological Survey, 2019) and according to the data supplied by Ahmed (2011) and the U.S. Geological Survey (2012), approximately 10% of the gypsum production correspond to all kind of gypsum waste materials (i.e. phosphogypsum and gypsum from demolition), although the lack of reporting makes it difficult to quantify exact figures (Lèbre et al., 2017), and there are no specific record for gypsum mining spoil. Nonetheless, large quantities of gypsum mining spoil are expected to be annually produced and accumulated in mining sites, resulting in both storage and environmental problems (Al-Farajat, 2009; Ballesteros et al., 2017).

Gypsum outcrops are related to arid and semiarid conditions. Consequently, Spain has the biggest outcrop area in Europe and, thus, it is one of the main gypsum producers worldwide (Escavy et al., 2012). As a result, recent research about gypsum mining spoil potential in rehabilitation of degraded habitats has been made in the country (Ballesteros, 2018), but as far as our knowledge reaches, there is no available scientific information on the use of gypsum mining spoil in the remediation of polluted soils.

Gypsum mining spoil is mainly formed of gypsum and clay materials. On one hand, gypsum is a calcium and sulfate-rich mineral ($CaSO_4{\cdot}2H_2O$) with the capacity to improve soil texture by promoting soil aggregation, decreasing bulk density and, so, increasing water percolation (Franzen et al., 2006; Tirado-Corbalá et al., 2017); to decrease soil crusting (Amezketa et al., 2005; Franzen et al., 2006); to adjust pH by raising it in acidic soils or decreasing it in alkaline ones (Wallace and Wallace, 1995; Franzen et al., 2006); to reclaim sodic and saline-sodic soils by promoting drainage and, so, leaching out excessive sodium (Sharma et al., 1974; Franzen et al., 2006); to rise plant essential nutrient availability by providing calcium and sulphur over time due to its relatively low dissolution rate (Franzen et al., 2006; Tirado-Corbalá et al., 2017); and to reduce PHE availability by forming precipitates and, so, decreasing PHE toxicity (Franzen et al., 2006; Kim et al., 2018). On the other hand, the clay minerals mixed with gypsum mining spoil also play a significant role in PHE sorption due to their ability to bind metal ions (Kabata-Pendias and Pendias, 2000), as major contributor to soil cation exchange capacity (Hooda, 2010). They are a source of negative surface charges and, therefore, an important solid phase for retaining positively charged ions through electrostatic sorption. Moreover, clay size particles are also responsible for retaining nutrients and water, and for stabilizing humus in the soil (Manjaiah et al., 2010). Despite all the benefits described, some authors observed that elevated doses of gypsum limited the uptake of other essential nutrients (i.e. Mg, K or P) by plants as a result of high concentrations of Ca^{2+} and SO_4^{2-}, and the associated salinity (Syed-Omar et al., 1991; Elrashidi et al., 2010); as well, Ca^{2+} could compete for soil sorption sites with metal cations (i.e. Cd and Cu), increasing their leachability and availability (Garrido et al., 2005) if pH decreased in the soil surface (Sherene, 2010). Consequently, preliminary studies should be accomplished to test the suitability of gypsum mining spoil as soil amendment, as well as the effective dose to be used.

2.2.2. Marble sludge

Marble sludge is a waste material that derives from cutting and polishing marble stones. Considering that marble is the largest produced natural stone in the world, and that at least 20-30% of marble become powder during cutting processes, large amounts of this low-cost waste material are annually produced (Rayed et al., 2019) which, if incorrectly discarded, can generate negative effects on the ecosystem (Al-Hamaiedh, 2010; El-Sayed et al., 2018). Marble sludge is very rich in calcium carbonate, which plays a key role in buffering soil pH through the dissolution of calcite and in PHE immobilization in soils (Fernández-Caliani and Barba-Brioso, 2010). Moreover, it promotes water retention and tends to decrease soil electrical conductivity (Sánchez et al., 2010; González et al., 2012). This amendment has been previously used and tested by other authors, with promising results for pH increase and PHE toxicity reduction in soils affected by mining activities (Pérez-Sirvent et al., 2007; Fernández-Caliani and Barba-Brioso, 2010; Del-Moral-Torres et al., 2010; González et al., 2012, 2013, 2017). Nevertheless, relatively low doses of marble sludge could not effectively reduce PHE availability in highly acidic soils if $CaCO_3$ particles end up coated by iron hydroxy sulphates and gypsum (Simón et al., 2010); and on the other hand, excessive pH increase as a result of the marble liming effect could reduce As fixation in soils (Simón et al., 2010), suggesting that the dose of this amendment should be carefully studied.

2.2.3. Vermicompost

Vermicompost is an organic amendment that derives from composting organic wastes, boosted by earthworms. In our case, vermicompost arises from composting horse manure with *Eisenia Andrei*. The use of earthworms is considered to optimize the composting process and thus to enhance the remediation potential of the final product (Huang et al., 2016). Just one average size horse generates ca. 8 tons of manure yearly (Kenny et al., 2019), what has encouraged its use as soil amendment after composting. The use of organic amendments such as horse manure can 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 PHE toxicity, what will promote the recovery of vegetation (Pérez-Esteban et al., 2012). Sierra-Aragón et al. (2019) demonstrated in an *ex-situ* laboratory experiment that the use of vermicompost improved soil conditions by increasing OC content and pH, and reduced the availability of certain pollutants (Cu, Zn, Cd and Pb), although they also observed that it did not have a positive effect on Pb and As immobilization. Considering that the role of organic matter in the restoration of polluted soils with PHEs is controversial (Gadepalle et al., 2007), its application should be studied with caution.

2.2.4. Organo-mineral amendments

Some authors have pointed out that the combination of organic and calcium-rich amendments is also a good and common technique to rise pH, immobilize PHE, improve soil conditions and to facilitate revegetation (Clemente et al., 2003).

The use of these waste materials would diminish the environmental and industrial impacts caused by their improper disposal and management, at the same time we enhance the rehabilitation of degraded ecosystems such as those in the Guadiamar Green Corridor (CMA, 2003) after the Aznalcóllar mining spill (Grimalt and Macpherson, 1999).

3. Thesis aims

In this thesis, we aim at assessing the role of gypsum mining spoil as amendment of soils affected by acidification, salinization and Potentially Harmful Elements (PHEs), in order to revalue this mining waste material, at the same time we assist the natural rehabilitation of polluted soils and their associated plant communities. The specific goals of this thesis are summarized in Table i.1.

Table i.1. Thesis specific goals and chapters in which they are addressed.

SPECIFIC GOALS	CHAPTERS
Assess the suitability of gypsum mining spoil as amendment of soils affected by PHEs, reducing their availability and toxicity	2, 3
Assess the suitability of gypsum mining spoil as amendment of soils affected by acidification	2, 3
Assess the effect of gypsum mining spoil on soil salinity	2, 3
Assess the role of gypsum mining spoil in promoting seed emergence and plant survival	2, 4
Evaluate the distribution of residual pollution and PHE availability in the sector of the Guadiamar Green Corridor (GGC) most affected by the Aznalcóllar mine spill	1, 3
Test the suitability of gypsum mining spoil compared to other soil amendments in the Assisted Natural Remediation of soils affected by residual pollution in the GGC	3, 4
Test the effectiveness of gypsum mining spoil compared to other soil amendments in enhancing native plant species propagation, vegetation growth and species richness in the GGC polluted soils	4
Test the effectiveness of gypsum mining spoil compared to other soil amendments in reducing PHE uptake by plants in the GGC polluted soils	2, 4

In order to achieve all the goals listed in Table i.1, we selected the closest sector to the Aznalcóllar mine, located in the Guadiamar Green Corridor (GGC) (SW Spain), as study area for two main reasons. On one hand, the presence of polluted, acidic and salinized soils is common and can be used as an outdoor laboratory, and on the other hand, there is a large list of previous research studies (Madejón et al., 2018) that would be very helpful in order to have a full and comprehensive understanding of our own results. Hence, in **Chapter 1**, we assessed the residual pollution in the uppermost sector of the GGC, PHE mobility, and the distribution of vegetation and PHE uptake pattern in relation to PHE availability in residually polluted soils; as

PHE toxicity is directly related to PHE availability (Romero-Freire et al., 2014; 2015), in this chapter we focused on assessing the mobility of PHE by the study of selective extractions and their relations to the main soil properties. Before using a new soil amendment in field, it is advisable to previously test its effects under controlled conditions (Gadepalle et al., 2007); therefore, in **Chapter 2**, after a deep bibliographical research about gypsum properties and environmental applications, in a greenhouse experiment we tested the role of gypsum mining spoil in improving soil properties, reducing PHE availability and promoting seed germination and plant growth and survival; for this experiment we used the most polluted soils found in the uppermost sector of GGC, which appeared as unvegetated soil patches. Then, we aimed at assessing the bioremediation potential of gypsum mining spoil under field conditions. In **Chapter 3**, we described a field experiment undertaken in the sector of the GGC most affected by residual pollution, where we tested the effect of gypsum mining spoil on the remediation of soil properties and the reduction of PHE mobility and toxicity. To compare and evaluate this effect, we also used other soil amendments and treatments previously used in the area or in similar scenarios (Fernández-Caliani and Barba-Brioso, 2010; García-Carmona et al., 2017; Madejón et al., 2018). As it has been reported that the residual pollution in these soils is limiting the recovery of vegetation (García-Carmona et al., 2019), in **Chapter 4**, we have focused on evaluating the effects of gypsum mining spoil on the spontaneous recovery of native vegetation and the PHE uptake pattern, also comparing these effects with those obtained by the other soil amendments and treatments tested in chapter 3.

The **General Discussion** summarizes the main findings and conclusions, highlighting those related to gypsum mining spoil.

4. References

Adriano, D.C., Wenzel, W., Vangronsvel, J., Bolan, N., 2004. Role of assisted natural remediation in environmental cleanup. Geoderma, 122 (2): 121-142. https://doi.org/10.1016/j.geoderma.2004.01.003

Ahmed, A., Ugai, K., Kamei, T., 2011. Investigation of recycled gypsum in conjunction with waste plastic trays for ground improvement. Constr. Build. Mater., 25: 208-217. https://doi.org/10.1016/j.conbuildmat.2010.06.036

Al-Farajat, M., 2009. Role of gypsum wastes in polluting groundwater and enhancing eutrophication in Coasts of Aqaba. Jordan J. Civ. Eng., 3 (3): 213-228.

Al-Hamaiedh, H., 2010. Reuse of marble sludge slime in ceramic industry. Jordan J. Civ. Eng., 4 (3): 264-271.

Aguilar, J., Dorronsoro, C., Fernández, E., Fernández, J., García, I., Martín, F., Sierra, M., Simón, M., 2007. Remediation of As-contaminated soils in the Guadiamar river basin (SW, Spain). Water Air Soil Pollut., 180: 109-118. https://doi.org/10.1007/s11270-006-9254-3

Amezketa, E., Aragues, R., and Bazol, R., 2005. Efficiency of sulfuric acid, mined gypsum and two gypsum byproducts in soil crusting prevention and sodic soil reclamation. Agron. J., 97: 983-989. https://doi.org/10.2134/agronj2004.0236

Azubuike, C.C., Chikere, C.B., Okpokwasili, G.C., 2016. Bioremediation techniques–classification based on site of application: principles, advantages, limitations and prospects. World J. Microb. Biot., 32: 180. https://doi.org/10.1007/s11274-016-2137-x

Ballesteros, M., Cañadas, E., Marrs, R., Foronda, A., Martín-Peinado, F., Lorite, J., 2017. Restoration of gypsicolous vegetation on quarry slopes: guidance for hydroseeding under contrasting inclination and aspect. Land Degrad. Dev., 28(7): 2146-2154. https://doi.org/10.1002/ldr.2740

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

Boletín Oficial del Estado (BOE) 114, 1995. Resolución 11463 por la que se aprueba el Plan Nacional de Recuperación de Suelos Contaminados. Ministerio obras públicas, transporte y medio ambiente, 114: 13926-13930.

Boletín Oficial del Estado (BOE) 15, 2005. Real Decreto 9/2005, de 14 de enero, por el que se establece la relación de actividades potencialmente contaminantes del suelo y los criterios y estándares para la declaración de suelos contaminados. Ministerio de la Presidencia, BOE-A-2005-895.

Burgos, P., Pérez-de-Mora, A., Madejón, P., Cabrera, F., Madejón, E., 2008. Trace elements in wild grasses: a phytoavailability study on a remediated field. Environ. Geochem. Health, 30: 109-114. https://doi.org/10.1007/s10653-008-9135-3

Campbell, A., 2008. Managing Australia's Soils - a policy discussion paper. Prepared for the National Committee on Soil and Terrain.

Clemente, R., Walker, D.J., Roig, A., Bernal, M.P., 2003. Heavy metal bioavailability in a soil affected by mineral sulphides contamination following the mine spillage at Aznalcóllar (Spain). Biodegradation, 14: 199-205. https://doi.org/10.1023/A:1024288505979

Clemente, R., Pardo, T., Madejón, P., Madejón, E., Bernal, M.P., 2015. Food byproducts as amendments in trace elements contaminated soils. Food Res. Int. 73: 176-189. https://doi.org/10.1016/j.foodres.2015.03.040

CMA (Consejería de Medio Ambiente), 2003. Ciencia y restauración del río Guadiamar. PICOVER 1998-2002. Junta de Andalucía, Sevilla, Spain.

De-Deyn, G.B., Cornelissen, J.H.C., Bardgett. R.D., 2008. Plant functional traits and soil carbon sequestration in contrasting biomes. Ecol. Lett., 11: 516-531. https://doi.org/10.1111/j.1461-0248.2008.01164.x

Del-Moral-Torres, F., González, V., García, I., Sánchez, J.A., de-Haro, S., Simón, M., 2010. Effect of organic and inorganic residues on the solubility of trace elements and phytotoxicity of mining waste. Fresen. Environ. Bull., 19: 2071-2076.

Ehrenfeld, J.G., Ravit, B., Elgersma, K., 2005. Feedback in the plant-soil system. Annu. Rev. Env. Resour., 30: 75-115. https://doi.org/10.1016/S0009-2541(02)00124-9

Introduction

El-Sayed, H.A., Farag, A.B., Kandeel, A.M., Younes, A.A., Yousef, M.M., 2018. Characteristics of the marble processing powder waste at Shaq El-Thoaban industrial area, Egypt, and its suitability for cement manufacture. HBRC J., 14 (2): 171- 179. https://doi.org/10.1016/j.hbrcj.2016.06.002

Elrashidi, M.A., West, L.T., Seybold, C.A., Benham, E.C., Schoeneberger, P.J., Ferguson, R., 2010. Effects of gypsum addition on solubility of nutrients in soil amended with Peat. Soil Sci., 175 (4): 162-172. https://doi.org/10.1097/SS.0b013e3181dd51d0

Escavy, J.I., Herrero, M.J., Arribas, M.E., 2012. Gypsum resources of Spain: Temporal and spatial distribution. Ore Geol. Rev., 49: 72-84. https://doi.org/10.1016/j.oregeorev.2012.09.001

FAO and ITPS, 2015. Status of the World's Soil Resources (SWSR) - Main Report. Food and Agriculture Organization of the United Nations and Intergovernmental Technical Panel on Soils, Rome, Italy.

Fernández-Caliani, J.C., Barba-Brioso, C., 2010. Metal immobilization in hazardous contaminated mine soils after marble slurry waste application. A field assessment at the Tharsis mining district (Spain). J. Hazard. Mater., 181: 817-826. https://doi.org/10.1016/j.jhazmat.2010.05.087

Franzen, D., Rehm, G., Gerwing, J., 2006. Effectiveness of gypsum in the North-Central Region of the U.S. NDSU Extension Service, North Dakota.

Gadepalle, V.P., Ouki, S.K., Van Herwijnen, R., Hutchings, T., 2007. Immobilization of heavy metals in soil using natural and waste materials for vegetation establishment on contaminated sites. Soil Sediment. Contam., 16: 233-251. https://doi.org/10.1080/15320380601169441

Gall, J.E., Boyd, R.S., Rajakaruna, N., 2015. Transfer of heavy metals through terrestrial food webs: a review. Environ. Monit. Assess., 187: 201. https://doi.org/10.1007/s10661-015-4436-3

García-Carmona, M., Romero-Freire, A., Sierra Aragón, M., Martínez Garzón, F.J., Martín Peinado, F.J., 2017. Evaluation of remediation techniques in soils affected by residual contamination with heavy metals and arsenic. Journal Environ. Manage., 191: 228-236. http://doi.org/10.1016/j.jenvman.2016.12.041

Introduction

García-Carmona, M., García-Robles, H., Turpín Torrano, C., Fernández Ondoño, E., Lorite Moreno, J., Sierra Aragón, M., Martín Peinado, F.J., 2019. Residual pollution and vegetation distribution in amended soils 20 years after a pyrite mine tailings spill (Aznalcóllar, Spain). Sci. Total Environ., 650: 933-940. https://doi.org/10.1016/j.scitotenv.2018.09.092

Garrido, F., Illera, V., García-González, M.T., 2005. Effect of the addition of gypsum- and lime-rich industrial by-products on Cd, Cu and Pb availability and leachability in metal-spiked acid soils. Appl. Geochem., 20: 397-408. https://doi.org/10.1016/j.apgeochem.2004.08.001

González, V., García, I., Del Moral, F., Simón, M., 2012. Effectiveness of amendments on the spread and phytotoxicity of contaminants in metal-arsenic polluted soil. J. Hazard. Mater., 205-206: 72-80. https://doi.org/10.1016/j.jhazmat.2011.12.011

González, V., Simón, M., García, I., Sánchez, J.A., del Moral, F., 2013. Effectiveness of amendments to restore metal-arsenic-polluted soil functions using *Lactuca sativa* L. bioassays. J. Soils Sediments, 13: 1213-1222. https://doi.org/10.1007/s11368-013-0701-7

González, V., Salinas, J., García, I., del Moral, F., Simón, M., 2017. Using marble sludge and phytoextraction to remediate metal(loid) polluted soils. J. Geochem. Explor., 174: 29-34. http://doi.org/10.1016/j.gexplo.2016.03.008

Gondard, H., Jauffret, S., Aronson, J., Lavorel, S., 2003. Plant functional types: a promising tool for management and restoration of degraded lands. Appl. Veg. Sci., 6: 223-234. https://doi.org/10.1111/j.1654-109X.2003.tb00583.x

Greyson, J., 2007. An economic instrument for zero waste, economic growth and sustainability. J. Clean. Prod., 15(13-14): 1382-1390. https://doi.org/10.1016/j.jclepro.2006.07.019

Grimalt, J.O., Macpherson, E. (Eds.), 1999. The environmental impact of the mine tailing accident in Aznalcóllar. Sci. Total Environ. 242 (special volume), 1-337.

Hooda, P., 2010. Trace Elements in Soils, first ed. John Wiley and Sons Ltd, Chichester, West Sussex.

Huang, M., Zhu, Y., Li, Z., Huang, B., Luo, N., Liu, C., Zeng, G., 2016. Compost as a soil amendment to remediate heavy metal-contaminated agricultural soil: mechanisms, efficacy, problems, and strategies. Water Air Soil Poll., 227: 359. https://doi.org/10.1007/s11270-016-3068-8

Illera, V., Garrido, F., Serrano, S., García-González, M.T., 2004. Immobilization of the heavy metals Cd, Cu and Pb in an acid soil amended with gypsum- and lime-rich industrial by-products. Eur. J. Soil Sci., 55: 135-145. https://doi.org/10.1046/j.1365-2389.2003.00583.x

Kabata-Pendias, A., Pendias, H., 2000. Trace Elements in Soils and Plants, third ed. CRC Press, Boca Raton.

Kenny, L.B., Westendorf, M., Williams, C.A., 2019. Chapter 14 - Managing manure, erosion, and water quality in and around horse pastures, in: Sharpe, P., (Ed), Horse Pasture Management. Academic Press, pp. 245-259. https://doi.org/10.1016/B978-0-12-812919-7.00014-7

Kim, H.S., Seo, B.H., Kuppusamy, S., Lee, Y.B., Lee, J.H., Yang, J.E., Owens, G., Kim, K.R., 2018. A DOC coagulant, gypsum treatment can simultaneously reduce As, Cd and Pb uptake by medicinal plants grown in contaminated soil. Ecotoxicol. Environ. Saf., 148: 615-619. https://doi.org/10.1016/j.ecoenv.2017.10.067

Kumpiene, J., Antelo, J., Brännvall, E., Carabante, I., Ekc, K., Komárek, M., Söderbergc, C., Wårell, L., 2019. In situ chemical stabilization of trace element-contaminated soil - Field demonstrations and barriers to transition from laboratory to the field - A review. Appl. Geochem., 100: 335-351. https://doi.org/10.1016/j.apgeochem.2018.12.003

Jeffrey, D.W., 1987. Soil-plant relationships: An ecological Approach. Croom Helm Ltd, Beckenham, Kent.

Lèbre, E., Corder, G., Golev, A., 2017. The role of the mining industry in a circular economy. A framework for resource management at the mine site level. J. Ind. Ecol., 21(3). https://doi.org/10.1111/jiec.12596

Lijzen, J.P.A., Baars, A.J., Otte, P.F., Rikken, M.G.J., Swartjes, F.A., Verbruggen, E.M.J., Wezel, A.P., 2001. Technical evaluation of the intervention values for soil/sediment and groundwater; human and ecotoxicological risk assessment and derivation of risk limits for soil, aquatic sediment and groundwater. In RIVM report 711701 023.

Liu, L., Li, W., Song, W., Guo, M., 2018. Remediation techniques for heavy metal-contaminated soils: Principles and applicability. Sci. Total Environ., 633: 206-219. https://doi.org/10.1016/j.scitotenv.2018.03.161

Madejón, P., Domínguez, M.T., Madejón, E., Cabrera, F., Marañón, T., Murillo-Carpio, J.M., 2018. Soil-plant relationships and contamination by trace elements: A review of twenty years of experimentation and monitoring after the Aznalcóllar (SW Spain) mine accident. Sci. Total Environ., 625: 50-63. https://doi.org/10.1016/j.scitotenv.2017.12.277

Manjaiah, K.M., Kumar, S., Schdev, M.S., Sachdev, P. Datta, S.C. 2010. Study of clay-organic complexes. Curr. Sci., 98(7): 915-921.

Ministerio de Medio Ambiente y Medio Rural y Marino (MARM), 2007. II Plan Nacional Integrado de Residuos (PNIR), 2008-2015. Secretaría General del Estado, Madrid, España. Available at: http://www.icog.es/files/PNIR3.pdf

Muyessar, T., Linsheng Y., 2016. Trace Elements Contamination and Human Health Risk Assessment in Drinking Water from the Agricultural and Pastoral Areas of Bay County, Xinjiang, China. Int. J. Environ. Res. Public Health, 13(10): 938. https://doi.org/10.3390/ijerph13100938

Payá-Pérez, A., Rodríguez-Eugenio, N., 2018. Status of local soil contamination in Europe: Revision of the indicator 'Progress in the management Contaminated Sites in Europe'. EUR 29124 EN, Publications Office of the European Union, Luxembourg.

Pérez-Esteban, J., Escolástico, C., Masaguer, A., Moliner, A., 2012. Effects of sheep and horse manure and pine bark amendments on metal distribution and chemical properties of contaminated mine soils. Eur. J. Soil Sci., 63: 733-742. https://doi.org/10.1111/j.1365-2389.2012.01468.x

Pérez-Sirvent, C., García-Lorenzo, M.L., Martínez-Sánchez, M.J., Navarro, M.C., Marimón, J., Bech, J., 2007. Metal-contaminated soil remediation by using sludges of the marble industry: Toxicological evaluation. Environ. Int., 33: 502-504. https://doi.org/10.1016/j.envint.2006.11.003

Pilon-Smits, E., 2005. Phytoremediation. Annu. Rev. Plant Biol., 56: 15-39. http://doi.org/10.1146/annurev.arplant.56.032604.144214

Ponge, J. 2015. The soil as an ecosystem. Biol. Fertil. Soils, 51: 645-648. https://doi.org/10.1007/s00374-015-1016-1

Rahman, Z., Singh, V.P., 2019. The relative impact of toxic heavy metals (THMs) (arsenic (As), cadmium (Cd), chromium (Cr)(VI), mercury (Hg), and lead (Pb)) on the total environment: an overview. Environ. Monit. Assess., 191(7): 419. https://doi.org/10.1007/s10661-019-7528-7

Rayed, A., Omrane, B., Chokri, S., Mohamed-Amine, K., Abdeliazim-Mustafa M., 2019. Effects of incorporation of marble powder obtained by recycling waste sludge and limestone powder on rheology, compressive strength, and durability of self-compacting concrete. Adv. Mater. Sci. Eng., Volume 2019. https://doi.org/10.1155/2019/4609353

Rodríguez-Eugenio, N., McLaughlin, M., Pennock, D., 2018. Soil Pollution: a hidden reality. Rome, FAO, 142.

Rodríguez-Jordá, M.P., Garrido, F., García-González, M.T., 2012. Effect of the addition of industrial by-products on Cu, Zn, Pb and As leachability in a mine sediment. J. Hazard. Mater., 213-214: 46-54. https://doi.org/10.1016/j.jhazmat.2012.01.049

Romero-Freire, A., Sierra-Aragón, M., Ortiz-Bernand, I., Martín-Peinado, F., 2014. Toxicity of arsenic in relation to soil properties: implications to regulatory purposes. J. Soils Sediments, 14 (5): 968-979. https://doi.org/10.1007/s11368-014-0845-0

Romero-Freire, A., Martín Peinado, F., van Gestel, C.A.M., 2015. Effect of soil properties on the toxicity of Pb: assessment of the appropriateness of guideline values. J. Hazard Mater., 289: 46-53. https://doi.org/10.1016/j.jhazmat.2015.02.034

Sánchez, J.A., García, I., del Moral, F., de Haro, S., González, V., Simón, M., Martín, F., Iriarte, A., 2010. Utilización de lodos de corte y pulido del mármol en la recuperación de escombreras de mármol. Rev. Cienc. Agrar., 33 (1): 182-190.

Seybold, C.A., Herrick, J.E., Brejda, J.J., 1999. Soil resilience: a fundamental component of soil quality. Soil Sci., 164 (4): 224-234.

Sharma, A.K., Fehrenbacher, J.B., Jones, B.A., 1974. Effect of gypsum, soil disturbance and tile spacing on the amelioration of Huey silt loam, a nitric soil in Illinois. Soil Sci. Soc. Am. Pro., 38: 628-632. https://doi.org/10.2136/sssaj1974.03615995003800040029x

Sherene, T., 2010. Mobility and transport of heavy metals in polluted soil environment. Biol. Forum, 2(2): 112-121.

Sierra-Aragón, M., Mitsui-Nakamaru, Y., García-Carmona, M., Martínez-Garzón, F.J., Martín-Peinado, F.J., 2019. The role of organic amendment in soils affected by residual pollution of potentially harmful elements. Chemosphere, 237: 124549. https://doi.org/10.1016/j.chemosphere.2019.124549

Simon, L., 2014. Potentially harmful elements in agricultural soils, in: Claudio Bini, C., Bech, J., (Eds), PHEs, Environment and Human Health. Potentially harmful elements in the environment and the impact on human health, Chapter 3. Springer Science and Business Media, Dordrecht. https://doi.org/10.1007/978-94-017-8965-3_3

Simón, M., Diez, M., González, V., García, I., Martín, F., and de Haro, S., 2010. Use of liming in the remediation of soils polluted by sulphide oxidation: A leaching-column study. J. Hazard. Mater., 180: 241-246. https://doi.org/10.1016/j.jhazmat.2010.04.020

Syed-Omar, S.R., Sumner, M.E., 1991. Effect of gypsum on soil potassium and magnesium status and growth of alfalfa. Commun. Soil Sci. Plan., 22 (19-20): 2017-2028. https://doi.org/10.1080/00103629109368554

Tirado-Corbalá, R., Slater, B.K., Dick, W.A., Barker, D., 2017. Alfalfa responses to gypsum application measured using undisturbed soil columns. Plants, 6: 29. https://doi.org/10.3390/plants6030029

U.S. Geological Survey, 2012. Mineral commodity summaries 2012: U.S. Geological Survey, 70-71.

U.S. Geological Survey, 2019. Mineral commodity summaries 2019: U.S. Geological Survey, 74-75. https://doi.org/10.3133/70202434

Velenturf, A.P.M., Purnell, P., Macaskie, L.E., Mayes, W.M., Sapsford, D.J., 2019. A new perspective on a global circular economy, in: Macaskie, L.E., Sapsford, D.J., Mayes, W.M., (Eds.), Resource Recovery from Wastes: Towards a Circular Economy, Chapter 1. Royal Society of Chemistry, pp. 1-2. https://doi.org/10.1039/9781788016353-00001

Wallace, A., Wallace, G.A., 1995. Gypsum is almost a universal soil amendment, Soil amendments and amendment technologies, Wallace Laboratories, El Segundo.

Xiong, J., Madejón, P., Madejón, E., Cabrera, F., 2015. Assisted natural remediation of a trace element-contaminated acid soil: an eight-year field study. Pedosphere, 25: 250-262. https://doi.org/10.1016/S1002-0160(15)60010-8

Zak, D.R., Holmes, W.E., White, D.C., Peacock, A.D., Tilman, D., 2003. Plant diversity, soil microbial communities, and ecosystem function: are there any links? Ecology, 84 (8): 2042-2050.https://doi.org/10.1890/02-0433

MATERIAL AND METHODS

MATERIAL AND METHODS

1. Study area

The study area in this thesis is located at the Guadiamar Green Corridor (SW Spain), in the sector closest to the Aznalcóllar pyrite mine, where the presence of soils affected by residual pollution is frequent (Figure m.1).

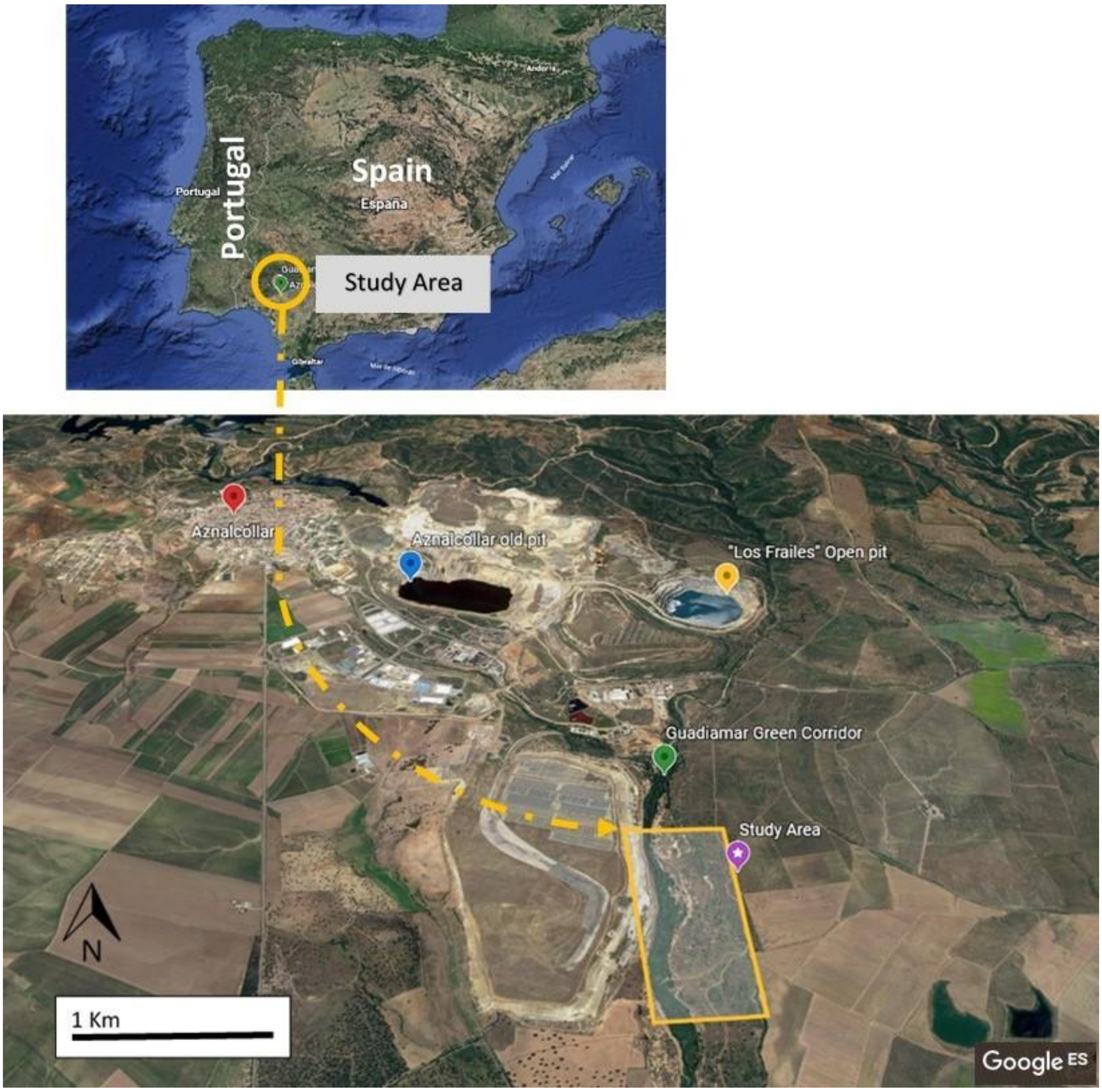


Figure m.1. Satellite image of the study area in Aznalcóllar (Seville, SW Spain).

Material and methods

The pyrite mine of Aznalcóllar (Seville, Spain) is located in the Iberian Pyrite Belt (IPB, Figure m.2), one of the largest massive sulphide deposits in the world, which has been exploited since the 3rd Millenium BC (Nocete et al., 2014). This orebody, whose reserves exceed 1,500 Mt (Sáez et al., 1999), is composed of 95% pyrite (FeS_2), chalcopyrite ($CuFeS_2$), sphalerite (ZnS), and accessory galena (PbS) and arsenopyrite (FeAsS), and 5% Cu-rich minerals (Chopin and Alloway, 2007). As a result, mining activities in this area have not only posed a potential pollution risk for the surrounding ecosystems, but have also led to the release of Cu, Zn, Pb and As into the environment for centuries (Chopin and Alloway, 2007).

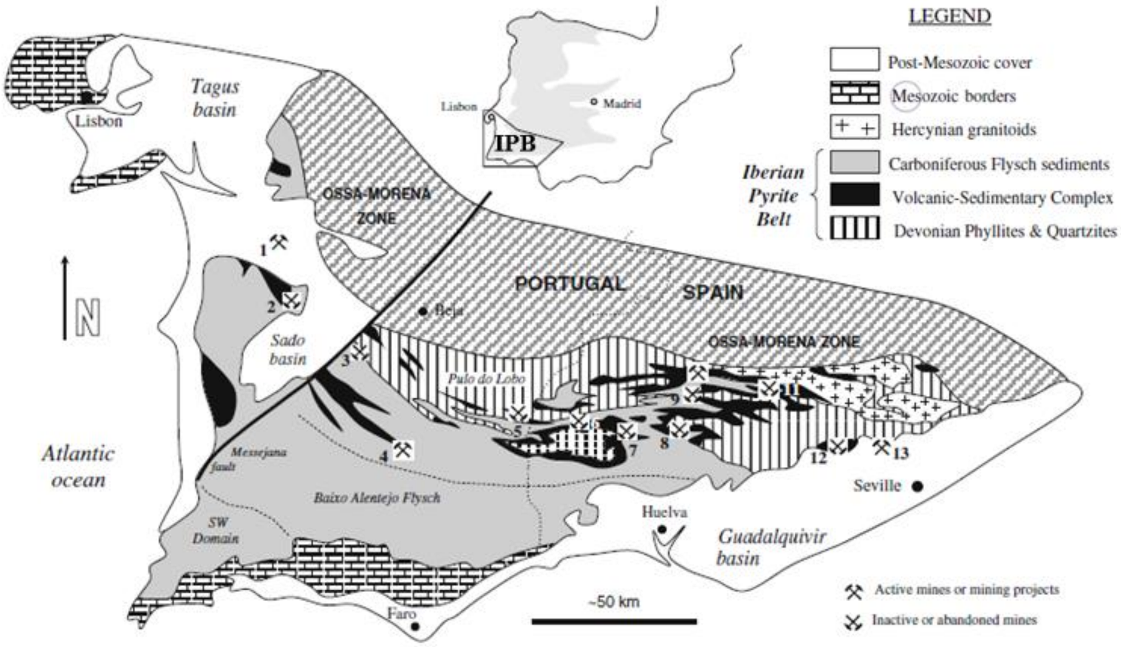


Figure m.2. Geological map of the southwestern Iberian Peninsula (adapted from Quesada et al. 1991 and Fernández-Caliani et al., 2009) showing the location of the major massive sulfide deposits in the Iberian Pyrite Belt (IPB): 1 Lagoa-Salgada, 2 Lousal, 3 Aljustrel, 4 Neves-Corvo, 5 São Domingos, 6 Herrerías, 7 Tharsis, 8 Sotiel-Migollas, 9 La Zarza-Perrunal, 10 Nueva Aguas Teñidas, 11 Riotinto, 12 Aznalcóllar-Los Frailes, 13 Las Cruces.

The current mine site (Figure m.3) occupies nearly 950 ha and contains around 80 million tonnes of polymetal sulphides (CMA, 2004). In 1996, a pond was placed next to the Agrio River, affluent of the Guadiamar River, which would contain the tailings from "Los Frailes" mining activity (Grimalt et al., 1999).

Figure m.3. Aznalcóllar mine site. Source: Andalusian Regional Government. Retrieved at: http://www.juntadeandalucia.es/economiainnovacioncienciayempleo/pam/img/aznalcollar.jpg

The Guadiamar valley is formed by Neogene to Quaternary sedimentary materials. The sector where we developed our study is formed by alluvial deposits, including two younger (lower) levels of Holocene age river terraces and an older (upper) level of Pliocene river terrace. These alluvial deposits are made of silty-sand materials with gravels dominated by quartzite, shales, and schists, with a basement consisting of thick Miocene blue marls (Manzano et al., 2000; Salvany et al., 2001). The dominant soil types in the area are associated with the fluvial dynamic of the Agrio and Guadiamar rivers. According to the FAO classification (IUSS, 2015), at the closest sector to the Aznalcóllar pyrite mine, the soils located close to the river channel are eutric Fluvisols, and outside the influence of periodic floodings are eutric Regosols. In all cases, soils have a neutral pH (6.5-7.5), organic matter content ranging from 0.8 to 2.9%, low content in calcium carbonate (usually below 1%), and texture varying from clay-loam to sandy-loam with high content in gravel (>10%) (Simón et al., 1998; Ordóñez et al., 2003). The climate of the area is typically Mediterranean, with cold wet winters, intense summer droughts (more than 6 months), annual rainfall ranging from 550 to 650 mm (Martín-Peinado, 2002), and a potential evapotranspiration of 900 mm (Simón et al., 2008). In terms of vegetation, the Guadiamar river floodplain was occupied mainly by croplands and orchards

(Madejón et al., 2003), but also by riverbank vegetation formed of *Fraxinus angustifolia*, *Populus alba*, *Populus nigra*, *Ulmus minor*, *Salix purpurea* and *Salix atrocinerea* as dominant species, and less frequently formed of a typical agrosylvopastoral system called "dehesa" (in Spain) with holm oak (*Quercus ilex*) and cork oak (*Quercus suber*), together with other typical Mediterranean species such as *Pyrus bougaeana, Retama sphaerocarpa, Daphne gnidum,* and *Thymus mastichina*, and annual pastureland filling the gaps in this open forest (Martín-Peinado, 2002).

On the 25th April 1998, the pond containing the toxic waste from the Aznalcóllar mining activity collapsed and a spill of ca. 6 hm^3 of tailings and acid waters spread 400 m wide to the river sides (Agrio and Guadiamar) and 43 km downstream to Doñana National Park (Grimalt and Macpherson, 1999) (Figure m.4). It is considered one of the most severe pollution accidents worldwide related to mining activities (Grimalt and Macpherson, 1999; Madejón et al., 2018; Sierra-Aragón et al., 2019).

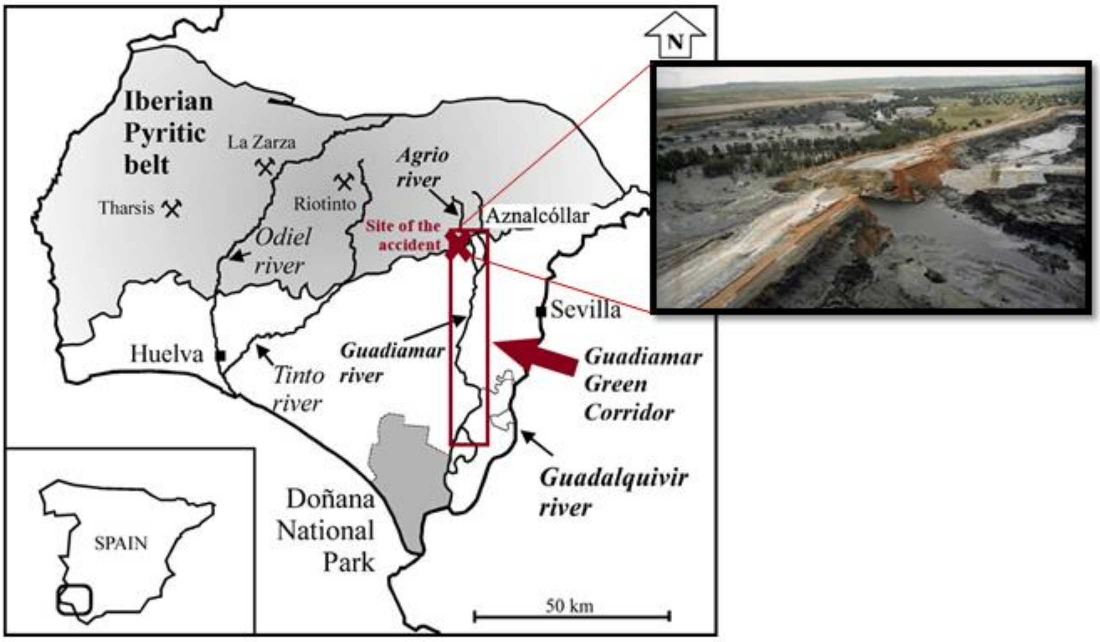


Figure m.4. Location map of the mining accident site, the area affected by the spill in red (Guadiamar Green Corridor), and Doñana National Park (adapted from Domènech et al., 2002). The picture shows the breakage of the Aznalcóllar tailing pond and the release of toxic sludge and acidic waters to the Agrio river. Retrieved at: https://www.elconfidencial.com/espana/andalucia/2018-04-25/aznalcollar-desastre-pudo-evitarse-ecologistas-alerta_1554645/

Material and methods

The content of Potentially Harmful Elements (PHEs) in the affected soils nearby the mine were measured 9 days after the accident, reporting concentrations of 8,063 mg kg^{-1} for Zn, 1,875 mg kg^{-1} for Cu, 30 mg kg^{-1} for Cd, 5,753 mg kg^{-1} for Pb, and 2,073mg kg^{-1} for As (Simón et al., 1999). A rapid and highly coordinated performance from the National and Regional Governments along with a pool of experienced scientists, prevented that the spill caused an irreversible damage in Doñana National Park, a key biodiversity hotspot and the largest reserve of birds in Europe (Grimalt et al., 1999). The first activities were focused on a rapid clean-up of the tailings and the uppermost layer of the soil (irreversibly polluted). This was followed by the application of soil amendments (organic and/or inorganic) to immobilize residual pollution in the soil, prevent groundwater pollution, and promote soil function recovery. Afterwards, an intense afforestation plan was implemented with native species, what would suppose the stabilization of the soil surface, through the accumulation of pollutants in the root system and a low root-to-shoot transfer of PHEs (phytostabilization); the main tree and shrub species used were: *Populus alba, Celtis australis, Fraxinus angustifolia, Quercus ilex, Quercus suber, Ceratonia siliqua,* and *Pinus pinea*, together with *Olea europaea, Myrtus communis, Retama sphaerocarpa, Rosmarinus officinalis, Tamarix gallica, Pistacea terebintus, and Phillyrea angustifolia* (Pastor-Jáuregui et al., 2020). Finally, the area was declared a natural protected area (Protected Landscape), named “The Guadiamar Green Corridor (GGC)” (CMA, 2003), in order to guarantee both population’s safety at first term, and its complete rehabilitation over time. Nonetheless, many years after the spill, the GGC is still affected by residual pollution conditioning the emergence, growth, and distribution of vegetation in some areas. According to the thresholds recommended by the Regional Government of Andalusia to declare a soil as potentially polluted (BOE, 2015), Pastor-Jáuregui et al. (2020) estimated that at least 11% of soils in the GGC exceeds the regulatory value for Pb, and at least 70% surpassed the regulatory value for As. Furthermore, in some areas closest to the mine (first 18 km downstream), an irregular distribution of the residual pollution is still observed (Figure m.5), with highly polluted and unvegetated patches (around 7% of the area according to Martín-Peinado et al., 2015) surrounded by less polluted and vegetated areas (Simón et al., 2008). Residual pollution is a threat for the ecosystem and indicates that urgent restoration activities are largely required (Martín-Peinado et al., 2015) to prevent the dispersal of pollutants towards the surrounding rehabilitated areas (García-Carmona et al., 2019). All things considered, the Guadiamar Green Corridor has become a large outdoor laboratory for pollution research (Madejón et al., 2018).

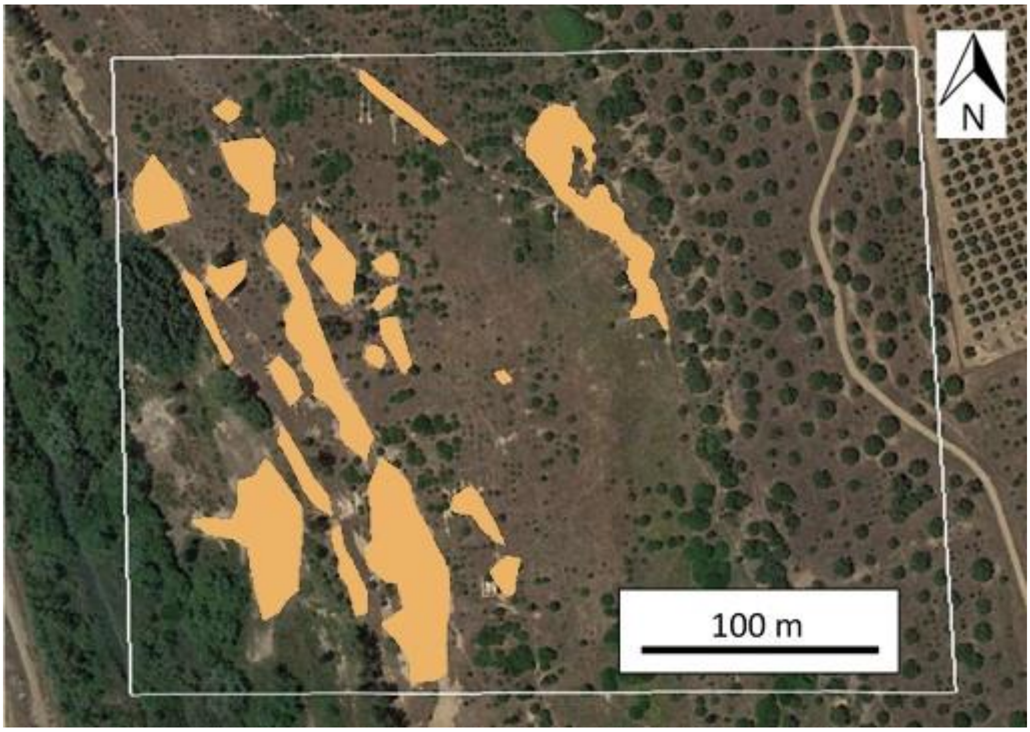


Figure m.5. Satellite image of an affected area in the closest sector to the Aznalcóllar mine in the Guadiamar Green Corridor. The rectangle (white line) represents 10 Ha, where highly polluted unvegetated patches (in orange) appeared surrounded by vegetation. In this area, the orange patches occupy around 0.8 Ha.

2. Experimental design

Within the study area, we measured the total concentrations of the main pollutants (Cu, Zn, Cd, Sb, As and Pb) in several unvegetated affected soils randomly selected. Pollutant concentrations were determined in field by X-ray fluorescence in a NITON XLt 792 analyzer (Figure m.6), with a 40 kV X-ray tube with Ag anode target excitation source, and a Silicon PIN-diode with a Peltier cooled detector. The procedure followed the manufacturer's instructions and the recommendations of the Method 6200 (US EPA, 1998). The accuracy of the method, the analytical precision and the detection limits were evaluated according to US EPA (2006) in Martín-Peinado et al. (2010). The patches with the highest load of pollutants were selected as experimental areas for the field experiments and as sampling areas to collect polluted soils for the greenhouse experiment.

Figure m.6. NITON XLt 792 analyzer.

2.1. Field study 1. Characterization of affected areas (Chapter 1)

In a preliminary study, we selected four residually polluted plots and divided them into three subareas according to a soil recovery gradient in terms of the presence of vegetation, from unvegetated soils (UV-AA) right in the centre, to two surrounding belt subareas characterized by a progressive vegetation appearance, an intermediate moderately re-vegetated belt (B1) and an outer and more recovered belt (B2) (Figure m.7).

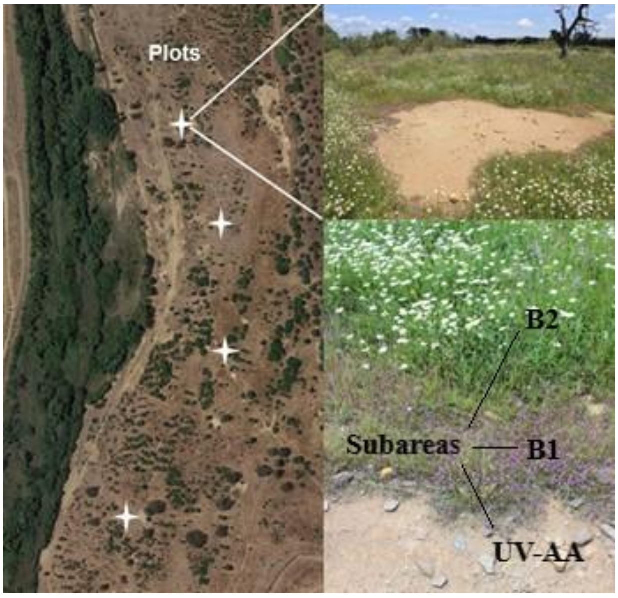


Figure m.7. Plot location in the Guadiamar Green Corridor on the left, and a detail of the different subareas according to herbaceous species composition and abundance (UV-AA: unvegetated affected soils; B1: belt 1; B2: belt 2) on the right.

2.2. Greenhouse experiment (Chapter 2)

Soil samples were collected at highly polluted unvegetated areas (UV-AA), from the uppermost 10 cm, and intensely homogenized into just one composite soil sample (C_0).

The experimental design (Figure m.8) was based on the addition of gypsum mining spoil (G) to the unvegetated polluted soils (C_0) in three different proportions (treatments): 10% G (T1), 20% G (T2), and 50% G (T3). Afterwards, we prepared 32 replicates per treatment (pot size= 6 cm x 5.6 cm x 8 cm), plus 32 control replicates (C= 0% G) with non-treated polluted soil (C_0), where seeds of two non-genetically related species (*Cynodon dactylon* Pers. and *Medicago sativa* L.) were sowed separately. Finally, pots with different treatments were randomly distributed in a greenhouse located at the Andalusian Institute of Agricultural Research and Training, IFAPA (Granada, SE Spain), which was equipped with an irrigation programmer, a nebulization system (30 l/h irrigation flow) and a temperature control sensor, in order to control water availability and temperature. The experiment lasted 82 days for *M. sativa* and 67 days for *C. dactylon*, and pots were watered five minutes daily.

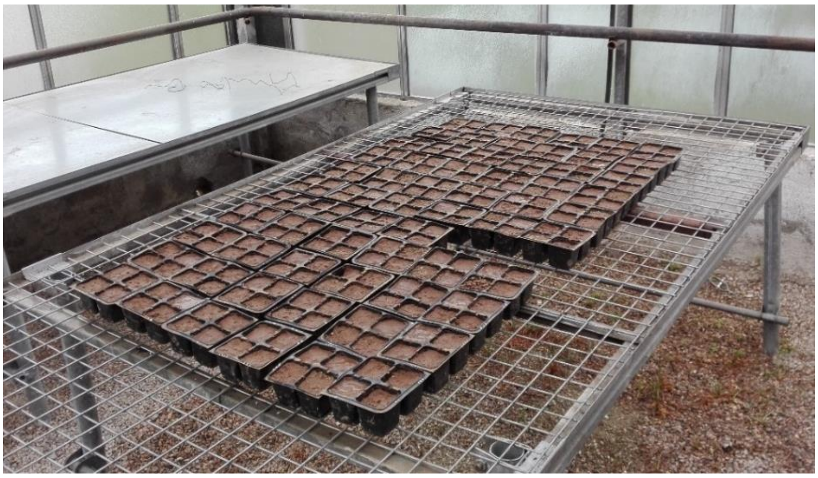


Figure m.8. Experimental design of the greenhouse experiment.

2.3. Field study 2. Experimental plots with soil treatments (Chapters 3 and 4)

Among the most polluted areas, we selected 6 unvegetated areas (UV-AA) to establish 6 experimental plots and test the remediation potential of different soil treatments (Figure m.9). As external controls, we also selected 6 neighbouring vegetated affected areas (V-AA) and an unaffected area (UA) within the Guadiamar Green Corridor, but where the spill did never reach.

Experimental plots (36 m^2) consisted in 9 subplots (4 m^2 each), where eight different treatments were applied, and a control subplot was also defined (UV-AA) (Figure m.9). Treatments consisted in: i) Simple treatments: 1. G: Addition of 5 kg m^{-2} of gypsum mining spoil; 2. M: Addition of 5 kg m^{-2} of marble sludge; 3. T: crust breaking through tillage; 4. V: mixture of V-AA and UV-AA (50% w/w) in the uppermost 10 cm of the soil; and ii) Mixed treatments (the same treatments mixed with vermicompost "v"): 5. Gv: G + v; 6. Mv: M + v; 7. Tv: T + v; 8. Vv: V + v. 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 V 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.

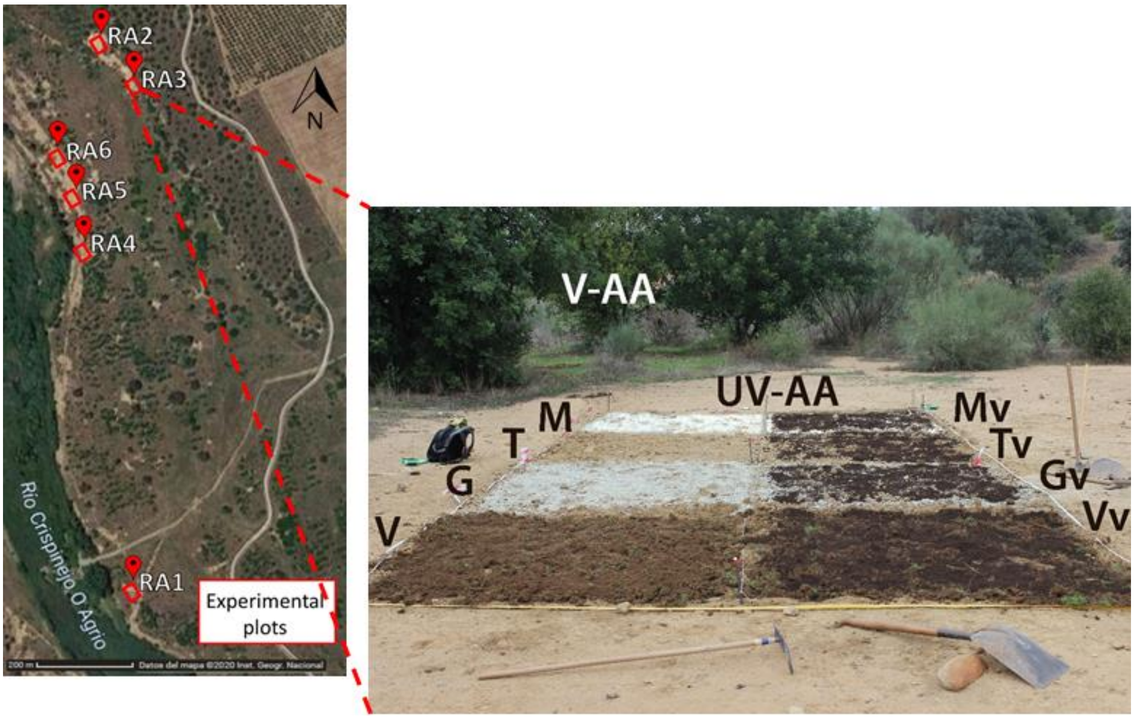


Figure m.9. Experimental plot with soil treatments: **G**: Addition of gypsum mining spoil; **M**: Addition of marble sludge; **T**: Tillage; **V**: mixture of UV-AA and V-AA; **Gv**: G plus vermicompost (v); **Mv**: M + v; **Tv**: T + v; **Vv**: V + v. **UV-AA**: Unvegetated Affected Area with control subplot. **V-AA**: Vegetated Affected Area.

The results of this experiment have been described in chapters 3 and 4. In chapter 3, we focused on the effects of treatments on soil properties and PHE mobility and toxicity, and in Chapter 4, on the effects on natural vegetation recovery and PHE uptake by plants.

3. Sampling

3.1. Plants

3.1.1. Field study 1. Characterization of affected areas (Chapter 1)

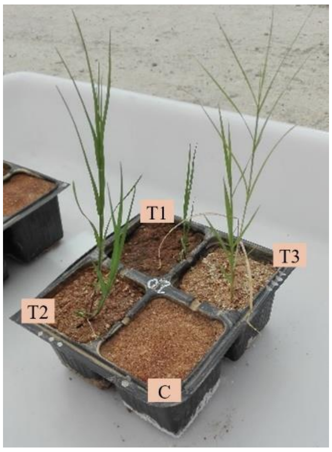
The sampling methods used for vegetation in field are based on species frequency variables (Bonham, 2013). Total and individual cover of herbaceous species were monitored using a 0.25 m^2 quadrat divided into 100 cells and expressed as the percentage of cells occupied by herbaceous species, both as total cover and as cover per species (Elzinga et al. 2015). The quadrat was randomly placed and replicated three times per experimental subplot. Herbaceous species richness was calculated as the average richness values per replicates of the same subplot.

In order to analyse PHE uptake by plants per subarea, three vegetation samples representing the whole community (composite sample) were collected in the two vegetated belts and in the four selected plots.

3.1.2. Greenhouse experiment (Chapter 2)

Plant emergence and survival of the two model species (*C. dactylon* and *M. sativa*) were monitored three times per week until the experiment was completed. We visually checked cotyledon protrusion for emergence and marked the first seedling to emerge in each pot (first individual, hereafter), for survival monitoring (Figure m.10). Following the same criteria, a second seedling was marked to ensure that enough individuals were available to assess growth, in case of early death of the first individual. When each pot had two seedlings, new emerging plants were immediately clipped after recording emergence. The second marked seedling in each pot was also clipped after 4 weeks if the first individual survived, to avoid competition between seedlings.

At the end of the experiment, all the plants were collected in order to measure biomass and analyse PHE content.



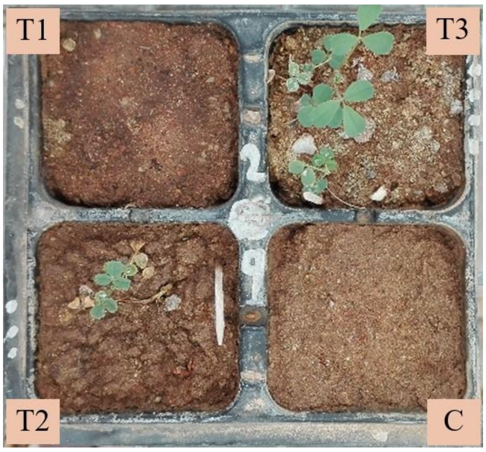


Figure m.10. Survival monitoring. On the left, a pot with *C. dactylon* plants. On the right, a pot with *M. sativa* plants. Treatments: C, non-treated soil (100% C_0); T1, 90% C_0 + 10% G; T2, 80% C_0 + 20% G; T3, 50% C_0 + 50% G. C_0: polluted soil. G: Gypsum mining spoil.

3.1.3. Field study 2. Experimental plots with soil treatments (Chapter 4)

Total and individual cover of herbaceous species were monitored using a 0.25 m^2 quadrat divided into 100 cells and expressed as the percentage of cells occupied by herbaceous species, both as total cover and as cover per species (Elzinga et al. 2015). The quadrat was replicated four times per experimental subplot and placed avoiding subplot borders to elude ecotones (Figure m.11).

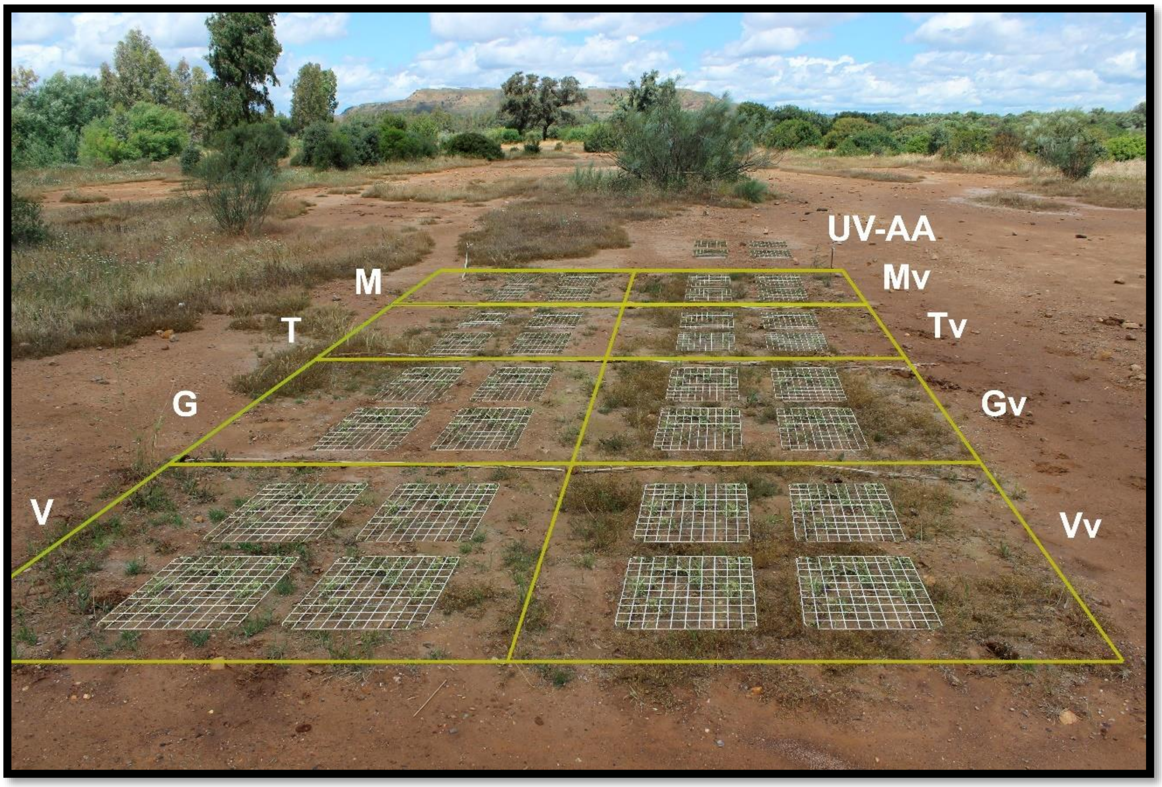


Figure m.11. Vegetation cover sampling in an experimental plot with quadrats superimposed. UV-AA: Unvegetated Affected Area; M: Addition of marble sludge; T: Tillage; G: Addition of gypsum mining spoil; V: mixture of UV-AA and V-AA; Mv: M plus vermicompost (v); Tv: T + v; Gv: G + v; Vv: V + v.

Herbaceous species richness was calculated in two ways: 1. Mean richness, calculated as the average richness values per replicates of the same treatment; 2. Total richness, calculated by counting the total number of different species that appeared at least once along all the replicates of one treatment.

In order to have an idea of the herbaceous plant diversity present in each soil type, the Shannon-Weiner Diversity Index (H', Shannon, 1948) was calculated as:

$$H' = -\sum_{I} pi * \ln pi$$

Where $pi = {}^{ni}/_{N}$

I is the total number of different species, *pi* is the proportion of individuals found in "*i*" species, *ni* is each species individual cover, and *N* is total species cover. As H' values can sometimes be difficult to interpret and to compare between different areas (Magurran, 2004), the Effective

Number of Species (ENS, MacArthur, 1965) that characterize a community was calculated for each treatment according to Jost (2006) as:

$$ENS = e^{H'}$$

In order to analyse PHE uptake by plants, one sample of the most frequent plant species was collected in each experimental plot (per treatment), in V-AA and in UA. In most cases, they were mixed samples formed of several individuals (same species), except where species abundance was limited to only one individual.

3.2. Soils

3.2.1. Field study 1. Characterization of affected areas (Chapter 1)

In order to characterize the soil from the unvegetated (UV-AA) and differently vegetated subareas (belts 1 and 2), we collected three soil samples per subarea and plot from the upper soil layer (0-10 cm) and intensively homogenized them to provide a single sample for each plot.

3.2.2. Greenhouse experiment (Chapter 2)

Soil samples were collected at highly polluted unvegetated areas (UV-AA) during the preliminary field study (field study 1). These samples were collected from the uppermost 10 cm and intensely homogenized into just one composite soil sample (C_0) to be used as substrate for the experiment. Moreover, soil samples from the area where the spill never reached (UA) were also collected to be used as background values.

At the end of the experiment we collected 5 soil samples per treatment (C_f, $T1_f$, $T2_f$, and $T3_f$) and species, to analyse soil properties and PHE content in plants.

3.2.3. Field study 2. Experimental plots with soil treatments (Chapter 3)

Aiming at assessing the evolution of treated soils, three soil samples per treatment and experimental plot (from the uppermost 10 cm) were collected every six months for 2 years (T0: right after the preparation of the experimental plots; T6: after the first 6 months; T12 after the first year of experiment; T18 after a year and a half after the start). Additionally, eighteen soil samples were collected in both the neighbouring vegetated affected (V-AA) and unaffected (UA) soils.

4. Laboratory analyses

4.1. Plants (Chapters 1, 2 and 4)

Plant samples were washed with distilled water, dried (70 °C for 48 h), divided per species into shoot and root, ground, weighed in a precision scale and digested separately in a microwave XP1500Plus (Mars®) in HNO_3:H_2O_2 (1:1) (Sah and Miller, 1992). To control the accuracy of the method, we used Standard Reference Material (ERM ® CD281) with PHE average recoveries ranging from 77% to 110%.

Concentration of micro and macronutrient elements in plants (Fe, Mn, Ca, Mg, K, Na) were measured (Chapter 1) by atomic absorption spectrometry (SpectrAA 220FS Varian) and potential pollutants (Cu, Zn, Cd, Sb, As and Pb) by ICP-MS in a PE SCIEX ELAN-5000A spectrometer.

In order to evaluate the capacity of plants to accumulate PHEs transferred from soils (Chapter 1 and 4), the Bioconcentration Factor (BAF) for each PHE was calculated according to Hobbs and Streit (1986) and Van der Ent et al. (2012) as:

$$BAF = {}^{Cshoot}/_{Csoil}$$

where '*Cshoot'* is the PHE concentration in shoot (mg kg^{-1} dry weight) and '*Csoil'* the PHE bioavailable concentrations (extracted by EDTA) in soil (mg kg^{-1} dry soil). Concentrations extracted by EDTA were selected for BAF calculation as this fraction represents the bioavailable forms of PHEs for plants (Parra et al., 2014). High BAF values (>1) can be associated to hyperaccumulation but it is not the only criteria (Van der Ent et al., 2012). For instance, Mehes-Smith et al. (2013) reported that a plant can be classified as hyperaccumulator only if it meets four criteria: i) $[PHE]_{Shoot}$/ $[PHE]_{root}$ >1; ii) $[PHE]_{Shoot}$/ $[PHE]_{soil}$ >1; iii) $[PHE]_{polluted\ plants}$/$[PHE]_{normal\ plants}$ >10-500; iv) $[Cu]_{polluted\ plants}$ = 300, $[Zn]_{polluted\ plants}$ = 3000, $[Pb]_{polluted\ plants}$ = 1000, (in mg kg^{-1}).

Then, to assess the capacity of plants to translocate PHEs from roots to shoots (Chapter 4), the Translocator Factor (TF) for each PHE was obtained according to Van der Ent et al. (2012) as:

$$TF = {}^{Cshoot}/_{Croot}$$

where '*Cshoot'* is the PHE concentration in shoot (mg kg^{-1} dry weight) and '*Croot'* the PHE concentration in root (mg kg^{-1} dry weight). TF values > 1 are related to PHE root-to-shoot translocation (Macnair, 2003).

Finally, the quotient (Q) between PHE concentrations accumulated in plants at affected and unaffected soils (Mehes-Smith et al., 2013) was calculated (Chapter 4), aiming at establishing a quantitative relation between the studied soil types. Values of the quotient bigger than 10-500 may indicate PHE hyperaccumulation (Mehes-Smith et al., 2013).

4.2. Soils

4.2.1. Field study 1. Characterization of affected areas (Chapter 1)

Soil samples were air dried at room temperature and sieved at 2-mm. Soil texture was determined by the Robinson pipette method (Soil Conservation Service, 1972); calcium carbonate ($CaCO_3$) content by volumetric method (Barahona, 1984); soil pH was measured in water and 0.1 M KCl in a ratio 1:2.5 with a 914 pH/Conductometer Metrohm; total organic carbon (OC) was analysed by a LECO® TruSpec CN (St. Joseph, MI, USA) after soil samples were acid-washed (HCl 1 mol/l for 24 h) to remove carbonates, following Ussiri and Lal (2008); soil:water extract (1:5) was prepared to determine the electrical conductivity (EC) using a Eutech CON700 conductivity-meter; cation exchange capacity (CEC) was determined according to the methodology of the Soil Conservation Service (1972). Amorphous iron and manganese oxides (Fe_o, Mn_o) were extracted according to Schwertmann and Taylor (1977) procedure and measured by atomic absorption spectroscopy (SpectrAA 220FS Varian).

In the laboratory, total concentrations of PHEs (Cu, Zn, Cd, As and Pb) were determined in soils by X-ray fluorescence in a NITON XLt 792 analyzer, with a 40 kV X-ray tube with Ag anode target excitation source, and a Silicon PIN-diode with a Peltier cooled detector. The procedure followed the manufacturer's instructions and the recommendations of the

Method 6200 (US EPA, 1998). The accuracy of the method, the analytical precision and the detection limits were evaluated according to US EPA (2006) in Martín Peinado et al. (2010).

Selective extractions were performed in order to assess the potential mobility and bioavailability of PHEs in soils, determining from mobile or available fractions to unavailable fractions. Soluble fraction (S) was extracted by distilled water from soil:water extract 1:5 according to Sposito et al. (1982). Exchangeable fraction (E) was obtained by 0.01M $CaCl_2$ extraction according to Novozamsky et al. (1993). Bioavailable fraction (B) for plants was extracted using 0.05M EDTA (pH 7) as described by Quevauviller et al. (1998). Fraction bounded to amorphous Fe/Mn oxides (O) was extracted by 0.1M oxalic acid-ammonium oxalate extract (pH 3) as described by Schwertmann and Taylor (1997). Total concentrations (T) of main pollutants (Cu, Zn, Cd, As and Pb) were additionally obtained by strong acid digestion (HNO_3:HF, 3:1) in a microwave oven XP1500Plus (Mars®). The PHE content in all extracted fractions was analyzed by inductively coupled plasma-mass spectrometry (ICP-MS) in a PE SCIEX ELAN-5000A spectrometer. The accuracy of the method, the analytical precision and the detection limits are detailed in Romero-Freire et al. (2016).

Residual fraction (R) was calculated as the difference between the total fraction (T) and the fraction with the most effective extractant for each element. The oxalic acid-ammonium oxalate reagent was generally the most effective for all the elements. According to Ure (1995), this reagent can extract the fraction of the elements bound to oxides and secondary clay minerals, the organically bound, exchangeable and water- soluble forms.

4.2.2. Greenhouse experiment (Chapter 2)

Both the initial composite soil sample and the soil samples from treatments were prepared (air-dried, sieved -2mm mesh- and finely ground with a soil mill, Retsch MM 400) to analyze their chemical properties (MAPA, 1994) and main PHEs (Cu, Zn, Cd, Sb, As and Pb). Total concentrations (T) were obtained through microwave-assisted (XP1500Plus, Mars®) acid digestion (HNO_3:HF, 3:1), water-soluble fraction (S) was extracted by distilled water from soil:water extract 1:5 according to Sposito et al. (1982), and bioavailable fraction (B) was extracted using 0.05 M EDTA (pH 7) as described by Quevauviller et al. (1998). PHEs were measured in all the extracted forms by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) (PE SCIEX ELAN-5000 spectrometer).

4.2.3 Field study 2. Experimental plots with soil treatments (Chapter 3)

We analysed the main physicochemical properties (pH, EC, OC and $CaCO_3$), according to standard methods (MAPA, 1994), and PHE (Cu, Zn, As and Pb) content in all soil types (unvegetated: UV-AA, vegetated: V-AA and unaffected: UA). Soil samples were sieved (2mm), finely ground and digested in strong acids (HNO_3:HF, 3:1) in a microwave oven XP1500Plus (Mars®) to analyse the total concentrations (T) of PHEs. Then, water-soluble (S) and bioavailable (B) fractions were extracted as described by Sposito et al. (1982) and Quevauviller et al. (1998), respectively. In all cases, PHE concentrations in soil samples were measured by inductively coupled plasma-mass spectrometry (ICP-MS) in a PE SCIEX ELAN-5000 spectrometer. The accuracy of the method was confirmed through the analysis of the Standard Reference Material "CRM025-050"; the average recoveries for Cu, Zn, As and Pb ranged between 78% and 100% of the certified reference values, being inside either the Confidence Interval (Cu, Zn and Pb) or the Prediction interval (As).

In this work, we apply the term "soluble" to the fraction of pollutants soluble in water, or in other words, to the pollutant concentrations that are easily mobile (short-term) from the soil to the plant or to groundwater through leaching. Similarly, we apply the term "bioavailable" to refer to the fraction of pollutants extracted by EDTA and that could be considered a potentially available fraction for plants (Beckett, 1989), as plants could absorb them from the soil in the long-term evolution.

In order to compare the relative mobility and bioavailability of PHEs in the different soils types (UA, UV-AA and V-AA) and treatments, bioavailability and solubility ratios were calculated for each PHE as the relation between the bioavailable (or soluble) and total concentration of each element. Higher values indicate that the solubility/bioavailability of the element is higher in relation to total concentrations, posing a higher risk for the ecosystem.

5. Toxicity bioassay (Chapter 3)

In order to assess the effectiveness of the soil treatments in the *in-situ* experiment, a toxicity bioassay was made using *Lactuca sativa* L. In Petri dishes containing 5 ml of 1:5 soil:water extract, 15 seeds of *L. sativa* were placed and incubated at 20 ± 1 º C during 120 h following OECD (2003) and US EPA (1996) recommendations. Then, *L. sativa* germination and root elongation were measured. Results were expressed as root elongation in the sample in relation to water controls; values obtained ranged from 0 to 100, where 0 indicates no elongation (maximum toxicity) and 100 indicates same elongation (no toxicity) compared to the control. Values above 100 indicate overstimulation of the elongation in relation to the control (hormesis) and were considered as no toxic response (value 100).

6. Data analysis

6.1. Plants

6.1.1. Field study 1. Characterization of affected areas (Chapter 1)

A Principal Components Analysis (PCA) was made to assess relationships among Potentially Harmful Elements (PHEs), and a non-metric multidimensional scaling (NMDS) ordination diagrams were performed to determine the effects of PHE concentration in the vegetation response. It was performed with a confidence level of 95% by the non-metric multidimensional scaling (NMDS) with Rstudio 2015 (Rstudio Team, Boston, USA).

6.1 2. Greenhouse experiment (Chapter 2)

All statistical analyses were performed using R version 3.4.2 (R Core Team, 2017). We assessed the effects of gypsum mining spoil (G) on plant emergence, survival and biomass production by applying generalized linear models (GLMs), through the "stats" package (R Core Team, 2017). Model suitability was assessed by graphical exploration of the residuals (Zuur et al. 2010). After that, final models were fitted assuming gaussian distribution and identity-link function for biomass production. For plant emergence and total survival, we fitted GLMs assuming binomial distribution and logit-link function. To evaluate differences among treatments on seedling survival rates over time, we used Cox proportional hazard models and, for data visualization, Kaplan-Meier curves (Bewick et al., 2004) using "survival" package v2.44-1.1 (Therneau, 2015). Pairwise comparisons between soil treatments in terms of plant emergence, survival and growth were performed with Tukey's *post-hoc* tests using "multcomp" package (Hothorn et al., 2008). Graphs and confidence intervals were obtained with R "ggplot2" v3.1.1 (Wickham, 2016) and "sciplot" v1.1-1 (Morales, 2017). Mean values and standard deviation were calculated by using ddply function (R "plyr" package, v1.8.4; Wickham, 2011).

6.1.3. Field study 2. Experimental plots with soil treatments (Chapter 4)

All statistical analyses were performed using R version 3.4.2 (R Core Team, 2017). We applied Linear Mixed Models (LMMs) and Generalized Linear Mixed Models (GLMMs) by means of "lme4" package (Bates et al., 2015) to assess the effectiveness of soil treatments for the following response variables: vegetation cover, plant species richness, plant diversity (H), Effective Number of Species (ENS), PHE uptake by plants, PHE accumulation in shoots and roots, Bioaccumulation Factor (BAF), Translocation Factor (TF) and quotient between PHE uptake by affected and unaffected plants (Q). In the models we included soil treatment as fixed factor, and residual areas, sampling period and species as random factors (in the case of cover, variable for which we had spatial pseudoreplication, the "subplot" factor was also included as random). GLMM models were fitted for species richness and ENS assuming poisson and gamma distributions, with log- and inverse-link functions, respectively. Plant cover, H, PHE uptake by plants, PHE accumulation in shoot and root, TF, BAF and Q were fitted with LMMs assuming gaussian distribution and identity-link function; in this case, the logarithmical transformation of variables (or arcsine square root transformation for percentages) was used to meet normality assumption. Model suitability in each case was assessed by graphical exploration of the residuals (Zuur et al. 2010). Then, pairwise comparisons between soil treatments were performed for all the variables with Tukey's *post-hoc* tests using "multcomp" package (Hothorn et al., 2008).

We calculated the variance explained by LMM and GLMM models using "r.squaredGLMM" function of "MuMIn" library (Barton 2016).

Graphs and confidence intervals were obtained with R "ggplot2" v3.1.1 (Wickham, 2016) and "sciplot" v1.1-1 (Morales, 2017). Mean values and standard deviation were calculated by using ddply function (R "plyr" package, v1.8.4; Wickham, 2011).

6.2. Soils

6.2.1. Field study 1. Characterization of affected areas (Chapter 1)

IBM SPSS Statistics for Windows, v.20.0 (IBM Corp., Armonk, N.Y., USA) was used to analyse the influence of soil properties on the selective extractions of PHE by performing Spearman´s correlations. Mean comparisons were made by means of Kruscall-Wallis test

(Theodorsson-Norheim, 1986), and significant differences were determined by Kruscall-Wallis posthoc test ($p < 0.05$).

6.2.2. Greenhouse experiment (Chapter 2)

All statistical analyses were performed using R version 3.4.2 (R Core Team, 2017). We assessed the effects of gypsum mining spoil (G) on soil properties and PHE immobilization by applying generalized linear models (GLMs). To fit GLMs for total/soluble/bioavailable PHEs in soils, soil pH and EC, the "stats" package was used (R Core Team, 2017). We also used GLMs to evaluate the differences between the polluted and non-polluted soils. Model suitability was assessed by graphical exploration of the residuals (Zuur et al. 2010). After that, final models were fitted assuming gamma/gaussian distribution and inverse/identity-link function for soil properties and PHE immobilization. Pairwise comparisons between soil treatments in terms of soil properties and PHE immobilization were performed with Tukey's *post-hoc* tests using "multcomp" package (Hothorn et al., 2008). Graphs and confidence intervals were obtained with R "ggplot2" v3.1.1 (Wickham, 2016) and "sciplot" v1.1-1 (Morales, 2017). Mean values and standard deviation were calculated by using ddply function (R "plyr" package, v1.8.4; Wickham, 2011).

6.2.3. Field study 2. Experimental plots with soil treatments (Chapter 3)

All statistical analyses were performed using R version 3.4.2 (R Core Team, 2017). By means of "lme4" package (Bates et al., 2015), we applied linear mixed models (LMMs) to assess the effectiveness of soil treatments in terms of soil properties (pH, EC, $CaCO_3$, OC), PHE immobilization in soils, and *Lactuca sativa* germination and root elongation in toxicity tests. We assumed gaussian error and identity-link function, and the logarithmical transformation of variables (or arcsine square root transformation for proportions and percentages) was used for $CaCO_3$, OC, germination, bioavailable and soluble fractions, and bioavailable and soluble ratios to meet normality assumption. Soil treatment was included as fixed factor, and residual areas and sampling period as random factors. To fit LMMs, "lme4" package was used (Bates et al., 2015). Then, model suitability was assessed by graphical exploration of the residuals (Zuur et al. 2010), and pairwise comparisons between soil treatments (including unaffected (UA), unvegetated (UV-AA) and vegetated (V-AA) affected soils) were performed for all the variables with Tukey's *post-hoc* tests using "multcomp" package (Hothorn et al., 2008).

We calculated the variance explained by LMMs using “r.squaredGLMM” function of “MuMIn” library (Barton, 2016).

A non-metric multidimensional scaling (NMDS) ordination analysis was performed by means of metaMDS function in the vegan package (Oksanen et al. 2013), in order to investigate the influence of soil properties and solubility ratios on PHE toxicity over *Lactuca sativa* (bioassays). The Bray-Curtis index was chosen as a dissimilarity measure between sites.

Graphs and confidence intervals were obtained with R “ggplot2” v3.1.1 (Wickham, 2016) and “sciplot” v1.1-1 (Morales, 2017). Mean values, standard errors and standard deviations were calculated by using ddply function (R “plyr” package, v1.8.4; Wickham, 2011).

7. References

Barahona, E., 1984. Determinación de carbonatos totales y caliza activa. Grupo de trabajo de normalización de métodos analíticos. In I Congreso Nacional de la Ciencia del Suelo, pp. 53-67. Madrid, Spain: Sociedad Española de la Ciencia del Suelo.

Barton, K., 2016. Multi-model inference. R package version 1.15.6. Available at: https://cran.r-project.org/web/packages/MuMIn/index.html

Bates, D., Maechler, M., Bolker, B., Walker, S., 2015. Fitting linear mixed-effects models using lme4. J. Stat. Softw., 67(1): 1-48. https://doi.org/10.18637/jss.v067.i01

Beckett P.H.T., 1989. The use of extractants in studies on trace metals in soils, sewage, sludge, and sludges-treated soils. Adv. Soil S., 9: 143-176.

Bewick, V., Cheek, L., Ball, J., 2004. Statistics review 12: Survival analysis. Crit. Care., 8(5): 389-394. https://doi.org/10.1186/cc2955

Boletín Oficial del Estado (BOE) 38, 2015. Decreto 18/2015 por el que se aprueba el reglamento que regula el régimen aplicable a los suelos contaminados. Consejería de Medioambiente y Ordenación del Territorio, 38: 28-64.

Bonham, C.D., 2013. Frequency, in: Measurements for terrestrial vegetation. Chapter 5, second edition. John Wiley & Sons.

Cabrera, F., Clemente, L., Cordón, R., Hurtado, M.D., López, R., Madejón, P., Marañón, T., Moreno, F., Murillo, J.M., Nagel, I., 2005. Effect of remediation on trace metal pollution in soils of Guadiamar river valley, in: Del Valls, T.A., Blasco, J. (Eds.), Integrated Assessment and Management of the Ecosystems Affected by the Aznalcóllar Mining Spill (SW, Spain). Cátedra UNESCO/Unitwin, Cádiz, pp. 33-40.

Cabrera, F., Ariza, J.L., Madejón, P., Madejón, E., Murillo, J.M., 2008. Mercury and other trace elements in soils affected by the mine tailing spill in Aznalcóllar (SW Spain). Sci. Total Environ., 390: 311-322. https://doi.org/10.1016/j.scitotenv.2007.10.002

Chopin, E.I.B., Alloway, B.J., 2007. Distribution and mobility of trace elements in soils and vegetation around the mining and smelting areas of Tharsis, Ríotinto and Huelva, Iberian Pyrite

Belt, SW Spain. Water Air Soil Pollut., 182: 245-261. https://doi.org/10.1007/s11270-007-9336-x

CMA (Consejería de Medio Ambiente), 2003. Ciencia y restauración del río Guadiamar. PICOVER 1998-2002. Junta de Andalucía, Sevilla, Spain.

CMA (Consejería de Medio Ambiente), 2004. Informational dossier: Aznalcóllar mine. VERSION: DA2 - 02/2014. Junta de Andalucía, Spain. Available at: http://www.juntadeandalucia.es/economiainnovacionyciencia/pamdoc/_archivos_/aznalcollar/dossier_minas_english.pdf

Domènech, C., de Pablo, J., Ayora, C., 2002. Oxidative dissolution of pyritic sludge from the Aznalcóllar mine (SW Spain). Chem. Geol., 190: 339-353. https://doi.org/10.1016/S0009-2541(02)00124-9

Elzinga, C.L., Salzer, D.W., Willoughby, J.W., 2015. Measuring and monitoring plant populations. Denver: U.S. Dept. of the Interior, Bureau of Land Management Nature Conservancy.

Fernández-Caliani, J.C., Barba-Brioso, C., González, I., Galán, E., 2009. Heavy Metal Pollution in Soils Around the Abandoned Mine Sites of the Iberian Pyrite Belt (Southwest Spain). Water Air Soil Pollut., 200: 211-226. https://doi.org/10.1007/s11270-008-9905-7

García-Carmona, M., Romero-Freire, A., Sierra Aragón, M., Martínez Garzón, F.J., Martín Peinado, F.J., 2017. Evaluation of remediation techniques in soils affected by residual contamination with heavy metals and arsenic. Journal Environ. Manage., 191: 228-236. http://doi.org/10.1016/j.jenvman.2016.12.041

García-Carmona, M., García-Robles, H., Turpín Torrano, C., Fernández Ondoño, E., Lorite Moreno, J., Sierra Aragón, M., Martín Peinado, F.J., 2019. Residual pollution and vegetation distribution in amended soils 20 years after a pyrite mine tailings spill (Aznalcóllar, Spain). Sci. Total Environ., 650: 933-940. https://doi.org/10.1016/j.scitotenv.2018.09.092

Grimalt, J.O., Macpherson, E. (Eds.), 1999. The environmental impact of the mine tailing accident in Aznalcóllar. Sci. Total Environ. 242 (special volume): 1-337.

Grimalt, J.O., Ferrer, M., Macphersonc, E., 1999. The mine tailing accident in Aznalcóllar. Sci. Total Environ., 242: 3-11. https://doi.org/10.1016/s0048-9697(99)00372-1

Hobbs, R.H., Streit, B., 1986. Heavy metal concentrations in plants growing on a copper mine spoil in the Grand Canyon, Arizona. Am. Midl. Nat., 115: 277-281. https://www.jstor.org/stable/2425864

Hothorn, T., Bretz, F., Westfall, P., 2008. Simultaneous inference in general parametric models. Biom. J., 50 (3): 346-363. https://doi.org/10.1002/bimj.200810425

IBM Corp., Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.

IUSS Working Group WRB. 2015. World Reference Base for Soil Resources 2014, update 2015. International soil classification system for naming soils and creating legends for soil maps. World Soil Resources Reports No. 106. FAO, Rome.

Jost, L., 2006. Entropy and diversity. Oikos, 113: 363-375.

MacArthur, R.H., 1965. Patterns of Species Diversity. Biol. Rev., 40 (4): 510-533.

Macnair, M.R., 2003. The hyperaccumulation of metals by plants. Adv. Bot. Res., 40: 63-105. https://doi.org/10.1016/S0065-2296(05)40002-6

Madejón, P., Murillo, J.M., Marañón, T., Cabrera, F., Soriano, M.A., 2003. Trace element and nutrient accumulation in sunflower plants two years after the Aznalcóllar mine spill. Sci. Total Environ., 307: 239-257. https://doi.org/10.1016/S0048-9697(02)00609-5

Madejón, P., Domínguez, M.T., Madejón, E., Cabrera, F., Marañón, T., Murillo-Carpio, J.M., 2018. Soil-plant relationships and contamination by trace elements: A review of twenty years of experimentation and monitoring after the Aznalcóllar (SW Spain) mine accident. Sci. Total Environ., 625: 50-63. https://doi.org/10.1016/j.scitotenv.2017.12.277

Magurran, A.E., 2004. Measuring Biological Diversity. Blackwell.

Manzano, M., Custodio, E., Ayora, C., Navarrete, P., 2000. Contamination of the Guadiamar river aquifer after the Aznalcóllar mine accident, SW Spain. Boletín Geológico y Minero, 111 (6): 93-106.

MAPA, 1994. Métodos oficiales de análisis. Tomo III: Métodos oficiales de análisis de suelos y aguas para el riego. Ministerio de Agricultura, Pesca y Alimentación. Servicio de Publicaciones, Madrid, pp. 205-285.

Martín-Peinado, F.J., 2002. Contaminación de suelos por el vertido de una mina de pirita (Aznalcóllar, España). Granada: Doctoral Thesis, Universidad de Granada, 2001. Available at: http://hdl.handle.net/10481/28793

Martín-Peinado, F.M., Ruano, S.M., González, M.B., Molina, C.E., 2010. A rapid field procedure for screening trace elements in polluted soil using portable X-ray fluorescence (PXRF). Geoderma, 159(1-2): 76-82. https://doi.org/10.1016/j.geoderma.2010.06.019

Martín-Peinado, F.J., Romero-Freire, A., García-Fernández I., Sierra-Aragón, M., Ortiz-Bernad, I., Simón-Torres, M., 2015. Long-term contamination in a recovered area affected by a mining spill. Sci. Total Environ., 514: 219-223. https://doi.org/10.1016/j.scitotenv.2015.01.102

Mehes-Smith, M., Nkongolo, K., Cholewa, E., 2013. Coping mechanisms of plants to metal contaminated soils, in: Silvern, S., Young, S., (Eds.), Environmental change and sustainability. Chapter 3. InTech. pp. 53. https://doi.org/10.5772/55124

Morales, M., 2017. Sciplot: Scientific graphing functions for factorial designs. Available at: https://cran.r-project.org/web/packages/sciplot/index.html

Nocete, F., Sáez, R., Bayona, M.R., Nieto, J.M., Peramo, A., López, P., Gil-Ibarguchi, J.I., Inácio, N., García, S., Rodríguez, J., 2014. Gold in the southwest of the Iberian Peninsula during the 3rd millennium BC. J. Archaeol. Sci. 41, 691-704. https://doi.org/10.1016/j.jas.2013.10.006

Novozamsky, I., Lexmond, T.H., Houba V.J., 1993. A single extraction procedure of soil for evaluation of uptake of some heavy metals by plants. Int. J. Environ. An. Ch., 51: 47-58. https://doi.org/10.1080/03067319308027610

OECD (Organization for Economic Cooperation and Development), 2003. Test No. 208: Terrestrial plant test: Seedling emergence and seedling growth test, in: Guideline for the testing of Chemicals, Section 2, OECD Publishing, Paris, p. 208. https://doi.org/10.1787/9789264070066-en

Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Szoecs, E., Wagner, H., 2013. Vegan: community ecology package. R package version 2.4-3. https://CRAN.R-project.org/package=vegan

Ordóñez, A., Guerra, J., Cano, M.J., 2003. The bryophyte diaspore bank in soils affected by the Aznalcóllar (Seville, SW Spain) mine disaster. Anales de Biología, 25: 37-48.

Parra, A., Zornoza, R., Conesa, E., Gómez-López, M.D., Faz, A., 2014. Seedling emergence, growth and trace elements tolerance and accumulation by Lamiaceae species in a mine soil. Chemosphere, 113: 132-140. https://doi.org/10.1016/j.chemosphere.2014.04.090

Pastor-Jáuregui, R., Paniagua-López, M., Martínez-Garzón, J., Martín-Peinado, F., Sierra-Aragón, M., 2020. Evolution of the residual pollution in soils after bioremediation treatments. Appl. Sci., 10(3): 1006. https://doi.org/10.3390/app10031006

Quesada, C., Bellido, F., Dallmeyer, R.D., Gil-Ibarguchi, J.I., Oliveira, J.T., Pérez-Estaun, A., 1991. Terranes within the Iberian Massif: Correlations with West African sequences, in: Dallmeyer, R. D., Lécorché, J. P., (Eds.), The West African orogens and circum-Atlantic correlations. Berlin: Springer, pp. 267-293. https://doi.org/10.1007/978-3-642-84153-8_11

Quevauviller, P., Lachica, M., Barahona, E., Gomez, A., Rauret, G., Ure, A., Muntau, H., 1998. Certified reference material for the quality control of EDTA-and DTPA-extractable trace metal contents in calcareous soil (CRM 600). Fresen. J. Anal. Chem., 360(5): 505-511.

R Core Team, 2017. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. Available at: https://www.r-project.org

RStudio Team, 2015. RStudio: Integrated Development for R. RStudio, Inc., Boston, MA URL http://www.rstudio.com/

Romero-Freire, A., Sierra Aragón, M., Martínez Garzón, F.J., Martín Peinado, F.J., 2016. Is soil basal respiration a good indicator of soil pollution? Geoderma, 263: 132-139. http://doi.org/10.1016/j.geoderma.2015.09.006

Sáez, R., Pascual, E., Toscano, M., Almodóvar, G., 1999. The Iberian type of volcano-sedimentary massive sulphide deposits. Miner. Deposita, 34(5-6): 549-570. https://doi.org/10.1007/s001260050220

Sah, R.N., Miller, R.O., 1992. Spontaneous reaction for acid dissolution of biological tissue in closed vessels. Anal. Chem., 64: 230-233. https://doi.org/10.1021/ac00026a026

Salvany, J.M., Mediavilla, C., Mantecón, R., Manzano, M., 2001. Geología del Valle del Guadiamar y áreas colindantes. Boletín Geológico y Minero. Vol. Especial, 57-68.

Schwertmann, U., Taylor, R.M., 1977. Iron oxides, in: Dixon, J.B., Webb, S.B. (Eds.), Minerals in soils environments. Soil Science Society of America Madison. pp. 148-180.

Shannon, C., 1948. A mathematical theory of communication. Bell System Tech. J., 27: 379-423, 623-656.

Sierra-Aragón, M., Mitsui-Nakamaru, Y., García-Carmona, M., Martínez-Garzón, F.J., Martín-Peinado, F.J., 2019. The role of organic amendment in soils affected by residual pollution of potentially harmful elements. Chemosphere, 237: 124549. https://doi.org/10.1016/j.chemosphere.2019.124549

Simón, M., Ortiz, I., García, I., Fernández, E., Fernández, J., Dorronsoro, C., Aguilar, J., 1998. El desastre ecológico de Doñana. Edafología, 5: 153-161.

Simón, M., Ortiz, I., García, I., Fernández, E., Fernández, J., Dorronsoro, C., Aguilar, J., 1999. Pollution of soils by the toxic spill of a pyrite mine Aznalcóllar, Spain. Sci. Total Environ., 242: 105-115. https://doi.org/10.1016/S0048-9697(99)00378-2

Simón, M., García, I., Martín, F., Díez, M., del Moral, F., Sánchez, J.A., 2008. Remediation measures and displacement of pollutants in soils affected by the spill of a pyrite mine. Sci. Total Environ., 407: 23-39. https://doi.org/10.1016/j.scitotenv.2008.07.040

Soil Conservation Service, 1972. Methods and procedures for collecting soil samples. Washington, DC: Soil Survey Laboratory, USDA.

Sposito G., Lund L.J., Chang A.C., 1982. Trace metal chemistry in arid-zone field soils amended with sewage sludge. I: fractionation of Ni, Cu, Zn, Cd and Pb in solid phases. Soil Sci. Soc. Am. J., 46: 260-264.

Theodorsson-Norheim, E., 1986. Kruskal-Wallis test: BASIC computer program to perform nonparametric one-way analysis of variance and multiple comparisons on ranks of several independent samples. Comput. Methods Programs Biomed., 23(1): 57-62.

Therneau, T., 2015. A Package for Survival Analysis in S. R Package Version 2.44-1.1. Available at: https://CRAN.R-project.org/package=survival

Ure, A.M., 1995. Methods of analysis for heavy metals in soils, in: Alloway, B. J. (Ed.), Heavy metals in soils, second ed. Blackie Academic and Professional. Glasgow. UK, pp. 58-102.

US EPA (US Environmental Protection Agency), 1996. Ecological effects test guidelines. Seed germination/root elongation toxicity test. OPPTS 850.4200. National Service Center for Environmental Publications: Washington, DC, USA, p. 8.

US EPA (US Environmental Protection Agency), 1998. Field portable x-ray fluorescence spectrometry for the determination of elemental concentrations in soil and sediment. Method 6200.

US EPA (US Environmental Protection Agency), 2006. XRF technologies for measuring trace elements in soil and sediment. NitonXLt 700 Series XRF Analyzer. Innovative technology verification report EPA/540/R-06/004.

Ussiri, D.A.N., Lal, R., 2008. Method for determining coal carbon in the reclaimed mine soils contaminated with coal. Soil Sci. Soc. Am. J., 72: 231-237. https://doi.org/10.2136/sssaj2007.0047

Van der Ent, A., Baker, A.J.M., Reeves, R., Pollard A.J., Schat, H., 2012. Hyperaccumulators of metal and metalloid trace elements: Facts and fiction. Plant Soil, 362 (1-2): 319-334. https://doi.org/10.1007/s11104-012-1287-3

Wickham, H., 2011. The split-apply-combine strategy for data analysis. J. Stat. Softw., 40(1). https://doi.org/10.18637/jss.v040.i01

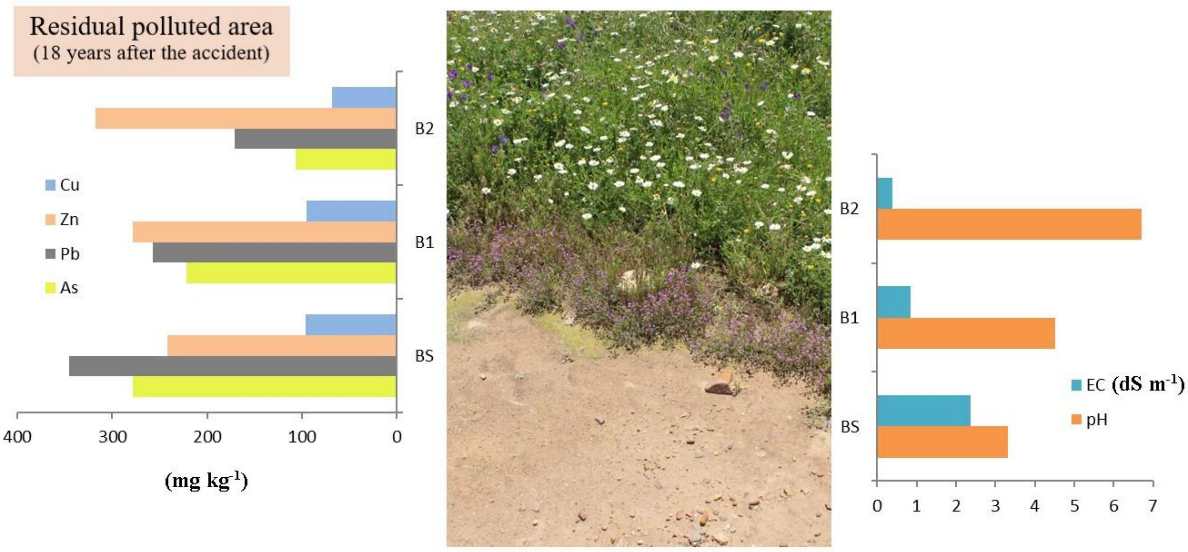
Wickham, H., 2016. ggplot2: Elegant graphics for data analysis. Springer-Verlag, New York.

Zuur, A.F., Ieno, E.N., Elphick, C.S., 2010. A protocol for data exploration to avoid common statistical problems. Methods Ecol. Evol., 1: 3-14. https://doi.org/10.1111/j.2041-210X.2009.00001.x

- CHAPTER 1 -

RESIDUAL POLLUTION AND VEGETATION DISTRIBUTION AFTER SOIL REMEDIATION

García-Carmona, M., García-Robles, H., Turpín Torrano, C., Fernández Ondoño, E., Lorite Moreno, J., Sierra Aragón, M., Martín Peinado, F.J.



Science of the Total Environment 650 (2019): 933-940

ABSTRACT

The present work assesses the residual pollution in the Guadiamar Green Corridor (SW, Spain) after a long-term aging process (18 years) since the accident of the Aznalcóllar pyrite mine. We have focused on the study of Potentially Harmful Elements (PHEs) in soils, their fractionation and the transference to the surrounding vegetation. The residual polluted areas are characterized by scattered plots with absence of vegetation, presenting high concentrations of PHEs, acidic pH and low organic carbon content. Surrounding these polluted plots, two vegetation gradient belts are clearly identified by changes in plant cover and richness. The inhibition of plant growth in the unvegetated soils is related to the highest mobility of soluble and exchangeable Cu, Zn and Cd forms, which significantly decrease with the distance to the polluted plots. Plant richness and cover show differences between belts, and PHE bioaccumulation in plants also differs, with preferential accumulation in roots. Despite the low bioavailability of As and Pb in soils, bioaccumulation factors in plants for these elements are significantly higher in belt 1 in relation to belt 2. High Cu and Cd potential toxic concentrations in aerial parts of vegetation are found, posing a risk for livestock and a potential entrance to the food chain. On the other hand, *Lamarckia aurea* (L.) Moench (in belt1) and *Trifolium campestre* Schreb. (in belt2) were the most dominant species in severely polluted soils. Elevated concentrations of PHEs in the vegetation growing in the area indicate plant adaptation mechanisms to live in these severely polluted soils, which can be used as a good bioindicator of pollution in similar polluted areas.

Keywords: Soil pollution, Potentially Harmful Elements fractionation, bioaccumulation, *Lamarckia aurea*, *Trifolium campestre*.

Chapter 1

1. INTRODUCTION

The capacity of Potentially Harmful Elements (PHEs) to degrade ecosystems, and their significant toxicological risk for human health through the food-chain are widely known. Otherwise, soil is an essential component of ecosystems, with a great capacity to cope with pollution and, thus, to protect the other components, both abiotic (air and water) and biotic ones (living organisms). There are many processes that influence toxicity in soils over time (Lock and Jannsen, 2003); soil properties largely control the mobility, bioavailability and, consequently, the potential toxicity of PHEs in the environment. Since total concentrations of PHEs provide limited information in relation to their toxic effects, selective extractions are a more suitable approach to assess the PHE fractionation in soils and, thus, their bioavailability for living organisms (Quevauviller, 1998; Marguí et al., 2007). Plants absorb PHEs from the soil in their bioavailable forms or solubilize them with their root exudates. Some PHEs are essential for plant growth and development, such as Cu and Zn, but when their concentrations surpass a certain threshold, they become toxic for plants, as it happens with non-essential elements. Hence, the potential PHE toxicity for plants primarily depends on phytoavailability. This strongly relies on the uptake capacity of each plant species, and on PHE concentration and bioavailability in soils (Liu et al., 2013), where soil physicochemical properties like pH, Eh, water regime, clay content, SOM, CEC and nutrient balance are key factors in the control of PHE mobility (Kabata-Pendias, 2011).

Plants can act as bioindicators of soils polluted with PHEs due to their capacity of interacting with them. To cope with PHE toxicity, plants adopt different strategies, including exclusion mechanisms and prevention of transfer to aerial parts, or accumulation in the above-ground tissues (Raskin et al., 1994; Sarwar et al., 2017). Plants that concentrate PHEs above normal values are called hyperaccumulators (Chaney, 1983). From a restoration point of view, the ability to grow in highly polluted soils is interesting for phytomanaging soil pollution (Moreno-Jiménez et al., 2009), since it is a cost-effective and environmentally friendly technique to decrease environmental risk in soils affected by mine tailings (Parraga-Aguado et al., 2014). The restoration of vegetation cover can stabilize and control the dispersion of pollutants throughout the ecosystem, which can also avoid soil erosion (Madejón, P. et al., 2006). However, accumulation of PHEs in excess on plant tissues can create an exposure pathway into the food-chain (McLaughlin, 1999; Christou et al., 2017).

A remarkable example of the damage that this type of pollution can produce is the mine spill occurred in 1998 in Aznalcóllar (SW Spain), where 45 x 10^5 m^3 of acidic waters and toxic tailings were spilled into the Agrio and Guadiamar rivers (Simón et al., 2001), finally affecting 45 km^2 of soils, mainly with agricultural use. However, the environmental impact was highly minimized thanks to the high buffer capacity of soils (Aguilar et al., 2007; Simón et al., 2008) and the rapid rehabilitation of the affected area. During the following three years after the accident, an important rehabilitation program was implemented to recover the affected area, which involved the removal of tailings and heavily polluted soils, the extensive application of organic and inorganic amendments, and the general phytostabilization of the area (Aguilar et al., 2004; Simón et al., 2008). The rehabilitation concluded with the affected area reconverted in a natural protected area, the Guadiamar Green Corridor (CMA, 2003). Multiple monitoring studies have been conducted on the affected area since then, deepening the knowledge about long-term PHE pollution and the evolution of the remediation (Madejón et al., 2018). Eighteen years after the accident, despite the aging process decreased pollutant concentrations and bioavailability in soils, residual pollution is still found in the area (Martín Peinado et al., 2015), posing a toxic risk to living organisms (Romero-Freire et al., 2016a; García-Carmona et al., 2017). Residual pollution is easily identified in soils by the total absence of vegetation; those areas are surrounded by soils with a gradual change in plant cover and richness, which indicates an interaction between pollutants and vegetation that should be monitored over time.

Unvegetated affected soils in Aznalcóllar are a synonym of severe pollution, posing a risk for the safety of the surrounding ecosystem (García-Carmona et al., 2017). More information is needed on PHE mobility in long-term polluted soils and their influence in the soil-plant system. In this study, we aimed at investigating the fractionation of the main potentially harmful elements in highly polluted soils and the influence of soil properties, in order to determine PHE phytoavailability and its effect on vegetation distribution. Therefore, physicochemical soil properties and constituents, mobility and bioavailability of pollutants (Cu, Zn, Cd, As and Pb) using selective extraction methods, and species richness and cover were measured in the area. In addition, PHE bioaccumulation in plants was analysed in order to study their response against highly polluted soils and, thus, to assess the potential environmental risk for the ecosystem.

2. MATERIAL AND METHODS

2.1. Soil and vegetation samples

Soils with residual pollution were detected in previous works (Martín Peinado et al., 2015) using satellite images from Google Earth for the identification and quantification of the unvegetated soil surface. Soils without vegetation were concentrated in the first 18 km downstream the tailing pond, representing about 7% of the total area affected by the spill. The residual polluted areas were randomly distributed and had a highly variable size, with unvegetated spots occupying from <1 to >200 m^2.

Four residual polluted plots were selected and divided into three subareas according to a soil recovery gradient in terms of vegetation presence. Soils were sampled from the unvegetated soil parts (UV-AA) right in the centre, to the two surrounding belt subareas characterized by a progressive vegetation appearance, an intermediate moderately re-vegetated belt (B1) and an outer and more recovered belt (B2) (Figure 1.1). Three soil samples were taken from the upper soil layer (0-10cm) per subarea and plot, each one composed of 5 soil sub-samples (centre and four corners from a square meter) and intensively homogenized to provide a single sample for each plot.

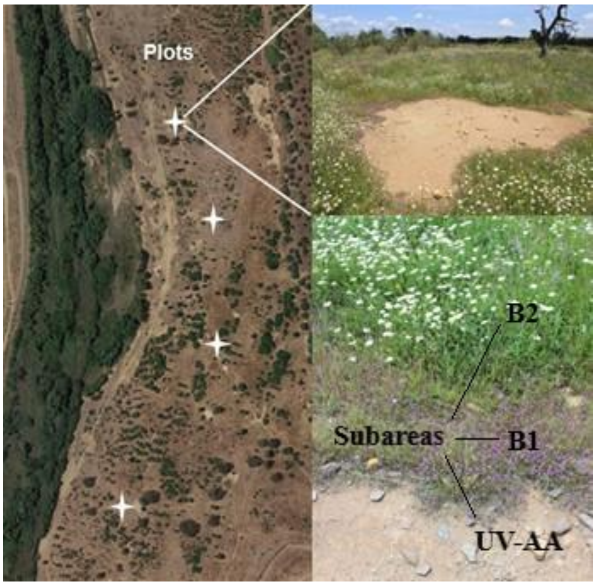


Figure 1.1. Plot location in the Guadiamar Green Corridor on the left, and a detail of the different subareas according to herbaceous species composition and abundance (UV-AA: unvegetated affected soils; B1: belt 1; B2: belt 2) on the right.

Vegetation samples representing the whole community were collected in the two vegetated belts for the four selected plots. A 50 x 50 cm^2 grid divided into 100 cells was used for the study of plant cover and species richness.

2.2. Analytical methods

2.2.1. Soil analysis

Soil samples were air dried at room temperature and sieved at 2-mm. Soil texture was determined by the Robinson pipette method (Soil Conservation Service, 1972); calcium carbonate content by volumetric method (Barahona, 1984); soil pH was measured in water and 0.1 M KCl in a ratio 1:2.5 with a 914 pH/Conductometer Metrohm; total organic carbon (OC) was analysed by a LECO® TruSpec CN (St. Joseph, MI, USA) after acidly washing soil samples (HCl 1 mol/l for 24 h) to remove carbonates, following Ussiri and Lal (2008) method; soil:water extract (1:5) was prepared to determine the electrical conductivity (EC) using a Eutech CON700 conductivity-meter; and cation exchange capacity (CEC) was determined according to the methodology of the Soil Conservation Service (1972). Amorphous iron and manganese oxides (Fe_o, Mn_o) were extracted according to Schwertmann and Taylor (1977) procedure and measured by atomic absorption spectroscopy (SpectrAA 220FS Varian).

Total concentrations of PHEs (Cu, Zn, Cd, As and Pb) were determined in soils by X-ray fluorescence in a NITON XLt 792 analyser, with a 40 kV X-ray tube with Ag anode target excitation source, and a Silicon PIN-diode with a Peltier cooled detector. The procedure followed the manufacturer's instructions and the recommendations of the Method 6200 (US EPA, 1998). The accuracy of the method, the analytical precision and the detection limits were evaluated according to US EPA (2006) in Martín Peinado et al. (2010).

2.2.2. Potentially Harmful Element fractionation

Selective extractions were performed to assess the potential mobility and bioavailability of potentially harmful elements in soils, determining from mobile or available fractions to unavailable fractions. Soluble fraction (S) was extracted by distilled water from soil:water extract 1:5 according to Sposito et al. (1982). Exchangeable fraction (E) was obtained by 0.01M $CaCl_2$ extraction according to Novozamsky et al. (1993). Bioavailable fraction (B) for plants was extracted using 0.05M EDTA (pH 7) as described by Quevauviller et al. (1998). Fraction bounded to amorphous Fe/Mn oxides (O) was extracted by 0.1M oxalic acid-ammonium oxalate extract (pH 3) as described by Schwertmann and Taylor (1997). Total concentrations (T) of main pollutants (Cu, Zn, Cd, As and Pb) were additionally obtained by strong acid digestion (HNO_3:HF, 3:1) in a microwave oven XP1500Plus (Mars®). The PHE content in all extracted fractions was analysed by inductively coupled plasma-mass spectrometry (ICP-MS) in a PE SCIEX ELAN-5000A spectrometer. The accuracy of the method, the analytical precision and the detection limits are detailed in Romero-Freire et al. (2016b).

Residual fraction (R) was calculated as the difference between the total fraction (T) and the fraction with the most effective extractant for each element. The oxalic acid- ammonium oxalate reagent was generally the most effective for all the elements. According to Ure (1995), this reagent can extract the fraction of the elements bound to oxides and secondary clay minerals, and the organically bound, exchangeable and water- soluble forms.

2.2.3. Plant analysis

Collected plants were divided into aerial parts and roots to analyse them separately. The different parts were washed with distilled water, dried (70 °C for 48 h), ground and digested in a microwave XP1500Plus (Mars®) in HNO_3:H_2O_2 (1:1) (Sah and Miller, 1992). The concentration of micro and macronutrients in plants (Fe, Mn, Ca, Mg, K, Na) were measured by atomic absorption spectrometry (SpectrAA 220FS Varian), and potential pollutants (Cu, Zn, Cd, As and Pb) by ICP-MS in a PE SCIEX ELAN-5000A spectrometer.

The bioaccumulation factor (BAF) for the uptake of Potentially Harmful Elements (PHEs) by vegetation was calculated through dividing concentrations in plants (mg kg^{-1} dry weight) by PHE bioavailable concentrations (extracted by EDTA) in the tested soils (mg kg^{-1} dry soil) (Wang et al., 2006; Kidd et al., 2007). PHE concentrations extracted by EDTA were selected for BAF calculation, as this fraction represents PHE bioavailable forms for plants (Marguí et al., 2007; Parra et al., 2014).

Richness and plant cover were also estimated. Richness was calculated as the number of species found per grid, and species cover as the percentage of the total surface covered by a given specie. The 50 x 50 cm^2 grid was randomly placed and replicated three times per sampling site.

2.3. Data analysis

Non-parametric Kruscall-Wallis test for the analysis of mean comparison was chosen due to sample size (Theodorsson-Norheim, 1986). Significant differences were determined by Kruscall-Wallis *post-hoc* test ($p < 0.05$). In order to analyse the influence of soil properties on the selective extractions of Potentially Harmful Elements (PHEs), Spearman´s correlations were performed. A Principal Components Analysis (PCA) was made to assess relationships among PHEs, and a non-metric multidimensional scaling (NMDS) ordination diagram was performed to determine the effects of PHE concentrations in vegetation response. All these analyses were performed with a confidence level of 95% by using IBM SPSS Statistics for Windows, v.20.0 (IBM Corp., Armonk, N.Y., USA), and the non-metric multidimensional scaling (NMDS) with Rstudio 2015 (Rstudio Team, Boston, USA).

3. RESULTS AND DISCUSSION

3.1. Soil properties

Soil properties analysed in unvegetated soils (UV-AA) and in the surrounding vegetated areas (B1 and B2) showed significant differences in most parameters (Table 1.1). Unvegetated soils were extremely acidic (pH<4), showed high EC (>2.3 dS m^{-1} in a 1:5 soil:water extract) and an elevated content in total iron (Fe_T). These conditions, along with the high presence of Potentially Harmful Elements (PHEs), are a consequence of the continued pollution process characterized by the oxidation of the remaining tailings in soils, which were left after a rapid and deficient first extensive clean-up operation that mixed the tailings with the soil in depth (Simón et al., 2008). Strong acidity, high EC and low OC have a severe impact in the mobility of PHEs (Ivezić et al., 2012; Rodríguez-Vila et al., 2017), being these parameters directly related to seed emergence inhibition in UV-AA. In contrast, this situation was different in the subareas surrounding the unvegetated soils (B1 and B2), where pH rose from 4.5 in B1 to 6.7 in B2, and $CaCO_3$ progressively increased. Calcium carbonate was intensely applied in the affected soils during rehabilitation actions, what enhanced soil pH and enabled the re-establishment of vegetation (Aguilar et al., 2004). The EC significantly decreased in belt areas (B1 and B2) in relation to unvegetated soils (UV-AA), also promoting favourable conditions for the establishment of vegetation. The presence of vegetation over time produced a significant increase of CEC and OC in both belt areas (B1 and B2) in relation to the unvegetated soils (UV-AA).

Table 1.1. Main soil properties in the subareas: unvegetated soils (UV-AA), belt1 (B1) and belt2 (B2). EC: electrical conductivity; OC: organic carbon content; $CaCO_3$: calcium carbonate content; CEC: cation exchange capacity; V: base saturation percentage; Fe_T/Mn_T: total concentration of Fe and Mn; Fe_o/Mn_o: amorphous concentration of Fe and Mn. Lowercase letters represent significant differences among areas (Kruscall Wallis test p<0.05).

	UV-AA		**B1**		**B2**	
	mean	*sd*	mean	*sd*	mean	*sd*
Clay (%)	24.23	*4.98*	19.6	*6.66*	22.89	*5.03*
Coarse silt (%)	13.37b	*2.00*	13.00b	*0.32*	11.19a	*0.78*
Fine silt (%)	17.47	*1.28*	16.13	*3.25*	18.95	*2.82*
Sand (%)	44.94	*6.46*	51.28	*9.63*	46.97	*7.74*
Gravel (%)	3.97	*1.77*	3.41	*1.21*	4.67	*2.21*
pH (H_2O)	3.32a	*0.28*	4.51b	*0.40*	6.71c	*1.16*
pH (KCl)	3.40a	*0.27*	4.19b	*0.35*	6.33c	*0.87*
EC (dS m^{-1})	2.37b	*0.41*	0.84a	*0.83*	0.39a	*0.13*
$CaCO_3$ (%)	0.68a	*0.05*	0.67a	*0.09*	1.39b	*0.67*
OC (%)	0.79a	*0.15*	2.01b	*0.70*	2.42b	*0.28*
Ca^{2+} ($cmol_+ kg^{-1}$)	6.33a	*1.6*	7.53a	*1.61*	11.01b	*0.60*
Mg^{2+} ($cmol_+ kg^{-1}$)	2.08b	*0.61*	0.84a	*0.68*	0.62a	*0.07*
K^+ ($cmol_+ kg^{-1}$)	0.03	*0.01*	0.04	*0.01*	0.04	*0.01*
Na^+ ($cmol_+ kg^{-1}$)	0.07a	*0.02*	0.11ab	*0.02*	0.12b	*0.02*
CEC ($cmol_+ kg^{-1}$)	8.99a	*0.46*	10.55b	*0.94*	11.79b	*0.62*
V (%)	94.45ab	*11.10*	80.75a	*13.32*	100.00b	*0.00*
Fe_T (mg kg^{-1})	40802b	*1935*	33242a	*4801*	30818a	*2814*
Fe_O (mg kg^{-1})	5641b	*747*	3812a	*954*	4393ab	*653*
Mn_T (mg kg^{-1})	387.3a	*34.6*	365.3a	*33.8*	740.9b	*53.9*
Mn_O (mg kg^{-1})	181.1a	*53.7*	190.8a	*46.4*	574.9b	*90.9*

3.2. Potentially Harmful Elements in soils

The total concentrations of Potentially Harmful Elements (PHEs) measured in these residual polluted plots were compared with the background values reported by Simón et al., (1999) for the soils in the area that remained unaffected by the mine spill. In all cases, the concentration of PHEs studied exceeded the background values, with maximum values exceeding 19- and 10-fold in the case of As and Pb, respectively, and 3-, 2- and 2.5-fold in the case of Cu, Zn and Cd, respectively. Furthermore, As and Pb concentrations exceeded the established levels (36 and 275 mg kg^{-1}, respectively) used for declaring a soil as polluted by the Regional Government in Andalusia (Spain) (BOE, 2015).

Changes in soil PHE solubility over time within the affected area as a result of the influence of soil properties were previously reported (Martín Peinado et al., 2015; Romero-Freire et al., 2016a). The application of different amendments, including organic matter, iron-rich clayey materials and sugar-refinery scum (rich in calcium carbonate) modified the properties of treated soils, promoting changes in PHE mobility. To assess the current mobility and bioavailability of PHEs in the affected soils and, hence, the influence on vegetation distribution, soil PHE fractionations were carried out. PHE fractions were analysed from mobile or available forms, what involves soluble in water, exchangeable and bioavailable forms; to immobile or unavailable fractions, what involves those bounded to amorphous Fe/Mn oxides and the residual fraction.

Statistically significant differences ($p<0.05$) were found among subareas for the different extracted fractions depending on the specific PHE mobility (Table 1.2). Previous studies in the area (Kraus and Wiegand, 2006; Simón et al., 2008) determined high mobility with strong leaching in depth under acidic conditions for Zn, Cd, and to a lesser extent, for Cu; while low mobility was detected for Pb and As, with significant accumulation in the uppermost part of the soil. In our results, the highest values measured were generally concentrated in UV-AA subareas, especially for soluble and exchangeable forms in the case of Cu, Zn and Cd, and bounded to Fe/Mn oxides forms for Cu and As, while B2 subareas showed the lowest values for PHE mobile forms, except for Pb extracted with EDTA, and bounded to Fe/Mn oxides. According to soil properties, the mobility of Cu, Zn and Cd was mainly negatively related to pH and OC content and positively to EC for soluble and exchangeable fractions, while no significant correlation with any soil properties was found for As and Pb mobile forms (Spearman analysis, Appendix 1.1).

Soluble and exchangeable Cu fractions decreased significantly in B2 from UV-AA subareas, 74- and 54-fold, respectively. According to our findings, the reduction of Cu mobility was related to the presence of OC and to the rise in pH. Both properties have been reported as highly significant in Cu retention (Kabata-Pendias, 2011); while acidic conditions with pH below 5.5 enhance Cu mobility in soils (Martínez and Motto, 2000). Copper bounded to Fe/Mn oxides reached the highest percentage extracted in relation to total concentrations in all areas (ranged between 68.04-61.65%), followed by Cu extracted by EDTA (33.18-47.13%), although without statistical differences among subareas. Copper associated with organic matter could be extracted by EDTA (Kidd et al., 2007), even from organic complexes characterized by their low bioavailability (Violante et al., 2010). According to our results, the main soil components related to Cu retention are iron oxides and organic matter, being even more important than clay minerals (McLaren et al., 1981).

Table 1.2. PHE concentrations (mg kg^{-1} dry soil) for the different soil fractions among subareas: unvegetated soils (UV-AA), belt1 (B1) and belt2 (B2) (R: residual fraction; O: bounded to amorphous Fe/Mn oxides fraction; B: bioavailable fraction; E: exchangeable fraction; S: soluble fraction; T: total concentration). Lowercase letters represent significant differences among areas (Kruscall Wallis test P<0.05). <LOD: below detection limit.

		UV-AA		**B1**		**B2**	
		mean	*sd*	mean	*sd*	mean	*sd*
Cu	**Cu_T**	96.60b	*12.71*	95.58b	*20.17*	68.20a	*7.70*
	Cu_S	0.74b	*0.79*	0.03a	*0.02*	0.01a	*0.00*
	Cu_E	9.41c	*9.92*	0.93b	*0.64*	0.18a	*0.05*
	Cu_B	37.01	*12.74*	31.72	*7.53*	32.14	*2.72*
	Cu_O	65.73b	*18.69*	58.92ab	*13.99*	42.12a	*5.44*
	Cu_R	30.86ab	*6.25*	36.64b	*7.88*	26.09a	*4.54*
Zn	**Zn_T**	242.33	*57.06*	277.73	*37.66*	317.80	*76.03*
	Zn_S	6.77b	*4.19*	1.47a	*1.63*	0.07a	*0.04*
	Zn_E	70.42c	*52.41*	23.66b	*7.65*	1.52a	*2.79*
	Zn_B	89.10	*48.37*	56.22	*8.41*	43.09	*15.78*
	Zn_O	97.78	*54.29*	59.52	*16.46*	75.49	*24.52*
	Zn_R	142.75a	*26.05*	193.48b	*22.80*	242.31b	*52.15*

Cd	**Cd_T**	0.86	*0.34*	0.80	*0.09*	0.87	*0.32*
	Cd_S	0.03	*0.02*	0.01	*0.01*	<LOD	-
	Cd_E	0.34b	*0.21*	0.15b	*0.04*	0.02a	*0.03*
	Cd_B	0.54	*0.25*	0.45	*0.04*	0.57	*0.22*
	Cd_O	0.81	*1.28*	0.24	*0.06*	0.47	*0.58*
	Cd_R	<LOD	-	0.37	*0.06*	0.06	*0.56*
As	**As_T**	278.08b	*58.03*	222.05b	*50.55*	106.98a	*30.90*
	As_S	<LOD	-	<LOD	-	0.01	*0.00*
	As_E	0.08	*0.10*	0.02	*0.02*	0.03	*0.02*
	As_B	2.15	*1.99*	0.87	*0.40*	1.37	*0.95*
	As_O	88.20b	*27.11*	59.52ab	*16.46*	36.56a	*13.41*
	As_R	189.89b	*36.65*	162.54b	*38.07*	70.45a	*17.86*
Pb	**Pb_T**	345.13b	*80.68*	256.58ab	*64.27*	171.23a	*26.07*
	Pb_S	<LOD	-	0.01	*0.01*	0.01	*0.01*
	Pb_E	0.02	*0.03*	0.01	*0.01*	0.01	*0.00*
	Pb_B	0.61a	*0.09*	2.48b	*1.99*	18.91c	*6.97*
	Pb_O	5.54a	*1.44*	6.89ab	*4.18*	16.07b	*5.72*
	Pb_R	339.57b	*79.54*	249.67b	*65.86*	154.11a	*31.86*

A similar behaviour was found in Zn fractionation. Soluble and exchangeable Zn concentrations were significantly higher in UV-AA, while decreased in the adjacent belts (100- and 46-fold lower, B1 and B2, respectively). The residual fraction was the most abundant form in revegetated areas (69.67% in B1 and 76.25% in B2), and also in unvegetated soils (58.91% of the total concentration). pH is the most important parameter for determining Zn mobility, which is very relevant at acidic conditions (Lock and Janssen, 2003; Romero-Freire et al., 2016b). In addition, organic matter is a natural sink of Zn in soils that easily absorbs this element (Kumpiene et al., 2008). The correlation between Zn and these two parameters (Appendix 1.1) was shown for both revegetated areas (B1 and B2), where the increase in pH and OC content promoted Zn retention in soils.

Similarly to Cu and Zn, soluble and exchangeable Cd fractions showed a strong reduction from UV-AA to B2 (76-fold for soluble Cd and 18-fold for exchangeable). Cd extracted by EDTA was high in all subareas (61.91% in UV-AA, 55.67% in B1 and 64.79% in B2), being the predominating fraction in both vegetated belts in relation to the total concentration, whereas

Cd bounded to Fe/Mn oxides was the most abundant fraction in UV-AA, reaching 93.16%, indicating that Cd in unvegetated soils could be retained in different forms from those that appear in soils with vegetation. Hence, Cd showed higher bioavailability in the vegetated soils (B1 and B2) than in unvegetated soils compared to the other elements, with a very low percentage of residual fraction, especially in B2. Soluble and exchangeable forms were controlled by pH, confirming that Cd is very mobile under acidic conditions even after a long-term ageing process; similar results were also showed by Clemente et al. (2008) for soils related to metallurgical activity. Moreover, Kirkham (2006) indicated that pH was the most important factor controlling Cd bioavailability for plants. Along with pH, OC is highly related to the reduction of Cd mobility in soils (Bur et al., 2010; Kabata-Pendias et al., 2011); in our study area, OC was significantly correlated with the retention of soluble and exchangeable Cd in the subareas with a significant increase in organic carbon (B1 and B2) compared to the unvegetated soils (UV-AA) (Appendix 1.1).

Arsenic presented a very low mobility in relation to total concentrations in all cases (less than 2% for mobile fractions, and even less than 0.03% for soluble As); meanwhile, the residual fraction predominated in all areas (68.29% in UV-AA, 73.20% in B1 and 65.85% in B2), followed by the fraction bounded to Fe/Mn oxides (ranging between 26.80-34.17% of total concentrations of As). Arsenic bounded to Fe/Mn oxide was mainly related to amorphous oxides forms (Appendix 1.1); the retention of As by soil oxides, corroborated by many authors (Manaka, 2006; Acosta et al., 2015), is due to the anionic behaviour of As, what promotes its binding to soil Fe/Mn oxides. The presence of poorly crystalline forms of iron oxides was previously reported in these soils (Aguilar et al., 2007) and provides the main sorption sites for As; along with aging, these crystalline forms can co-precipitate with As, finally immobilizing this pollutant in the solid phase (Suda and Makino, 2016).

The residual fraction of Pb predominated in all subareas too (ranging from 98.39% in UV-AA to 90.01% in B2), whereas the percentage of soluble and exchangeable forms were negligible (<0.01% of total concentrations in all cases), suggesting low availability for plants. Lead is a very immobile element in soils, being clay minerals, pH and organic matter, the most important factors determining Pb fixation (Kabata-Pendias, 2011; Romero-Freire et al., 2015). Generally, Pb reaches high accumulation in the uppermost part of soils, mainly due to Pb sorption by OM, forming stable complexes and, thus, reducing phytotoxicity at low soil pH (Udom et al., 2004). However, in our study, Pb extracted by EDTA and bounded to Fe/Mn amorphous oxides showed a significant increase in B2, from 0.18% and 1.16% of total concentrations in UV-AA to 11.04% and 9.38% in B2, respectively. This could be related to the increase of $CaCO_3$ content in B2, where Pb could be forming co-precipitates with Fe easily extracted by EDTA and oxalic acid-ammonium oxalate reagents. Simón et al. (2005) reported that Pb co-precipitates with Fe, coating the surface of the calcium carbonate used as an amendment of these soils; moreover, their findings showed that the quantity of Pb precipitated decreased at pH>6.5, increasing its bioavailability. Therefore, the addition of calcium carbonate is a suitable remediation technique to raise pH in polluted soils, but monitoring studies are strongly recommended after every remediation program to prevent future mobilizations.

3.3. Relationship between PHEs and vegetation

3.3.1. Vegetation distribution

A total of 27 species were identified within the studied area. Species richness showed significant differences among subareas, nearly twice higher in B2 than in B1, both values far from those in UV-AA (Figure 1.2). Total cover showed a similar pattern, reaching the highest values in B1 and B2 (95.75% - 100%, respectively) and the lowest in UV-AA (4%). Therefore, cover and richness were significantly higher in all cases for the surrounding belts and drastically low in UV-AA.

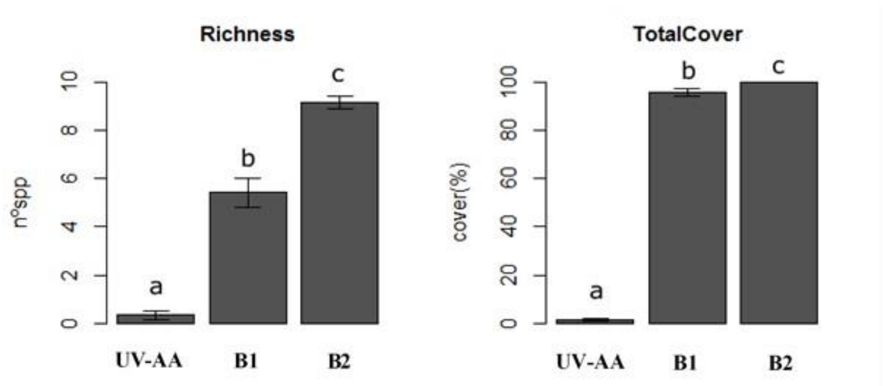


Figure 1.2. Vegetation richness and total cover per subarea (unvegetated -UV-AA- and vegetated subareas -B1 and B2-). Lowercase letters represent significant differences among subareas (Kruscall Wallis test P<0.05).

To assess the distribution of the different species among the three subareas, an NMDS analysis was performed (R^2=0.98) (Figure 1.3). The graphic, in which line length represents the abundance of species, showed an inverse relationship between pollutants and richness. Whereas in UV-AA rarely appeared a species (only *Lamarckia aurea* (L.) Moench and *Spergularia rubra* (L.) J. Presl & C. Presl, both with 4% cover), the two vegetated belts concentrated most of the species, although there were significant differences in terms of species distribution between them.

Important differences were found among subareas regarding the percentage of cover per specie. In UV-AA, only *L. aurea* and *S. rubra* were found with a low cover. In B1, *L. aurea* was dominant (80%) and appeared associated with other species such as *Anthemis arvensis* (L.), *Hypochaeris glabra* (L.), *S. rubra* and *Vulpia membranácea* (L.) Dumort. In contrast, in B2, the dominant species was *Trifolium campestre* Schreb., with more than 70% cover, together with *Leontodon longirostris* (Finch & P.D. Sell) Talavera, *Avena sterilis* (L.), *Chrysanthemum coronarium* (L.) or *Anagallis arvensis* (L.). Therefore, the dominant species were different in each belt and, thus, their distribution. Consequently, *L. aurea* (dominant species in B1), rarely appeared in B2 and was replaced by *T. campestre* as dominant species.

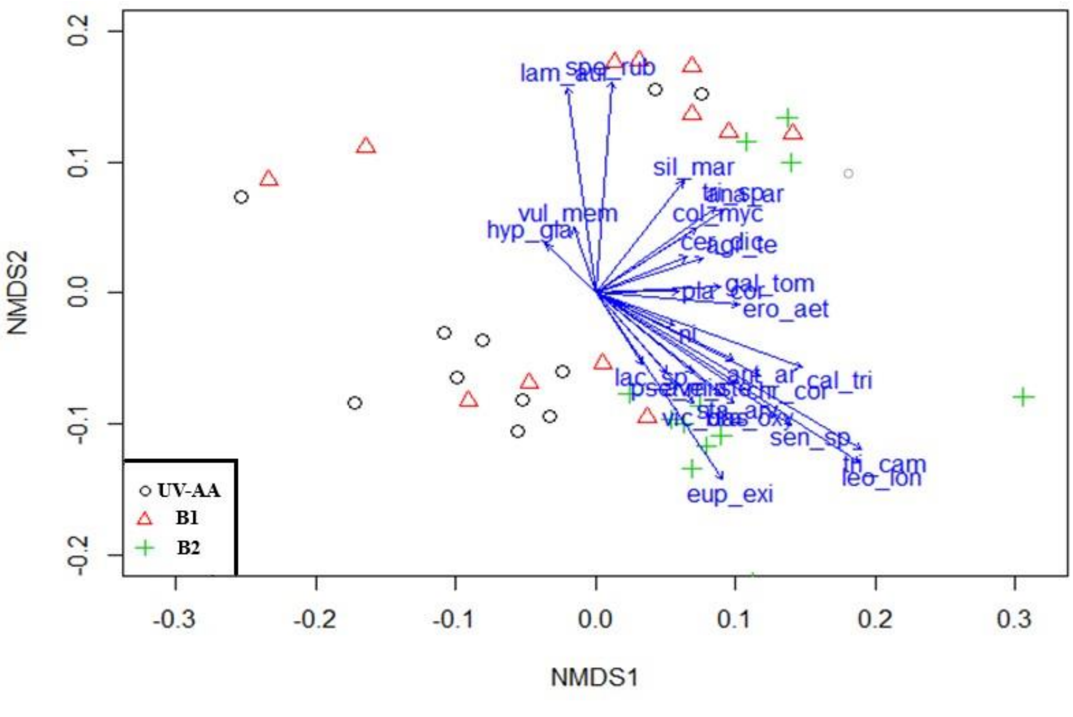


Figure 1.3. NMDS analysis representing plant species distribution among subareas. Unvegetated soils (UV-AA): black circles, belt1 (B1): red triangles, and belt2 (B2): green cross. Species (in blue): agr_te = *Agrostis tenerrima*; ana_ar = *Anagallis arvensis*; ant_ar = *Anthemis arvensis*; ave_ste = *Avena sterilis*; bra_oxy = *Brassica oxyrrhina*; cal_tri = *Calendula tripterocarpa*; cer_dic = *Cerastium dichotomum*; chr_cor = *Chrysanthemum coronarium*; col_myc = *Coleostephus myconis*; ero_aet = *Erodium aethiopicum*; eup_exi

= *Euphorbia exigua*; gal_tom = *Galactites tomentosa*; hyp_gla = *Hypochaeris glabra*; sta_arv = *Stachys arvensis*; lac_sp = *Lactuca* sp.; lam_aur = *Lamarckia aurea*; leo_lon = *Leontodon longirostris*; ni = No identified; pla_cor = *Plantago coronopus*; sen_sp = *Senecio lividus*; sil_mar = *Silybum marianum*; spe_rub = *Spergularia rubra*; tri_cam = *Trifolium campestre*; tri_sp = *Trifolium* sp.; pse_min = *Pseudorlaya minuscula*; vic_das = *Vicia dasycarpa*; vul_mem = *Vulpia membranacea*.

L. aurea showed the highest tolerance to PHE toxicity, even more than other species also tolerant to this kind of pollution in B1. According to Madejón, E. et al. (2006), *L. aurea* can accumulate high quantities of Cu, Pb and Zn (the latter one with concentrations over phytotoxic levels) without affecting their nutrient absorption capacity. *S. rubra*, the other species that accompanies *L. aurea*, is also able to cope with high levels of PHEs beyond normal values (Hernández and Pastor, 2008). On the other hand, *T. campestre* also showed a good ability to cope with elevated levels of this kind of pollution in B2, results that fit with those of Bidar et al. (2007), who reported that this species could grow in soils highly polluted with Cd, Pb and Zn, by accumulating them in roots. Consequently, both *L. aurea* and *T. campestre* were the species best adapted to this kind of soil pollution; moreover, the high PHE concentrations in vegetation indicates that these plants may have developed adaptation mechanisms that allow them to live under these stressful conditions. Hence, an in-depth study about the bioaccumulation capacity of these species per subareas could help identify potential species for the phytoremediation of both the study area and other similar polluted areas.

3.3.2. PHE bioaccumulation

Differences in PHE accumulation in plants were found between roots and aerial parts and between belts in some cases (Appendix 1.2). In B1, roots contained significantly more Cu and Zn than aerial parts, and in B2, roots accumulated significantly more Cu and As than aerial parts. In the rest of cases, there were no significant differences between parts, although roots tended to accumulate more PHEs in both subareas. Comparing between belts, vegetation in B1 accumulated significantly more As and Pb than vegetation in B2, despite Pb bioavailable fraction in B2 soils was higher than in B1 (Table 1.2). In this sense, an antagonistic influence was identified between Ca-Mg and As-Pb (PCA analysis, Appendix 1.3); a higher presence of Ca in B2 could be related to the decrease in As and Pb bioaccumulation in roots. The protective action of calcium in vegetation against the presence of As has been previously reported in these soils by Madejón, E. et al. (2006). Other studies have reported a reduction in Pb toxicity due to the presence of Ca, blocking Pb transport into roots and, therefore, Pb toxicity (Kim et al., 2002).

The bioaccumulation factor (BAF) using EDTA extraction of soils was calculated to evaluate the transfer of PHEs into plants (Table 1.3). Since total concentrations have been commonly used to predict PHE transfer from soil to plant without considering the bioavailability of PHEs in soils, several authors highly recommend the EDTA extraction to predict this potential for bioaccumulation (Wang et al., 2006). In most cases, BAF values were above 1, indicating accumulation in plants, preferentially in roots despite the high variability of values. Cu BAF was significantly higher in roots than in aerial parts, with values above 1 in both B1 and B2 subareas, indicating high transfer of Cu from soil to plants; according to Kabata-Pendias (2011), Cu is easily absorbed by plant’s roots due to its high solubility in soils, although this element usually shows low mobility inside the plant, being strongly retained in roots (Ali et al., 2002). Despite no differences were shown for Zn between roots and aerial parts, high values of BAF indicated high transference of Zn into plants, also due to its high solubility in soils (Kabata-Pendias, 2011). BAF values for As and Cd in roots and aerial parts revealed the transfer of these two elements from soils to plants; furthermore, in B2, As and Cd BAF were also significantly higher in roots than in aerial parts. Due to the high solubility of Cd in soils (Kabata-Pendias, 2011), plants easily absorbed it, and seemed to tolerate relatively high concentrations of this element (Chan and Hale, 2004). Arsenic and Lead belong to the group of potentially harmful elements that are hardly translocated to aerial parts even once absorbed by plants; nevertheless, our findings suggest the opposite. Our results showed transfer and accumulation of As in roots; moreover, comparing our results with the findings reported by Del Río et al. (2002), translocation into aerial parts exits and seems to have been maintained over time. As well, the increase of Pb extracted by EDTA in the vegetated areas, especially in B2 soils, resulted in higher transfer into plants, with translocation from roots to aerial parts. In this sense, Kabas et al. (2012) reported the influence of organic matter in increasing the accumulation of Pb in vegetation through the formation of soluble organic ligands related to root exudates, what would increase the uptake of PHEs.

Table 1.3. Bioaccumulation Factor (BAF) calculated for PHEs dividing concentrations in plants (mg kg^{-1} dry weight) by concentration extracted by EDTA (mg kg^{-1} dry soil), for the different belts (B1 and B2) and different part of the plants (root and aerial). Lowercase letters represent significant differences between part of the plants (Kruscall Wallis test $p<0.05$). Capital letters represent significant differences between belts (Kruscall Wallis test $p<0.05$).

	B1				**B2**			
	Root		**Aerial**		**Root**		**Aerial**	
	mean	*sd*	mean	*sd*	mean	*sd*	mean	*sd*
CuBAF	2.34b	*0.71*	0.71a	*0.22*	1.85b	*0.16*	0.54a	*0.10*
ZnBAF	4.45	*0.85*	3.32	*0.55*	4.93	*1.61*	2.60	*0.26*
AsBAF	31.49B	*15.57*	10.93	*11.37*	8.20Ab	*4.82*	1.12a	*0.67*
CdBAF	4.79	*1.97*	2.86	*1.24*	3.40b	*1.72*	1.50a	*0.97*
PbBAF	33.25B	*30.55*	17.467B	*17.37*	1.43A	*1.17*	0.65A	*0.22*

Comparing between belts, only As and Pb BAF showed significant differences; higher BAF was observed in plant's roots from B1 for As and Pb, also for aerial parts in B1 for Pb. Despite their usually low mobility in soils, corroborated by the limited percentage of As and Pb extracted by EDTA, both elements reached the highest bioaccumulation factor of all PHEs, with significantly higher transfer from soil to plant in B1 than in B2. Therefore, both elements could have a greater influence on the differences in vegetation distribution between B1 and B2 soils.

PHE bioaccumulation values in the aerial parts of our plants exceeded the normal values reported by Chaney (1983); therefore, the plants growing in these polluted soils would have certain degree of tolerance. Furthermore, the concentrations of Cu and As in the aerial parts in B1, and the concentration of Zn in B1 and B2 were in the phytotoxic range defined by Kabata-Pendias (2011); it was also the case of Fe, with concentrations in plants far exceeding the maximum value reported (1000 mg kg^{-1}), maybe with toxicity implication for vegetation. Chaney (1983) established the maximum dietary levels of PHEs tolerated by domestic livestock (cattle, sheep, swine and chicken). According to these results, Cu in the aerial parts of our plants exceeded the maximum levels chronically tolerated by sheep (25 mg kg^{-1}) in B1, and by all types of livestock in the case of Cd (limit value 0.5 mg kg^{-1}) in both subareas. That let us conclude there is a potential risk of pollutant transference through the food-chain, being necessary to take action and control polluted soils in the area to safeguard the ecosystem and human health.

4. CONCLUSIONS

Eighteen years after the Aznalcóllar mine spill and the implementation of the rehabilitation program, residual pollution is still found in the area. As a consequence of the high content of Potentially Harmful Elements (PHEs), unvegetated soil patches are surrounded by soils with two different vegetation gradients depending on the level of soil pollution. These soils pose a high risk of pollution dispersion towards the most recovered areas.

The lack of vegetation was influenced by the most mobile elements, Cu, Zn and Cd, which reached the highest mobility in the unvegetated soils. The surrounding vegetated belts registered a significant decrease in PHE concentration and mobilization due to the change in soil conditions, especially the increase of pH and organic matter content. Regarding PHE bioaccumulation in plants, PHEs were preferentially accumulated in roots, especially in the case of As and Pb, for which the bioaccumulation factor was significantly higher in plant's roots than in aerial parts, and in Belt 1 compared to Belt 2, even if As and Pb bioavailability in B1 soils was lower. Hence, As and Pb could be possibly conditioning the different distribution of vegetation in belts.

Vegetation is a good indicator of soil pollution. In our study, richness and plant cover changed according to soil pollution patterns. Furthermore, many species can tolerate high concentrations of potentially harmful elements in soils through different strategies, like exclusion or accumulation. *Lamarckia aurea* and *Trifolium campestre* have demonstrated the highest capacity to cope with severe soil pollution. However, the PHEs bioaccumulated in plants can result in a risk for the ecosystem and living organisms through the food-chain. In this sense, high concentrations of Cu and Cd measured in aerial parts pose a risk to livestock. A continuous monitoring of PHEs in soils and their transfer to wild vegetation is necessary to ensure ecosystem safety in the area; moreover, assisted natural remediation is required to control bioavailability and toxicity of pollutants, especially in the unvegetated soils.

5. ACKNOWLEDGEMENTS

The authors would like to thank Fundación Tatiana Pérez De Guzmán El Bueno for funding Helena García-Robles' PhD, as well as Mr. David Nesbitt for the English corrections and comments to this paper.

6. REFERENCES

Acosta, J.A., Arocena, J.M., Faz, A., 2015. Speciation of arsenic in bulk and rhizosphere soils from artisanal cooperative mines in Bolivia. Chemosphere, 138: 1014-1020. https://doi.org/10.1016/j.chemosphere.2014.12.050

Aguilar, J., Bouza, P., Dorronsoro, C., Fernández, E., Fernández, J., García, I., Martín, F., Simón, M., 2004. Application of remediation techniques for immobilization of metals in soils contaminated by a pyrite tailing spill in Spain. Soil Use and Management, 20(4): 451-453. https://doi.org/10.1111/j.1475-2743.2004.tb00396.x

Aguilar, J., Dorronsoro, C., Fernández, E., Fernández, J., García, I., Martín, F., Sierra, M., Simón, M., 2007. Remediation of As-contaminated soils in the Guadiamar river basin (SW, Spain). Water, Air and Soil Pollution, 180: 109-118. https://doi.org/10.1007/s11270-006-9254-3

Ali, N.A., Bernal, M.P., Ater, M., 2002. Tolerance and bioaccumulation of copper in *Phragmites australis* and *Zea mays*. Plant and Soil, 239(1): 103-111. https://doi.org/10.1023/A:1014995321560

Barahona, E., 1984. Determinación de carbonatos totales y caliza activa. Grupo de trabajo de normalización de métodos analíticos. In I Congreso Nacional de la Ciencia del Suelo, pp. 53-67. Madrid, Spain: Sociedad Española de la Ciencia del Suelo.

Bidar, G., Garçon, G., Pruvot, C., Dewaele, D., Cazier, F., Douay, F., Shirali, P., 2007. Behavior of *Trifolium repens* and *Lolium perenne* growing in a heavy metal contaminated field: plant metal concentration and phytotoxicity. Environmental Pollution, 147(3): 546-553. https://doi.org/10.1016/j.envpol.2006.10.013

Boletín Oficial del Estado (BOE) 38, 2015. Decreto 18/2015 por el que se aprueba el reglamento que regula el régimen aplicable a los suelos contaminados. Consejería de Medioambiente y Ordenación del Territorio, 38: 28-64.

Bur, T., Probst, A., Bianco, A., Gandois, L., Crouau, Y., 2010. Determining cadmium critical concentrations in natural soils by assessing Collembola mortality, reproduction and growth. Ecotoxicology and Environmental Safety, 73(3): 415-422. https://doi.org/10.1016/j.ecoenv.2009.10.010

Chapter 1

Chan, D.Y., Hale, B.A., 2004. Differential accumulation of Cd in durum wheat cultivars: uptake and retranslocation as sources of variation. Journal of Experimental Botany, 55(408): 2571-2579. https://doi.org/10.1093/jxb/erh255

Chaney, R.L., 1983. Plant uptake of inorganic waste, in: Parr, J.F., Marsh, P.B., Kla, J.M., (Eds.), Land Treatment of Hazardous Wastes, Noyes Data Corporation, Park Ridge, 50-76. https://doi.org/10.4236/ijoc.2012.22022

Christou, A., Theologides, C.P., Costa, C., Kalavrouziotis, I.K., Varnavas, S.P., 2017. Assessment of toxic heavy metals concentrations in soils and wild and cultivated plant species in Limni abandoned copper mining site, Cyprus. Journal of Geochemical Exploration, 178: 16-22. https://doi.org/10.1016/j.gexplo.2017.03.012

Clemente, R., Dickinson, N.M., Lepp, N.W., 2008. Mobility of metals and metalloids in a multi-element contaminated soil 20 years after cessation of the pollution source activity. Environmental Pollution, 155(2): 254-261. https://doi.org/10.1016/j.envpol.2007.11.024

CMA (Consejería de Medio Ambiente)., 2003. Ciencia y restauración del río Guadiamar. PICOVER 1998-2002. Consejería de Medio Ambiente, Junta de Andalucía, Sevilla.

Del Río, M., Font, R., Almela, C., Vélez, D., Montoro, R., de Haro-Bailón, A., 2002. Heavy metals and arsenic uptake by wild vegetation in the Guadiamar river area after the toxic spill of the Aznalcóllar mine. J. Biotechnol., 98: 125-137. https://doi.org/10.1016/S0168-1656(02)00091-3

García-Carmona, M., Romero-Freire, A., Sierra Aragón, M., Martínez Garzón, FJ., Martín Peinado, F.J., 2017. Evaluation of remediation techniques in soils affected by residual contamination with heavy metals and arsenic. Journal of Environmental Management, 191: 228-236. https://doi.org/10.1016/j.jenvman.2016.12.041

Hernández, A.J., Pastor, J., 2008. Relationship between plant biodiversity and heavy metal bioavailability in grasslands overlying an abandoned mine. Environmental Geochemistry and Health, 30(2): 127-133. https://doi.org/10.1007/s10653-008-9150-4

IBM Corp., Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.

Ivezić, V., Almås, Å.R., Singh, B.R., 2012. Predicting the solubility of Cd, Cu, Pb and Zn in uncontaminated Croatian soils under different land uses by applying established regression models. Geoderma, 170: 89-95. https://doi.org/10.1016/j.geoderma.2011.11.024

Kabas, S., Faz, A., Acosta, J.A., Zornoza, R., Martínez-Martínez, S., Carmona, D.M., Bech, J., 2012. Effect of marble waste and pig slurry on the growth of native vegetation and heavy metal mobility in a mine tailing pond. Journal of Geochemical Exploration, 123: 69-76. https://doi.org/10.1016/j.gexplo.2012.07.008

Kabata-Pendias, A., 2011. Trace elements in soils and plants, fourth edition. CRC, Boca Raton.

Kidd, P.S., Domínguez-Rodríguez, M.J., Díez, J., Monterroso, C., 2007. Bioavailability and plant accumulation of heavy metals and phosphorus in agricultural soils amended by long-term application of sewage sludge. Chemosphere, 66: 1458-1467. https://doi.org/10.1016/j.chemosphere.2006.09.007

Kim, Y.Y., Yang, Y.Y., Lee, Y., 2002. Pb and Cd uptake in rice roots. Physiologia Plantarum, 116(3): 368-372. https://doi.org/10.1034/j.1399-3054.2002.1160312.x

Kirkham, M.B., 2006. Cadmium in plants on polluted soils: effects of soil factors, hyperaccumulation, and amendments. Geoderma, 137(1-2): 19-32. https://doi.org/10.1016/j.geoderma.2006.08.024

Kraus, U., Wiegand, J., 2006. Long-term effects of the Aznalcóllar mine spill-heavy metal content and mobility in soils and sediments of the Guadiamar river valley (SW Spain). Science of the Total Environment, 367(2): 855-871. https://doi.org/10.1016/j.scitotenv.2005.12.027

Kumpiene, J., Lagerkvist, A., Maurice, C., 2008. Stabilization of As, Cr, Cu, Pb and Zn in soil using amendments-a review. Waste Management, 28(1): 215-225. https://doi.org/10.1016/j.wasman.2006.12.012

Liu, X., Song, Q., Tang, Y., Li, W., Xu, J., Wu, J., Wang, F., Brookes, P.C., 2013. Human health risk assessment of heavy metals in soil-vegetable system: a multi-medium analysis. Science of the Total Environment, 463: 530-540. https://doi.org/10.1016/j.scitotenv.2013.06.064

Lock, K., Jannsen, C.R., 2003. Influence of ageing on zinc bioavailability in soils. Environmental Pollution, 126(3): 371-374. https://doi.org/10.1016/s0269-7491(03)00232-x

Madejón, E., De Mora, A.P., Felipe, E., Burgos, P., Cabrera, F., 2006. Soil amendments reduce trace element solubility in a contaminated soil and allow regrowth of natural vegetation. Environmental Pollution, 139(1): 40-52. https://doi.org/10.1016/j.envpol.2005.04.034

Madejón, P., Murillo, J.M., Marañón, T., Cabrera, F., 2006. Bioaccumulation of trace elements in a wild grass three years after the Aznalcóllar mine spill (South Spain). Environmental monitoring and assessment, 114(1): 169-189. https://doi.org/10.1007/s10661-006-2523-1

Madejón, P., Domínguez, M.T., Madejón, E., Cabrera, F., Marañón, T., Murillo, J.M., 2018. Soil-plant relationships and contamination by trace elements: A review of twenty years of experimentation and monitoring after the Aznalcóllar (SW Spain) mine accident. Science of the Total Environment, 625: 50-63. https://doi.org/10.1016/j.scitotenv.2017.12.277

Manaka, M., 2006. Amount of amorphous materials in relationship to arsenic, antimony, and bismuth concentrations in a brown forest soil. Geoderma, 136(1): 75-86. https://doi.org/10.1016/j.geoderma.2006.02.002

Marguí, E., Queralt, I., Carvalho, M.L., Hidalgo, M., 2007. Assessment of metal availability to vegetation (*Betula pendula*) in Pb-Zn ore concentrate residues with different features. Environmental Pollution, 145(1): 179-184. https://doi.org/10.1016/j.envpol.2006.03.028

Martín Peinado, F.M., Ruano, S.M., González, M.B., Molina, C.E., 2010. A rapid field procedure for screening trace elements in polluted soil using portable X-ray fluorescence (PXRF). Geoderma, 159(1-2): 76-82. https://doi.org/10.1016/j.geoderma.2010.06.019

Martín Peinado, F., Romero-Freire, A., García, I., Sierra, M., Ortiz, I., Simón, M., 2015. Long-term contamination in a recovered area affected by mining spill. Science of the Total Environment, 514: 219-223. https://doi.org/10.1016/j.scitotenv.2015.01.102

Martínez, C.E., Motto, H.L., 2000. Solubility of lead, zinc and copper added to mineral soils. Environmental Pollution, 107(1): 153-158. https://doi.org/10.1016/s0269-7491(99)00111-6

McLaren, R.G., Swift, R.S., Williams, J.G., 1981. The absorption of Copper by soil materials at low equilibrium solution concentrations. Journal of Soil Science, 32: 247-256. https://doi.org/10.1111/j.1365-2389.1981.tb01704.x

McLaughlin, M.J., Parker, D.R., Clarke, J.M., 1999. Metals and micronutrients-food safety issues. Field Crops Research, 60(1): 143-163. https://doi.org/10.1016/S0378-4290(98)00137-3

Moreno-Jiménez, E., Peñalosa, J.M., Manzano, R., Carpena-Ruiz, R.O., Gamarra, R., Esteban, E., 2009. Heavy metals distribution in soils surrounding an abandoned mine in NW Madrid (Spain) and their transference to wild flora. Journal of Hazardous Materials, 162(2): 854-859. https://doi.org/10.1016/j.jhazmat.2008.05.109

Novozamsky, I., Lexmond, T.H., Houba V.J., 1993. A single extraction procedure of soil for evaluation of uptake of some heavy metals by plants. International Journal of Environmental Analytical Chemistry, 51: 47-58. https://doi.org/10.1080/03067319308027610

Parra, A., Zornoza, R., Conesa, E., Gómez-López, M.D., Faz, A., 2014. Seedling emergence, growth and trace elements tolerance and accumulation by Lamiaceae species in a mine soil. Chemosphere, 113: 132-140. https://doi.org/10.1016/j.chemosphere.2014.04.090

Parraga-Aguado, I., González-Alcaraz, M.N., Álvarez-Rogel, J., Conesa, H.M., 2014. Assessment of the employment of halophyte plant species for the phytomanagement of mine tailings in semiarid areas. Ecological Engineering, 71: 598-604. https://doi.org/10.1016/j.ecoleng.2014.07.061

Quevauviller, P., Lachica, M., Barahona, E., Gomez, A., Rauret, G., Ure, A., Muntau, H., 1998. Certified reference material for the quality control of EDTA-and DTPA-extractable trace metal contents in calcareous soil (CRM 600). Fresenius' journal of analytical chemistry, 360(5): 505-511.

Raskin, I., Kumar, P.N., Dushenkov, S., Salt, D.E., 1994. Bioconcentration of heavy metals by plants. Current Opinion in biotechnology, 5(3): 285-290. https://doi.org/10.1016/0958-1669(94)90030-2

Rodríguez-Vila, A., Forjan, R., Guedes, R.S., Covelo, E.F., 2017. Effect of waste mixed with biochar as soil amendment on trace element solubility in a mine soil. Spanish Journal of Soil Science, 7(2): 109-121. https://doi.org/10.3232/SJSS.2017.V7.N2.03

Romero-Freire, A., Martín-Peinado, F.J., Van Gestel, C.A.M., 2015. Effect of soil properties on the toxicity of Pb: Assessment of the appropriateness of guideline values. J. hazard. Mater., 289: 46-53. https://doi.org/10.1016/j.jhazmat.2015.02.034

Romero-Freire, A., García, I., Simón, M., Martín, F., 2016a. Long-term toxicity assessment of soils in a recovered area affected by mining spill. Environmental Pollution, 208: 553-561. https://doi.org/10.1016/j.envpol.2015.10.029

Romero-Freire, A., Sierra, M., Martínez, F.J., Martín, F., 2016b. Is soil basal respiration a good indicator of soil pollution? Geoderma, 263: 132-139. https://doi.org/10.1016/j.geoderma.2015.09.006

RStudio Team, 2015. RStudio: integrated development for R. RStudio, Inc., Boston, MA URL http://www.rstudio.com/

Sah, R.N., Miller, R.O., 1992. Spontaneous reaction for acid dissolution of biological tissues in closed vessels. Analytical Chemistry, 64(2): 230-233. https://doi.org/10.1021/ac00026a026

Sarwar, N., Imran, M., Shaheen, M.R., Ishaq, W., Kamran, A., Matloob, A., Rehim, A., Hussain, S., 2017. Phytoremediation strategies for soils contaminated with heavy metals: Modifications and future perspectives. Chemosphere, 171: 710-721. https://doi.org/10.1016/j.chemosphere.2016.12.116

Schwertmann, U., Taylor, R.M., 1977. Iron oxides, in: Dixon, J.B., Webb, S.B. (Eds.), Minerals in soils environments. Soil Science Society of America Madison: 148-180

Simón, M., Ortiz, I., García, I., Fernández, E., Fernández, J., Dorronsoro, C., Aguilar, J., 1999. Pollution of soils by the toxic spill of a pyrite mine (Aznalcóllar, Spain). Science of the Total Environment, 242(1): 105-115. https://doi.org/10.1016/S0048-9697(99)00378-2

Simón, M., Martín, F., Ortiz, I., García, I., Fernández, J., Fernández, E., Aguilar, J., 2001. Soil pollution by oxidation of tailings from toxic spill of a pyrite mine. Science of the Total Environment, 279(1): 63-74. https://doi.org/10.1016/S0048-9697(01)00726-4

Simón, M., Martín, F., García, I., Bouza, P., Dorronsoro, C., Aguilar, J., 2005. Interaction of limestone grains and acidic solutions from the oxidation of pyrite tailings. Environmental Pollution, 135(1): 65-72. https://doi.org/10.1016/j.envpol.2004.10.013

Simón, M., Díez, M., García, I., Martín, F., 2008. Distribution of As and Zn in soils affected by the spill of a pyrite mine and effectiveness of the remediation measures. Water, Air and Soil Pollution, 198(1-4): 77-85. https://doi.org/10.1007/s11270-008-9827-4

Soil Conservation Service, 1972. Methods and Procedures for collecting soil samples. Washington, DC: Soil Survey Laboratory, USDA.

Sposito G., Lund L.J., Chang A.C., 1982. Trace metal chemistry in arid-zone field soils amended with sewage sludge. I: fractionation of Ni, Cu, Zn, Cd and Pb in solid phases. Soil Science Society of America Journal, 46: 260-264. https://doi.org/10.2136/sssaj1982.03615995004600020009x

Suda, A., Makino, T., 2016. Functional effects of manganese and iron oxides on the dynamics of trace elements in soils with a special focus on arsenic and cadmium: a review. Geoderma, 270: 68-75. https://doi.org/10.1016/j.geoderma.2015.12.017

Theodorsson-Norheim, E., 1986. Kruskal-Wallis test: BASIC computer program to perform nonparametric one-way analysis of variance and multiple comparisons on ranks of several independent samples. Computer methods and programs in biomedicine, 23(1): 57-62. https://doi.org/10.1016/0169-2607(86)90081-7

Udom, B.E., Mbagwu, J.S.C., Adesodun, J.K., Agbim, N.N., 2004. Distributions of zinc, copper, cadmium and lead in a tropical ultisol after long-term disposal of sewage sludge. Environment International, 30(4): 467-470. https://doi.org/10.1016/j.envint.2003.09.004

Ure, A.M., 1995. Methods of analysis for heavy metals in soils, in: Alloway, B.J. (Ed.) Heavy metals in soils, second ed. Blackie Academic and Professional. Glasgow. pp. 58-102

US EPA (US Environmental Protection Agency), 1998. Field portable x-ray fluorescence spectrometry for the determination of elemental concentrations in soil and sediment. Method 6200.

US EPA (US Environmental Protection Agency), 2006. XRF technologies for measuring trace elements in soil and sediment. NitonXLt 700 Series XRF Analyzer. Innovative technology verification report EPA/540/R-06/004.

Ussiri, D.A.N., Lal, R., 2008. Method for determining coal carbon in the reclaimed mine soils contaminated with coal. Soil Science Society of American Journal, 72: 231-237. https://doi.org/10.2136/sssaj2007.0047

Violante, A., Cozzolino, V., Perelomov, L., Caporale, A.G., Pigna, M., 2010. Mobility and bioavailability of heavy metals and metalloids in soil environments. Journal of soil Science and Plant Nutrition, 10(3): 268-292. https://doi.org/10.4067/S0718-95162010000100005

Wang, G., Su, M.Y., Chen, Y.H., Lin, F.F., Luo, D., Gao, S.F., 2006. Transfer characteristics of cadmium and lead from soil to the edible parts of six vegetable species in southeastern China. Environmental Pollution, 144(1): 127-135. https://doi.org/10.1016/j.envpol.2005.12.023

APPENDICES

Appendix 1.1. Spearman correlation coefficients of soil properties and PHE fractions (O: bounded to amorphous Fe/Mn oxides fraction; B: bioavailable fraction; E: exchangeable fraction; S: soluble fraction; T: total concentration). EC: electrical conductivity; OC: organic carbon content; CEC: cation exchange capacity; Fe_o: amorphous concentration of Fe. *Significant correlation p<0.05. ** Significant correlation p<0.01

	Clay	pH_{H2O}	EC	OC	CEC	Fe_o
Cu_T		-0.687*				
Cu_S		-0.825**	0.853**	-0.811**	-0.923**	
Cu_E		-0.876**	0.837**	-0.725**	-0.844**	
Cu_B						
Cu_O		-0.734**	0.622*			
Zn_T					0.601*	
Zn_S		-0.846**	0.874**	-0.657*	-0.755**	
Zn_E		-0.923**	0.755**	-0.818**	-0.776**	
Zn_B		-0.671*				
Zn_O						
Cd_T						
Cd_S		-0.928**	0.848**	-0.736**	-0.816**	
Cd_E		-0.930**	0.713**	-0.727**	-0.734**	
Cd_B						
Cd_O						
As_T		-0.713**	0.601*	-0.664*	-0.692*	
As_S						
As_E	0.636*					
As_B						
As_O		-0.720**	0.671*	-0.615*	-0.587*	0.762**
Pb_T		-0.727**	0.636*	-0.601*	-0.608*	0.699*
Pb_S						
Pb_E						
Pb_B		0.846**	-0.741**	0.888**	0.888**	
Pb_O		0.623*	-0.648*	0.694*	0.729**	

Appendix 1.2. Trace element bioaccumulation (mg kg^{-1} dry weight) for the different belts (B1 and B2) and different part of the plants (root and aerial). Lowercase letters represent significant differences between part of the plants (Kruscall-Wallis test P<0.05). Capital letters represent significant differences between belts (Kruscall-Wallis test P<0.05).

	B1				**B2**			
	Root		**Aerial**		**Root**		**Aerial**	
	mean	*sd*	mean	*sd*	mean	*sd*	mean	*sd*
FeBA	5679.22A	*1799.76*	3188.76	*1231.09*	3583.46B	*1351.79*	2903.05	*860.26*
MnBA	362.65	*44.82*	545.7B	*133.55*	287.18	*182.65*	155.10A	*82.44*
CaBA	2784.87A	*1937.72*	5517.11A	*1097.03*	6064.78Ba	*1297.37*	10805.05Bb	*1012.70*
MgBA	1199.90A	*108.79*	1881.68b	*325.84*	1744.42B	*376.27*	2189.69	*221.54*
NaBA	2308.33	*626.17*	3552.11	*1247.96*	3180.98	*605.88*	3294.84	*1818.35*
KBA	1967.76	*2135.11*	306.43A	*76.75*	156.79a	*24.87*	484.79Bb	*83.96*
CuBA	72.35b	*23.58*	22.56a	*9.79*	59.53b	*9.06*	17.33a	*2.15*
ZnBA	244.85b	*20.17*	183.71a	*21.08*	219.69	*124.44*	112.96	*48.09*
AsBA	23.12B	*8.85*	7.96	*7.78*	9.49Ab	*4.43*	1.49a	*1.46*
CdBA	2.14	*0.94*	1.26	*0.55*	2.19	*1.84*	0.98	*1.03*
PbBA	38.08B	*11.65*	19.02	*6.89*	21.04A	*7.69*	11.28	*3.44*

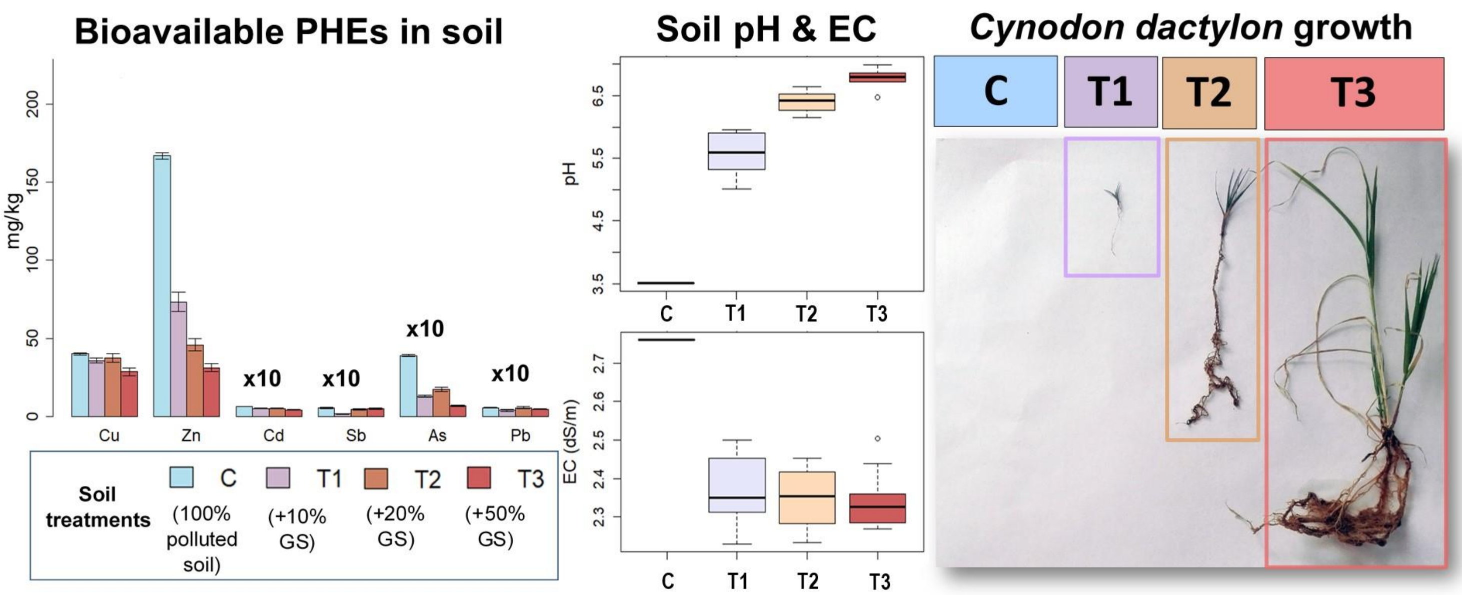
Appendix 1.3. Principal component analysis (PCA) after Varimax normalization method including trace element bioaccumulation.

	Component		
	1	**2**	**3**
FeBA	0.879		
MnBA			0.706
CaBA	-0.811		
MgBA	-0.630		
NaBA			0.785
KBA	0.735		
CuBA	0.804		
ZnBA		0.881	
AsBA	0.967		
CdBA		0.966	
PbBA	0.940		
Acc. Var (%)	46.95	66.77	81.43

- CHAPTER 2 -

GYPSUM MINING SPOIL IMPROVES PLANT EMERGENCE AND GROWTH IN SOILS POLLUTED WITH POTENTIALLY HARMFUL ELEMENTS

García-Robles, Helena; Melloni, Eliane G.P.; Navarro, Francisco B.; Martín-Peinado, Francisco José; Lorite, Juan.



Sent to Journal of Environmental Management

ABSTRACT

Soil pollution is a major problem worldwide and its restoration one of the biggest challenges of our society. In normal conditions, soils act as a buffer, filtering and immobilizing pollutants. However, some anthropogenic activities, such as mining, may exceed soil capacity, causing relevant health and ecosystem hazards. In this vein, the use of mineral amendments can help reduce soil pollution. Gypsum mining spoil (G) is a waste material produced in large quantities in gypsum mining industry, which can be very useful in the remediation of polluted soils. In this study, we carried out an *ex-situ* experiment to assess the capacity of G to both reduce the availability of potentially harmful elements (PHEs) in soils and promote seed emergence and plant performance. Soils affected by residual pollution after the Aznalcóllar mine spill were collected for this study, treated with G in different proportions, and sown with seeds of two non-genetically related species. Seed emergence and biomass production were monitored, and PHE content in soils and plants were analysed by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS). Results showed a strong and direct relation among G addition to polluted soils, reduction of PHE availability and plant emergence, biomass production and reduction of PHE uptake by plants. The most effective treatment of was the addition of 50% G, highlighting the promising results of G as soil amendment and the need to assess the effective dose of this material to be used in the remediation of polluted soils and vegetation recovery.

Keywords: Soil pollution; inorganic amendment; *Cynodon dactylon*; *Medicago sativa*

1. INTRODUCTION

Soils provide crucial environmental functions and services, being their productivity the most essential service for human survival and development (Kabata-Pendias and Pendias, 2000). Centuries of anthropogenic activities have resulted in the accumulation of pollutants in soils (Rodríguez-Eugenio et al., 2018). Hence, pollution is one of the main concerns affecting soils globally (FAO and ITPS, 2015; Payá-Pérez et al., 2018; Rodríguez-Eugenio et al., 2018). Among all chemical pollutants, Potentially Harmful Elements (PHEs), which include heavy metals and metalloids, are of major concern since they can persist in soils for a long period of time (Pilon-Smith, 2005) and produce negative cumulative effects on organisms. Consequently, they represent the main source of global environmental pollution with noxious implications for human health (Muyessar and Linsheng, 2016). PHEs are naturally present in soils, and some of them are essential micronutrients for plants, however, when concentrations exceed a specific threshold, they may cause toxicity (DalCorso et al., 2014; Higueras et al., 2016). For instance, high concentrations of PHEs can affect plant nutrition and fitness by displacing other essential nutrients, what may cause deficiencies limiting plant performance (DalCorso et al., 2014; Kabata-Pendias, 2011). Otherwise, some plants can stabilize PHEs in soils (phytostabilizers) or accumulate high concentrations of PHEs in their tissues (hyperaccumulators). However, PHEs accumulated in plants may become accessible for the subsequent links of the food chain, posing a significant hazard for the environment and living organism (Hooda, 2010; Nworie et al., 2019).

Remediation of soil pollution is essential to restore soil functions (Martín-Peinado et al., 2015). This usually entails the implementation of long-term strategies focused on the application of organic and/or inorganic amendments to reduce the mobility of pollutants (Hooda, 2010). Henceforth, detailed studies should be conducted to assess the interaction of PHEs with the amendment and the potential change in the behaviour of pollutants after the modification of the soil environment (García-Carmona et al., 2017).

Gypsum has been used as a soil fertilizer since its relatively low solubility makes it a long-lasting source of calcium (Shainberg et al., 1989; Toma et al., 1999). Moreover, gypsum can amend certain degraded soils such as acidified, salinized (with excessive sodium) or aluminium-polluted soils (Chen and Dick, 2011; Franzen et al., 2006), and some authors have tested the capacity of gypsum and other gypsum-like byproducts to bind heavy metals, such as Cu, Zn and Pb (Garrido et al., 2005; Illera et al., 2004; Sherene, 2010).

In 2018, gypsum world production reached 160 million tons (U.S. Geological Survey, 2019). Consequently, large quantities of gypsum mining spoil are annually produced and accumulated in mining sites, resulting in both storage and environmental problems (Al-Farajat, 2009; Ballesteros et al., 2017). Reusing and recycling gypsum waste is lately seen as a suitable management solution (Ahmed et al., 2011; Chandara et al., 2009) that perfectly fits the zero-waste strategy (Greyson, 2007). Gypsum mining spoil contains a high proportion of gypsum (50-70%), moderately high amounts of calcium carbonate (> 20%), and a mixture of fine (clay) and coarse (gravel) particles, which gives it a good potential to be used as an amendment in polluted soils.

The aim of this study is to assess the changes in soil properties by the use of gypsum mining spoil, to test the effectiveness in reducing PHE mobility and availability in soils, and to evaluate the influence of this amendment in enhancing plant performance.

2. MATERIALS AND METHODS

2.1. Experimental design

Soil samples were collected at the closest sector to the Aznalcóllar mine (Seville, SW Spain) in the Guadiamar Green Corridor (GGC, CMA 2003), area affected by the mine toxic spill since 1998 and where numerous restoration activities had been implemented (Madejón et al., 2018). Nevertheless, there are still residual polluted areas characterized by high concentrations of PHEs (mainly As, Pb, Zn, Cd, Cu, and Sb) and by the absence of vegetation (Martín-Peinado et al., 2015), the so-called "unvegetated affected area" hereafter (UV-AA). For this experiment, we selected five residual plots at the closest to the mine and most polluted area, after being measured *in-situ* with a NITON XLT 792 field portable X-Ray fluorescence analyser. Soil samples were collected from the uppermost 10 cm and intensely homogenized into just one composite soil sample (C_0). We also collected five samples of natural soils (Nat) in the surrounding unaffected area to be used as background values. As soil amendment, we used gypsum mining spoil (G) provided by Knauf-GmbH and extracted at a gypsum quarry in

Escúzar (Granada, SE Spain). Both the soil and amendment samples were sieved through 4 mm.

The experimental design was based on the addition of G to C_0 in three different proportions (treatments): 10% G (T1), 20% G (T2), and 50% G (T3). Afterwards, we prepared 32 replicates per treatment (pot size= 6 cm x 5.6 cm x 8 cm), plus 32 control replicates (C= 0% G) with non-treated polluted soil (C_0). We sowed seeds of alfalfa (*Medicago sativa* L.) in each pot (Figure 2.1), and the same experimental design was prepared for Bermuda grass (*Cynodon dactylon* (L.) Pers.). *M. sativa* and *C. dactylon* are two non-genetically related and native species present in the affected area, which are tolerant to PHEs and frequently used in pollution and phytoremediation studies (Madejón et al., 2002; Flores-Cáceres, 2013).

Finally, the 256 pots prepared (32 replicates x 4 treatments x 2 species) were randomly placed in a greenhouse equipped with an irrigation programmer, a nebulization system (30 l/h irrigation flow) and a temperature control sensor. The experiment lasted 82 days for *M. sativa* and 67 days for *C. dactylon*, and pots were watered five minutes daily.

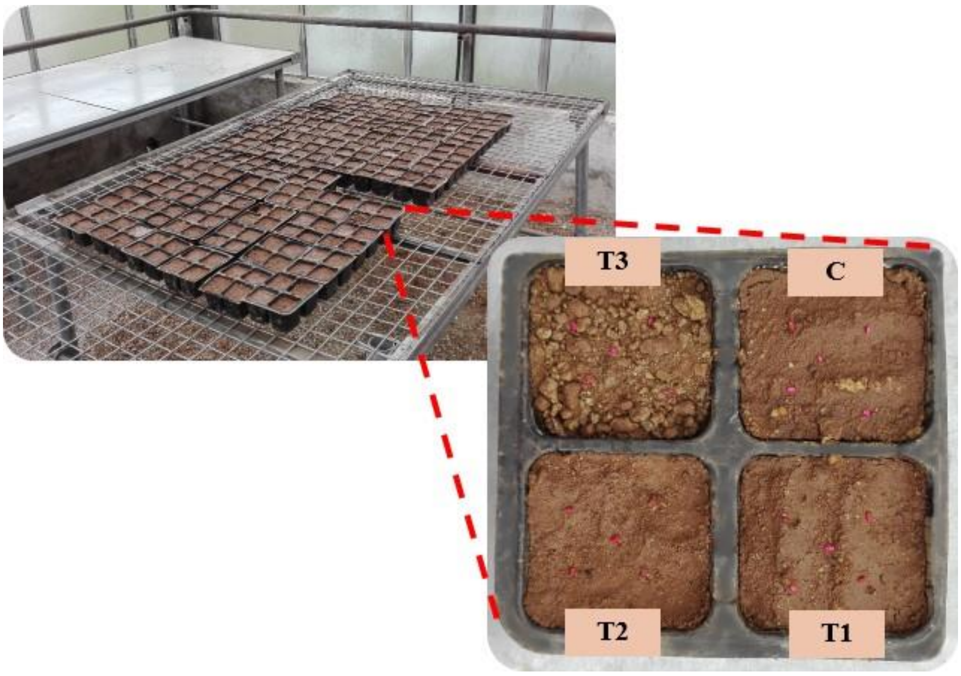


Figure 2.1. On the left, layout of experimental pots in the greenhouse. On the right, one pot with seeds of *Medicago sativa*. Treatments: C, non-treated soil (100% C_0); T1, 90% C_0 + 10% G; T2, 80% C_0 + 20% G; T3, 50% C_0 + 50% G. C_0: polluted soil. G: Gypsum mining spoil.

During the experiment, plant emergence and survival were monitored three times per week. At the end of the experiment all plants were collected, divided into shoots and roots, washed with distilled water, and dried in an oven (Memmert oven, Model 100-800) at 70ºC for 48 h. After stabilization at room temperature, we weighed biomass in a precision scale (GRAM PRECISION STA-310 S, ±0.001 g). We also collected 5 soil samples per treatment (C_f, $T1_f$, $T2_f$, and $T3_f$) and species.

2.2. PHE analyses in plants and soils

Plant samples were ground by means of a conventional mill. Due to the generalized low biomass produced, just one composite sample was prepared per treatment and species in most cases. Finally, they were digested with an acidic solution (HNO_3:H_2O_2, 1:1) in a microwave XP1500Plus (Mars®) (Sah and Miller, 1992) in order to measure PHE content in plant by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) (PE SCIEX ELAN-5000 spectrometer).

Soil samples were prepared (air-dried, sieved -2mm mesh- and finely ground with a soil mill, Retsch MM 400) to analyse their chemical properties (MAPA, 1994) and main PHEs (Cu, Zn, Cd, Sb, As and Pb). Total concentrations (T) were obtained through microwave-assisted (XP1500Plus, Mars®) acid digestion (HNO_3:HF, 3:1), water-soluble fraction (S) was extracted by distilled water from soil:water extract 1:5 according to Sposito et al. (1982), and bioavailable fraction (B) was extracted using 0.05 M EDTA (pH 7) as described by Quevauviller et al. (1998). PHEs were measured in all the extracted forms by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) (PE SCIEX ELAN-5000 spectrometer).

2.3. Data analysis

All statistical analyses were performed using R version 3.4.2 (R Core Team, 2017). We assessed the effects of gypsum mining spoil (G) on soil properties, PHE immobilization in soils, and on plant emergence, survival, and biomass production by applying generalized linear models (GLMs). To fit GLMs for seed emergence, plant survival and biomass, total/soluble/bioavailable PHEs in soils, soil pH and EC, the "stats" package was used (R Core Team, 2017). Model suitability was assessed by graphical exploration of the residuals (Zuur et al. 2010). After that, final models were fitted assuming gamma/gaussian distribution and inverse/identity-link function for soil properties, PHE immobilization in soils and biomass production. For plant emergence and total survival, we fitted GLMs assuming binomial distribution and logit-link function. We also used GLMs to evaluate the differences between the polluted and non-polluted soils. To evaluate differences among treatments on seedling survival rates over time, we used Cox proportional hazard models and, for data visualization, Kaplan-Meier curves (Bewick et al., 2004) using "survival" package v2.44-1.1 (Therneau, 2015). Pairwise comparisons between soil treatments in terms of soil properties, PHE immobilization in soils, and plant emergence, survival and growth were performed with Tukey's *post-hoc* tests using "multcomp" package (Hothorn et al., 2008). Graphs and confidence intervals were obtained with R "ggplot2" v3.1.1 (Wickham, 2016) and "sciplot" v1.1-1 (Morales, 2017). Mean values and standard deviation were calculated by using ddply function (R "plyr" package, v1.8.4; Wickham, 2011).

3. RESULTS

3.1. Plant performance and PHE uptake

3.1.1. Seed emergence

Our results show that in the polluted non-treated soils (C), seed emergence was totally inhibited, whereas it was promoted by the addition of gypsum mining spoil (G) at any proportion (T1, T2, T3), both for *Medicago sativa* (84 % in T1, 88 % in T2, and 100% in T3) and *Cynodon dactylon* (78 % in T1, 94 % in T2, and 100 % in T3), with no significant differences among treatments (Figure 2.2).

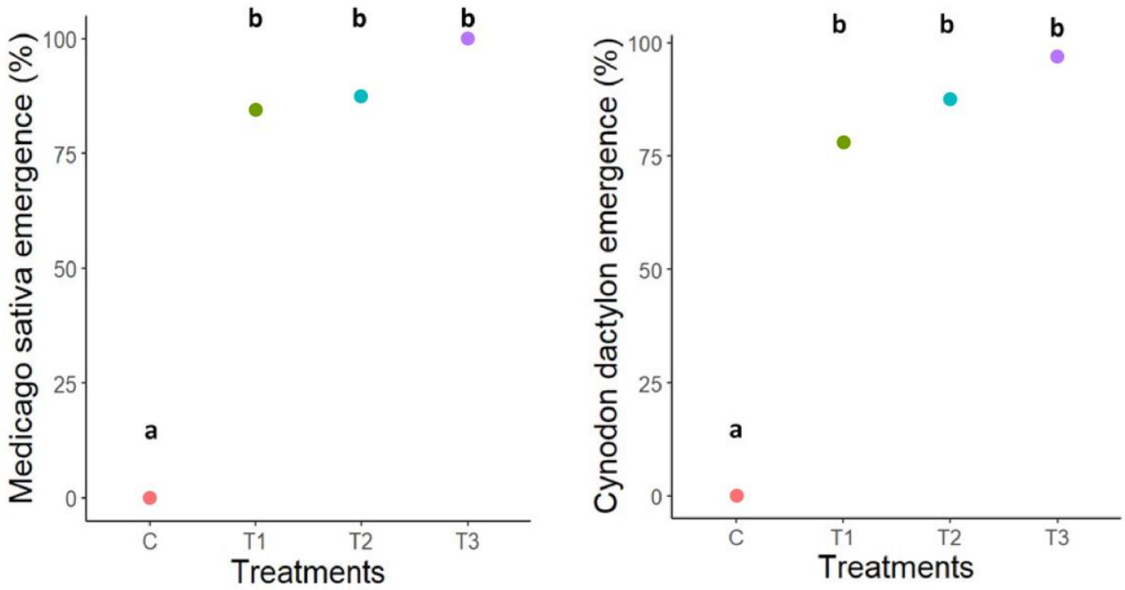


Figure 2.2. Percentage (mean) of plant emergence in experimental soils. Treatments: C, non-treated soil (100% C_0); T1, 90% C_0 + 10% G; T2, 80% C_0 + 20% G; T3, 50% C_0 + 50% G. C_0: polluted soil. G: Gypsum mining spoil. Different letters in the upper part of the figure indicate significant differences ($p<0.05$) for the *post-hoc* Tukey tests performed after the GLMs.

3.1.2. Survival and biomass production

M. sativa showed the highest survival rate in T3 treated soils (97 %), followed by T2 (64 %) and T1 (30 %), with statistically significant differences among all of them (Figure 2.3). On the other hand, *C. dactylon* registered high survival rates (above 90%) in all treated soils, with no statistically significant differences in any case (Figure 2.3). Results extracted from Kaplan-Meier survival curves and Cox proportional hazard models showed that plant survival across the experiment was rather stable for *M. sativa* in T3 and for *C. dactylon* in any treatment, while plant survival for *M. sativa* in T1 and T2 experienced a sharp decrease within the first 25 days and then got stabilized (Figure 2.3).

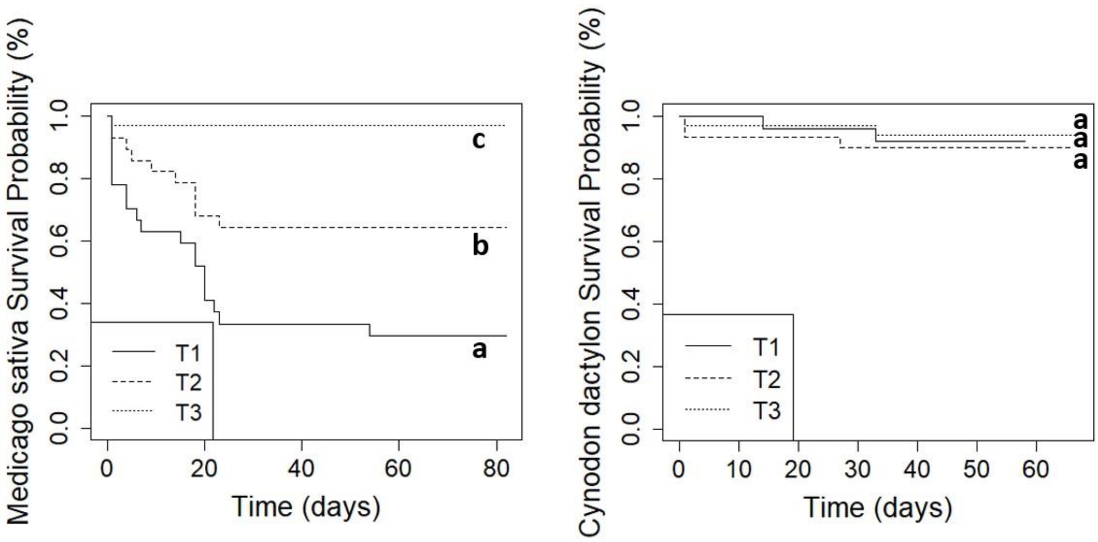


Figure 2.3. Kaplan-Meier survival curves representing plant survival rates along the experiment (in days) per treatment and species. The experiment lasted 82 days for Medicago sativa (on the left) and 67 days for Cynodon dactylon (on the right). Treatments: T1: 90% C_0 + 10% G; T2: 80% C_0 + 20% G; T3: 50% C_0 + 50% G. C_0: polluted soil. G: Gypsum mining spoil. Different letters represent statistically significant differences between treatments ($p < 0.05$).

The most effective treatments for biomass production (all components analyzed: shoot, root, and total biomass) were T2 and T3 for *C. dactylon* (no significant differences between them), and T3 for *M. sativa* (Figure 2.4). Seed emergence and growth only occurred in treated soils, and biomass production for *M. sativa* (any treatment) and *C. dactylon* in T1 was rather low in terms of shoot, root and total biomass (Figure 2.4).

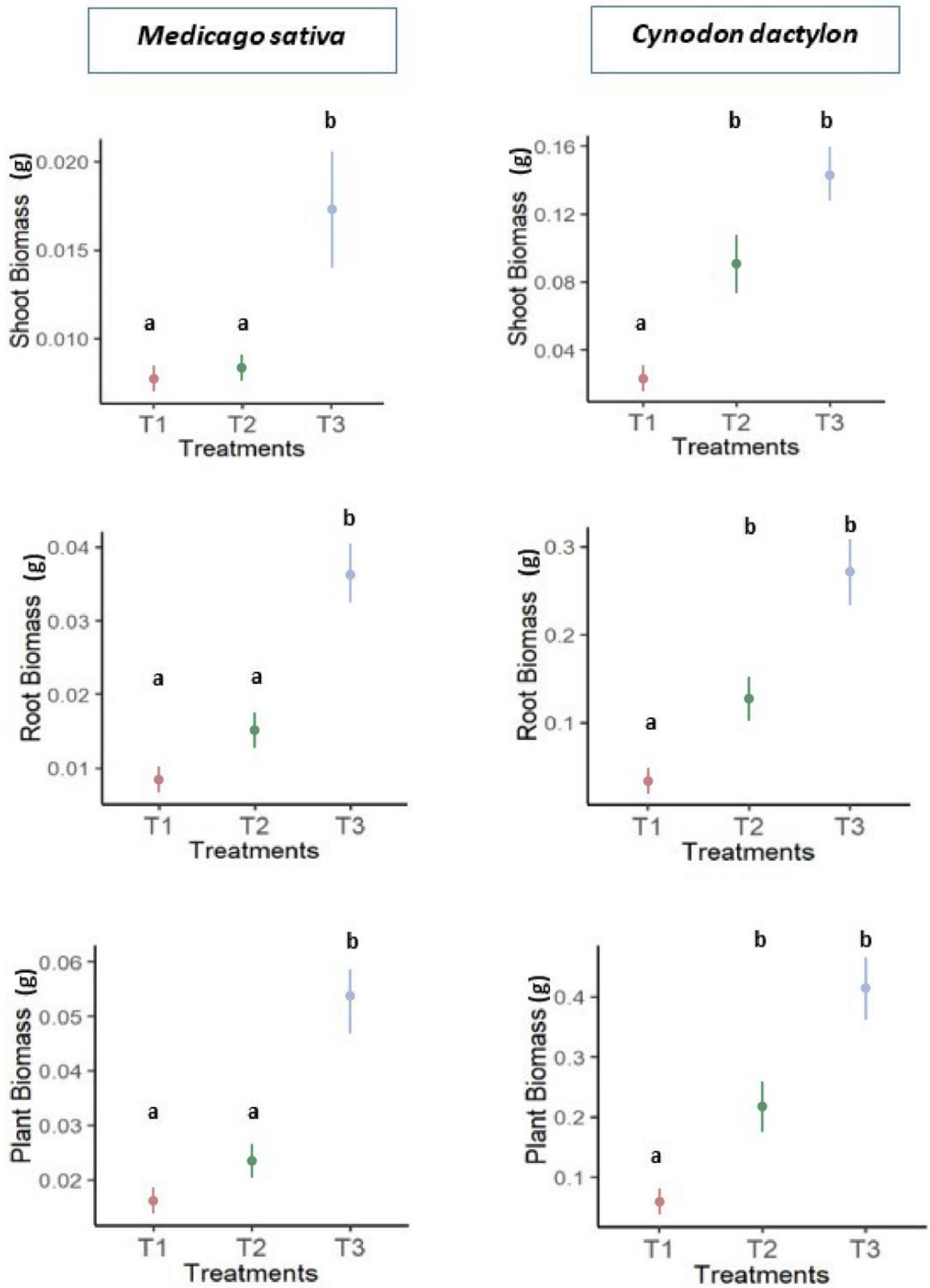


Figure 2.4. Shoot, root, and plant biomass (mean±SD in g) per treatment and species (*Medicago sativa* on the left and *Cynodon dactylon* on the right). Treatments: T1, 90% C_0 + 10% G; T2, 80% C_0 + 20% G; T3, 50% C_0 + 50% G. C_0: polluted soil. G: Gypsum mining spoil. Different letters represent statistically significant differences ($p<0.05$) for the *post-hoc* Tukey tests performed after the GLMs.

3.1.3. PHE uptake

As none of the seeds sown in the polluted non-treated soils (C) emerged, there was no PHE record for plant tissues in this case. The low biomass obtained for *M. sativa* in all treated soils (T1, T2 and T3) and for *C. dactylon* in T1 soils, compelled us to prepare only 1 mixed sample in these cases for chemical analyses; therefore, the concentrations are included as a single guiding value (Appendix 2.1). In our experiment, both species presented the lowest accumulation of PHEs (Appendix 2.1) in T3, followed by T2. Moreover, both species retained a great part of PHEs in their roots (Appendix 2.2). *C. dactylon* accumulated more than 80% of total Cu, Sb, As and Pb in roots in all the treated soils (similar values). *M. sativa* also registered the highest accumulation of those elements (and Cd) in roots, especially in T3 (less than 30% of PHEs were accumulated in shoots). Both *M. sativa* and *C. dactylon* accumulated Zn (and Cd in the case of *C. dactylon*) in similar quantities in roots and shoots.

3.2 . Soil properties and PHE content

The natural soils (Nat) collected in the unaffected area showed nearly neutral pH values (6.4 ± 0.4), low EC (0.09 ± 0.04 dS m^{-1}) and low concentrations of PHEs (Appendix 2.3). The polluted soils (C_0) collected for the experiment at the selected residual areas had a strong acidic pH (3.5 ± 0.1), high EC (2.76 ± 0.01 dS m^{-1}) and significantly higher total PHEs (Appendices 2.3 and 2.4) than Nat (with values more than 10 times higher in the case of As). Gypsum mining spoil (G) had nearly neutral pH values (7.5 ± 0.2), high EC (2.90 ± 0.04 dS m^{-1}) and negligible quantities of PHEs in all their forms (Appendices 2.3 and 2.4), except for soluble Sb, which was significantly higher than in Nat and C_0.

At the end of our experiment, non-treated soil samples (C_f, 0% G) still presented a strong acidic pH (3.8 ± 0.1) and high EC (2.24 ± 0.02 dS m^{-1}). In treated soils, the acidic pH was partially corrected in $T1_f$ (10% G) and $T2_f$ (20% G) (5.6 ± 0.2 and 6.4 ± 0.01, respectively) and neutralized (6.8 ± 0.1) in $T3_f$ (50% G). In terms of electrical conductivity (EC), the addition of G at any dose did not have a significant impact on salinity (EC > 2 dS m^{-1}), and the same happened for the cation exchange capacity (CEC, Appendix 2.5), which was even reduced (from 9.6 ± 1.0 cmol kg^{-1} in C_0, to 7.5 ± 0.6 cmol kg^{-1} in $T3_f$). Total calcium significantly rose with increasing doses of G, and the same happened for total sulphur, both being main components of gypsum mining spoil (Appendix 2.5).

With regard to PHEs, comparing initial non-treated soils (C_0) with final non-treated soils (C_f) (after having watered C_0 daily for 12 weeks and where no plant emergence occurred), the main PHEs lixiviated (leaching >50% of total Zn and Cd, and < 25% of total Cu, Sb and As), with the exception of Pb. As a result, when compared to C_0, total PHEs (Table 2.1) and their soluble (Table 2.2) fractions were significantly reduced in all the treated soils, especially in $T3_f$, except total Pb that was only reduced in $T3_f$, and soluble Sb that decreased in $T1_f$ but significantly increased in $T2_f$ and $T3_f$. On the other hand, the bioavailable fractions (Table 2.3) of Zn, Cd and As were reduced by G at any dose, especially in $T3_f$; nevertheless, bioavailable Cu was only reduced in $T3_f$, and bioavailable Sb and Pb only in $T1_f$.

Table 2.1. Mean values (±SD) of total content (mg kg^{-1}) of PHEs (Cu, Zn, Cd, Sb, As and Pb) present in non-treated soil samples before (C_0) and after the experiment (C_f) and in treated soil samples at the end of the experiment (C_f, $T1_f$, $T2_f$, $T3_f$). Letter "T" before PHEs refers to total content. Treatments: C, non-treated soil (100% C_0); T1, 90% C_0 + 10% G; T2, 80% C_0 + 20% G; T3, 50% C_0 + 50% G. C_0: polluted soil. G: Gypsum mining spoil. NGR (regional thresholds to declare potentially polluted soils for agricultural use in Andalusia, BOE 2015). Nat: Reference levels of total PHEs for unaffected soils within the Guadiamar Green Corridor). N: number of samples. Different letters (columns) represent statistically significant differences ($p < 0.05$) for the *post-hoc* Tukey tests performed after the GLMs. [1] <LOD = Under limit of detection.

Total PHEs in soil samples (*Cynodon dactylon*)

Sample	N	TCu	TZn	TCd	TSb	TAs	TPb
Nat	5	50.29± 22.25	81.08± 12.99	<LOD [1]	<LOD [1]	17.35± 7.63	46.69± 31.31
NGR	5	595	10000	25	90	36	275
C_0	5	103.50± 4.25 c	220.20± 11.45 c	0.77± 0.03 d	68.37± 4.56 c	198.53± 11.57 c	240.14± 17.71 ab
C_f	5	88.92± 3.55 b	69.80± 9.34 a	0.21± 0.04 a	67.24± 5.10 bc	155.36± 11.54 b	264.49± 14.65 b
$T1_f$	5	93.78± 4.77 b	150.50± 11.90 b	0.47± 0.04 bc	65.69± 3.62 bc	160.05± 10.70 b	264.31± 15.59 b
$T2_f$	5	88.96± 4.43 b	152.77± 3.12 b	0.49± 0.02 c	61.12± 1.75 b	149.45± 6.97 b	253.38± 8.73 ab
$T3_f$	5	77.65± 7.06 a	136.18± 13.05 b	0.42± 0.04 b	53.37± 4.62 a	125.13± 14.31 a	227.19± 28.41 a

Total PHEs in soil samples (*Medicago sativa*)

Sample	N	TCu	TZn	TCd	TSb	TAs	TPb
Nat	5	50.29± 22.25	81.08± 12.99	<LOD [1]	<LOD [1]	17.35± 7.63	46.69± 31.31
NGR	5	595	10000	25	90	36	275
C_0	5	103.50± 4.25 b	220.20± 11.45 c	0.77± 0.03 c	68.37± 4.56 c	198.53± 11.57 c	240.14± 17.71 b
C_f	5	97.42± 11.36 b	99.82± 10.04 a	0.39± 0.14 a	63.65± 7.02 bc	194.02± 18.68 bc	242.82± 45.87 b
$T1_f$	5	100.29± 6.46 b	225.60± 16.75 c	0.73± 0.03 c	61.32± 5.35 bc	185.53± 11.05 bc	230.98± 16.19 b
$T2_f$	5	90.21± 6.27 b	220.88± 14.88 c	0.68± 0.06 bc	55.77± 2.84 b	169.18± 9.69 b	208.39± 11.82 b
$T3_f$	5	72.24± 11.38 a	179.41± 32.05 b	0.54± 0.11 b	42.59± 7.99 a	132.35± 25.65 a	159.84± 31.64 a

Table 2.2. Mean values (±SD) of soluble PHEs (Cu, Zn, Cd, Sb, As and Pb) (mg kg^{-1}) in non-treated soil samples before (C_0) and after (C_f) the experiment and in treated soil samples ($T1_f$, $T2_f$, $T3_f$) at the end of the experiment. Letter "S" before PHEs refers to soluble fraction. Treatments: C, non-treated soil (100% C_0); T1, 90% C_0 + 10% G; T2, 80% C_0 + 20% G; T3, 50% C_0 + 50% G. C_0: polluted soil. G: Gypsum mining spoil. N: number of samples. Different letters (columns) represent statistically significant differences ($p < 0.05$) for the *post hoc* Tukey tests performed after the GLMs.

Soluble PHEs in soil samples (*Cynodon dactylon*)

Sample	N	SCu	SZn	SCd	SSb	SAs	SPb
C_0	5	123.72± 7.12 b	185.81± 16.84 c	0.540± 0.040 e	0.06± 0.01 b	0.12± 0.01 c	0.0400± 0.0100 b
C_f	5	3.38± 2.48 b	16.17± 9.31 c	0.140± 0.100 d	0.02± 0.00 a	0.05± 0.01 ab	0.0026± 0.0043 a
$T1_f$	5	0.16± 0.01 a	0.12± 0.05 b	0.009± 0.003 c	0.10± 0.01 c	0.04± 0.00 a	0.0001± 0.0001 a
$T2_f$	5	0.15± 0.02 a	0.06± 0.01 a	0.004± 0.000 b	0.16± 0.01 d	0.06± 0.00 b	0.0003± 0.0004 a
$T3_f$	5	0.11± 0.02 a	0.05± 0.03 a	0.002± 0.001 a	0.16± 0.02 d	0.06± 0.01 b	0.0004± 0.0006 a

Soluble PHEs in soil samples (*Medicago sativa*)

Sample	N	SCu	SZn	SCd	SSb	SAs	SPb
C_0	5	123.72± 7.12 c	185.81± 16.84 d	0.540± 0.040 c	0.06± 0.01 b	0.12± 0.01 c	0.0400± 0.0100 b
C_f	5	2.44± 1.41 b	8.34± 3.42 c	0.080± 0.020 b	0.02± 0.00 a	0.05± 0.01 b	0.0024± 0.0016 a
$T1_f$	5	0.13± 0.02 a	0.33± 0.15 b	0.013± 0.003 a	0.08± 0.02 b	0.03± 0.00 a	0.0028± 0.0010 a
$T2_f$	5	0.15± 0.01 a	0.08± 0.01 a	0.004± 0.001 a	0.17± 0.01 c	0.05± 0.00 b	0.0024± 0.0028 a
$T3_f$	5	0.11± 0.03 a	0.06± 0.02 a	0.002± 0.000 a	0.17± 0.01 c	0.05± 0.01 b	0.0002± 0.0001 a

Table 2.3. Mean values (±SD) of bioavailable PHEs (Cu, Zn, Cd, Sb, As and Pb) (mg kg^{-1}) in non-treated soil samples before (C_0) and after (C_f) the experiment and in treated soil samples at the end of the experiment ($T1_f$, $T2_f$, $T3_f$). Letter "B" before PHEs refers to bioavailable fraction. Treatments: C, non-treated soil (100% C_0); T1, 90% C_0 + 10% G; T2, 80% C_0 + 20% G; T3, 50% C_0 + 50% G. C_0: polluted soil. G: Gypsum mining spoil. N: number of samples. Different letters (columns) represent statistically significant differences (p < 0.05) for the *post hoc* Tukey tests performed after the GLMs.

Bioavailable PHEs in soil samples (*Cynodon dactylon*)

Sample	N	BCu	BZn	BCd	BSb	BAs	BPb
C_0	5	40.06± 1.36 b	166.87± 4.69 e	0.62± 0.02 c	0.54± 0.06 b	3.90± 0.18 d	0.55± 0.05 a
C_f	5	34.92± 3.75 ab	12.57± 3.71 a	0.17± 0.05 a	0.54± 0.21 b	1.47± 0.18 bc	0.46± 0.08 a
$T1_f$	5	35.83± 3.36 ab	73.34± 13.74 d	0.51± 0.05 b	0.17± 0.05 a	1.29± 0.18 b	0.39± 0.12 a
$T2_f$	5	37.33± 6.06 b	45.92± 8.33 c	0.51± 0.07 b	0.45± 0.09 b	1.75± 0.32 c	0.55± 0.16 a
$T3_f$	5	28.72± 5.65 a	31.25± 5.80 b	0.42± 0.06 b	0.51± 0.08 b	0.68± 0.14 a	0.47± 0.02 a

Bioavailable PHEs in soil samples (*Medicago sativa*)

Sample	N	BCu	BZn	BCd	BSb	BAs	BPb
C_0	5	40.06± 1.36 b	166.87± 4.69 c	0.62± 0.02 c	0.54± 0.06 b	3.90± 0.18 c	0.55± 0.05 bc
C_f	5	35.84± 3.48 b	17.18± 10.46 a	0.22± 0.12 a	0.39± 0.05 b	1.50± 0.33 b	0.43± 0.06 ab
$T1_f$	5	37.32± 3.32 b	55.69± 5.93 b	0.53± 0.04 c	0.23± 0.04 a	1.50± 0.33 b	0.40± 0.05 a
$T2_f$	5	40.13± 0.71 b	57.71± 6.53 b	0.58± 0.02 c	0.49± 0.01 b	1.99± 0.11 b	0.64± 0.19 c
$T3_f$	5	22.49± 6.38 a	25.22± 7.52 a	0.35± 0.09 b	0.42± 0.15 b	0.63± 0.12 a	0.54± 0.07 bc

The ratio between soluble and total PHEs (Appendix 2.6) decreased with the addition of G for Cu, Zn, Cd and Pb, especially in $T3_f$, and for As, especially in $T1_f$. On the contrary, this ratio for Sb progressively increased in $T2_f$ and $T3_f$, and was maintained in $T1_f$. Finally, the ratio between bioavailable and total PHEs (Appendix 2.7) was reduced in $T3_f$ for Cu (*M. sativa*), in $T2_f$ and $T3_f$ for Zn, in all treatments for As, and only in $T1_f$ for Sb. For Cd and Pb, it depended on the species and no clear results were registered in this regard, observing an increase of the ratio for Pb in the soils where *M. sativa* grew and an increase for Cd in the soils with *C. dactylon*.

4. DISCUSSION

4.1. Plant performance and PHE uptake

4.1.1. Seed emergence

In this *ex-situ* experiment, none of the species could emerge in the polluted non-treated soils (C) as a result of the strong surface crust, the acidic pH, and the high concentration of salts and PHEs, resulting in a lack of available essential nutrients. In fact, Delgado-Caballero et al. (2017) found that low pH together with high concentrations of Cd, Pb and Zn could have increased the solubility and toxicity of these elements, inhibiting seed germination (Undersander et al., 2011; Tiller and Merry, 1981). In this context, gypsum mining spoil had a crucial role to reinitiate plant colonization, which had been arrested for 20 years.

Thus, after having amended polluted soils with gypsum mining spoil at any proportion, high emergence rates were observed for both model species (*Medicago sativa* and *Cynodon dactylon*). This could be mainly due to the improvement of soil properties, including pH increase (with values above 4), what would have promoted the reduction of PHE toxicity by decreasing their bioavailability, being of particular importance the reduction of As, Cd, and Pb bioavailable fractions (Kabata-Pendias, 2011; Delgado-Caballero et al., 2017). Seed germination and emergence are the very first and crucial stages in plant cycle (Fenner & Thompson, 2005); however, after emergence, survival and growth must be monitored to evaluate the effectiveness of this amendment.

4.1.2. Survival and growth

In our experiment, gypsum mining spoil proved to favour plant survival and growth. Madejón et al. (2006) also saw that the application of inorganic amendments to polluted soils enabled seedling growth under similar conditions. Moreover, both survival and growth were higher in the treatments with a greater proportion of gypsum mining spoil. In fact, plant survival decreased (sharply in the case of *M. sativa*) where a low proportion of amendment was applied. Similarly, seedling growth was low for both species in the treatment with the lowest dose of gypsum mining spoil. These could have been due to the excessive concentration of PHEs accumulated in plant tissues, causing not only a limited growth but also plant death (Chibuike and Obiora, 2014; Kabata-Pendias, 2011). In this sense, these negative effects could have been especially important in the case of arsenic, which was accumulated at phytotoxic levels (Kabata-Pendias, 2011) and whose deleterious effects have been previously reported (Madejón et al. 2002; Kabata-Pendias, 2011; Kumpiene et al., 2019).

The differential survival of the two model species highlights the importance of using tolerant species to remediate heavily polluted soils (Nirola et al. 2016).

4.1.3. PHE uptake

According to our results, the addition of gypsum mining spoil (calcium-rich amendment, Ballesteros et al. 2018) limited PHE uptake, especially at the highest dose of amendment, probably due to the protective action of calcium (Carbonell et al., 1998). As calcium is one of the main antagonistic elements against some PHE sorption and metabolism (i.e. Pb), its presence in the soil solution enhances the selectivity in the uptake of metabolic important elements against unwanted ones (Kabata-Pendias, 2011). Moreover, sulphur may have also reduced the availability of some PHEs such as arsenic (Kabata-Pendias, 2011). Being the incorporation of PHEs to the food web and their biomagnification a great environmental concern, these protective effects offered by the amendment are of paramount relevance (Gall et al. 2015).

After PHE uptake, both species retained a great part of PHEs in their roots. *C. dactylon* accumulated most Cu, Sb, As and Pb in their roots at any dose of gypsum mining spoil, suggesting this species could act as phytostabilizer (Abou-Shanab et al., 2007; Sekabira et al., 2011). *M. sativa* mostly accumulated Cu, Cd, Sb, As and Pb in its roots, especially in the soils with the highest dose of amendment, indicating that this species could be dose-dependent. Nevertheless, the addition of gypsum mining spoil was less effective in immobilizing Zn in roots for both species (and Cd too for *C. dactylon*), making this element more bioavailable for herbivores. Hence, our results suggest that gypsum mining spoil addition, reduces PHE uptake and retains them mainly in plant roots, reducing the potential risk for the ecosystem (Freitas et al., 2004; Kumpiene et al., 2019).

4.2. Soil properties and PHE content

The polluted soils of this experiment are characterized by a strong acidic pH, salinity and high concentrations of PHEs. The addition of gypsum mining spoil increased pH towards neutrality, but no change was observed on EC values, probably because gypsum solubility may have resulted in more available salts (Casas-Castro and Casas-Barba, 1999).

The addition of gypsum mining spoil, especially at its highest dose, produced a dilution effect of total PHEs. Nevertheless, total concentrations of Cu, Cd and Zn in treated soils were still substantially higher than the background concentrations in the surrounding unaffected soils (Nat). Otherwise, total concentrations of As far exceeded the regional threshold for agricultural soils (NGR, BOE 2015) (36 mg kg^{-1}), and the concentrations of Sb and Pb were still too close (Sb= 90 and Pb= 275 mg kg^{-1}).

Leaching was very significant for the most mobile elements (Zn and Cd) in non-treated soils when comparing initial and final (at the end of the experiment) values of PHEs, posing a high environmental risk (Page et al., 2014). On the contrary, the addition of gypsum mining spoil promoted PHE immobilization in soil. Soluble and bioavailable forms of Cu, Zn and Cd were reduced with the addition of the amendment due to pH rise (Hooda, 2010; Kabata-Pendias, 2011). Moreover, the presence of gypsum mining spoil could have promoted the formation of Al-hydroxy polymers in the polluted soil, immobilizing them (Garrido et al., 2005). As well, Ca and S seem to have significantly reduced Zn solubility (Kabata-Pendias, 2011). Soluble As did not increased along with pH as it could be expected (Hooda, 2010), but decreased with the lowest dose of gypsum mining spoil. Moreover, bioavailable As decreased in all treated soils, especially with the highest dose, probably because arsenate toxicity diminished as a result of its adsorption by iron hydroxysulphates (O'Neill, 1995). Therefore, gypsum mining spoil proved its effectiveness in reducing the toxicity of Cu, Zn, Cd and As. On the contrary, its effect on Sb and Pb was controversial. Bioavailable Sb only descended with the lowest dose of the amendment and soluble Sb increased with the addition of higher doses. This fact could have been promoted by both the presence of organic matter (Nakamaru and Martín-Peinado, 2017) as a result of seed emergence and growth, and by a significant addition of soluble Sb present in the amendment. Lead bioavailable fraction did not present any change in most of the amended soils. Nevertheless, according to Pb bioavailability ratio, Pb bioavailability increased in the treatments with higher doses of gypsum mining spoil. However, Pb solubility was reduced with any dose of the amendment; in this sense, it has been reported that calcium ions could reduce Pb bioavailability by direct competence (Li et al., 2014), that sulphate ions could promote the precipitation of Pb as anglesite ($PbSO_4$) (Rehman et al., 2017), and that the formation of Al-hydroxy polymers could promote Pb sorption (Garrido et al., 2003).

According to our results, the potential toxicity of some PHEs seem to depend on the dose of gypsum mining spoil; consequently, the right dose should be carefully studied, especially where complex mixtures of PHEs are present (Clemente et al., 2012; Madejón et al., 2018; Simón et al., 2010).

4.3. Applicability

PHE fixation is considered among the most effective treatments for a wide range of polluted soils (Vangronsveld and Cunningham, 1998), since chemical immobilization prevents the transport of pollutants into deeper soil layers and groundwater (Querol et al 2006). In this sense, considering our result, gypsum mining spoil could be a promising amendment material for the remediation of PHE-polluted soils.

Compared to other inorganic amendments frequently used in remediation of soils polluted with PHEs such as lime (Clemente et al., 2003, 2006; Madejón et al., 2006b; Pérez-de-Mora et al., 2006), Wallace and Wallace (1995) observed that gypsum was more effective in improving acid soils, especially because gypsum can reach the subsoil where lime cannot penetrate. On top of that, and contrary to organic amendments (McGrath et al., 1995), gypsum mining spoil has negligible quantities of PHEs and no pathogens.

The combination of gypsum mining spoil with organic amendments could improve its potential in soil remediation. In this sense, Alvarenga et al. (2008) showed that organo-mineral amendments could decreased Cu, Pb, and Zn mobile fractions in mining soils, and Jiménez-

Moraza et al. (2006) observed the immobilization of Zn and Cd after the application of sugar-beet lime.

Gypsum is a mineral in global demand (Herrero et al., 2013), and its extraction through mining produces great amounts of gypsum mining spoil (Ballesteros et al. 2018). Hence, the use of this waste as amendment would help to overcome this environmental issue. The costs associated to the use and management of gypsum mining spoil are usually low, and as no further processing is required, the expenses of this waste material are mainly related to its transport, what should be reflected in reduced market prices in comparison to other gypsum derived products such as phosphogypsum (Campbell et al., 2006). In this vein, further studies should be conducted on the applicability of this material, including the economic viability of its commercialisation as amendment for environmental and agricultural applications.

5. CONCLUSIONS

The presence of gypsum mining spoil (G) at any dose, and especially at 50%, enhanced seed emergence, biomass production (growth) and survival rates in our model species. This is directly related to the reduction of soil acidity, soil crust formation and of the availability of Potentially Harmful Elements (PHEs).

In comparison to other soil amendments, the use of G would bring the following benefits: i) Its relatively low solubility rates make it a great source of calcium over time; ii) It has negligible quantities of PHEs and no pathogens described; iii) It does not need to be processed prior to its application, reducing time and costs.

The next step would be to test the effectiveness of G in the field and assess its effects on a wider range of native plant species. As well, further studies should be conducted in order to enhance the potential of G as amendment of polluted soils. In this vein, it would be interesting to test a new mixed amendment containing G and an organic matter-rich amendment (i.e. olive mill waste compost, vermicompost, manure, etc.).

In summary, our findings suggest that G could be used as an effective amendment, when applied in the right dose, to recover soils polluted with PHEs and their associated vegetation. Moreover, the use of G as soil amendment, will simultaneously help mitigate two urgent environmental issues: (i) the sustainable management of mining waste material; and (ii) the effective remediation of degraded soils polluted with PHEs.

6. ACKNOWLEDGEMENTS

The authors would like to thank KNAUF-GmbH for the financial support. We also would like to thank Miguel Ballesteros, Cristian Turpin Torrano and IFAPA staff, especially Pepe Vilchez, for their help during the experiment, and IFAPA institute for lending us their facilities.

This work was supported by the Research Project RTI 2018-094327-B-I00 (Ministry of Science, Innovation and Universities).

Helena García Robles was financed by Tatiana-Pérez-de-Guzmán-el-Bueno Foundation PhD grant Programme 2016.

7. REFERENCES

Abou-Shanab, R., Ghanem, N., Ghanem, K., Al-Kolaibe, A., 2007. Phytoremediation Potential of Crop and Wild Plants for Multi-metal Polluted Soils. Res. J. Agric. & Biol. Sci., 3(5): 370-376.

Ahmed, A., Ugai, K., Kamei, T., 2011. Investigation of recycled gypsum in conjunction with waste plastic trays for ground improvement. Constr. Build. Mater., 25: 208-217. https://doi.org/10.1016/j.conbuildmat.2010.06.036

Al-Farajat, M., 2009. Role of Gypsum Wastes in Polluting Groundwater and Enhancing Eutrophication in Coasts of Aqaba. Jordan J. Civ. Eng., 3 (3): 213-228.

Alvarenga, P., Palma, P., Gonçalves, A.P., Baião, N., Fernandes, R.M., de Varennes, A., Vallini, G., Duarte, E., Cunha-Queda, A.C., 2008. Assessment of chemical, biochemical and ecotoxicological aspects in a mine soil amended with sludge of either urban or industrial origin. Chemosphere, 72: 1774-1781. https://doi.org/10.1016/j.chemosphere.2008.04.042

Ballesteros, M., Cañadas, E., Marrs, R., Foronda, A., Martín-Peinado, F., Lorite, J., 2017. Restoration of Gypsicolous Vegetation on Quarry Slopes: Guidance for Hydroseeding under

Contrasting Inclination and Aspect. Land Degrad. Dev., 28(7): 2146-2154. https://doi.org/10.1002/ldr.2740

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

Bewick, V., Cheek, L., Ball, J., 2004. Statistics review 12: Survival analysis. Crit. Care., 8(5): 389 - 394. https://doi.org/10.1186/cc2955

Boletín Oficial del Estado (BOE) 38, 2015. Decreto 18/2015 por el que se aprueba el reglamento que regula el régimen aplicable a los suelos contaminados. Consejería de Medioambiente y Ordenación del Territorio, 38: 28-64.

Campbell, C. G., Garrido, F., Illera, V., García-González, M. T., 2006. Transport of Cd, Cu and Pb in an acid soil amended with phosphogypsum, sugar foam and phosphoric rock. Appl. Geochem., 21(6): 1030-1043. https://doi.org/10.1016/j.apgeochem.2006.02.023

Carbonell, A.A., Aarabi, M.A., DeLaune, R.D., Gambrell, R.P., Patrick, W.H.J., 1998. Bioavailability and uptake of arsenic by wetland vegetation: Effects on plant growth and nutrition. J. Environ. Sci. Health, Part A, 33(1): 45-66. https://doi.org/10.1080/10934529809376717

Casas-Castro, A., Casas-Barba, E., 1999. El análisis de suelo-agua-planta y su aplicación en la nutrición de los cultivos hortícolas en la zona del sureste peninsular. Caja Rural De Almería.

Chandara, C., Azizlia, K.A.M., Ahmad, Z.A., Sakai, E., 2009. Use of waste gypsum to replace natural gypsum as set retarders in Portland cement. J. Waste Manag., 29: 1675-1679. https://doi.org/10.1016/j.wasman.2008.11.014

Chen, L., Dick, W.A., 2011. Gypsum as an agricultural amendment. The Ohio State University Extension, Ohio, Bulletin 945.

Chibuike, G.U., Obiora, S.C., 2014. Heavy Metal Polluted Soils: Effect on Plants and Bioremediation Methods. Appl. Environ. Soil Sci., 2014. https://doi.org/10.1155/2014/752708

Clemente, R., Walker, D.J., Roig, A., Bernal, M.P., 2003. Heavy metal bioavailability in a soil affected by mineral sulphides contamination following the mine spillage at Aznalcóllar (Spain). Biodegradation, 14: 199-205. https://doi.org/10.1023/A:1024288505979

Clemente, R., Almela, C., Bernal, M.M., 2006. A remediation strategy based on active phytoremediation followed by natural attenuation in a soil polluted by pyrite waste. Environ. Pollut., 143: 397-406. https://doi.org/10.1016/j.envpol.2005.12.011

Clemente, R., Walker, D.J., Pardo, T., Martínez-Fernández, D., Bernal, M.P., 2012. The use of a halophytic plant species and organic amendments for the remediation of a trace elements-polluted soil under semi-arid conditions. J. Hazard. Mater., 223-224: 63-71. http://doi.org/10.1016/j.jhazmat.2012.04.048

CMA (Consejería de Medio Ambiente), 2003. Ciencia y restauración del río Guadiamar. PICOVER 1998-2002. Junta de Andalucía, Sevilla, Spain.

DalCorso, G., Manara, A., Piasentin, S., Furini, A., 2014. Nutrient metal elements in plants. Metallomics, 6(10): 1770-1788. https://doi.org/10.1039/c4mt00173g

Delgado-Caballero, M.D.R., Alarcon-Herrera, M.T., Valles-Aragon, M.C., Melgoza-Castillo, A., Ojeda-Barrios, D.L., Leyva-Chavez, A., 2017. Germination of *Bouteloua dactyloides* and *Cynodon dactylon* in a multi-polluted soil. Sustainability, 9(1): 81. https://doi.org/10.3390/su9010081

FAO and ITPS, 2015. Status of the World's Soil Resources (SWSR) – Main Report. Food and Agriculture Organization of the United Nations and Intergovernmental Technical Panel on Soils, Rome, Italy.

Fenner, M.K., Thompson, K., 2005. The ecology of seeds. Cambridge University Press.

Flores-Cáceres, M.L., 2013. Caracterización de los mecanismos de respuesta antioxidante de *Medicago sativa* a metales pesados. Madrid: Universidad Autónoma de Madrid, 2013. Available at: http://hdl.handle.net/10486/14216

Franzen, D., Rehm, G., Gerwing, J., 2006. Effectiveness of gypsum in the North-Central Region of the U.S. NDSU Extension Service, North Dakota.

Freitas, H., Prasad, M.N.V., Pratas, J., 2004. Plant community tolerant to trace elements growing on the degraded soils of Saõ Domingos mine in the south east of Portugal: environmental implications. Environ. Int., 30: 65-72. https://doi.org/10.1016/S0160-4120(03)00149-1

Gall, J.E., Boyd, R.S., Rajakaruna, N., 2015. Transfer of heavy metals through terrestrial food webs: a review. Environ. Monit. Assess., 187: 201. https://doi.org/10.1007/s10661-015-4436-3

García-Carmona, M., Romero-Freire, A., Sierra-Aragón, M., Martínez-Garzón, F.J., Martín-Peinado, F.J., 2017. Evaluation of remediation techniques in soils affected by residual contamination with heavy metals and arsenic. J. Environ. Manage., 191: 228-236. https://doi.org/10.1016/j.jenvman.2016.12.041

Garrido, F., Illera, V., Vizcayno, C., García-González, M.T., 2003. Evaluation of industrial by-products as soil acidity amendments: chemical and mineralogical implications. Eur. J. Soil Sci., 54(2): 411-422. https://doi.org/10.1046/j.1365-2389.2003.00522.x

Garrido, F., Illera, V., García-González, M.T., 2005. Effect of the addition of gypsum- and lime-rich industrial by-products on Cd, Cu and Pb availability and leachability in metal-spiked acid soils. Appl. Geochem., 20: 397-408. https://doi.org/10.1016/j.apgeochem.2004.08.001

Greyson, J., 2007. An economic instrument for zero waste, economic growth and sustainability. J. Clean. Prod., 15(13-14): 1382-1390. https://doi.org/10.1016/j.jclepro.2006.07.019

Herrero, M.J., Escavy, J.I., Bustillo, M., 2013. The Spanish building crisis and its effect in the gypsum quarry production (1998–2012). Resour. Policy, 38(2): 123-129. https://doi.org/10.1016/j.resourpol.2013.02.005

Higueras, P.L., Sáez-Martínez, F.J., Reyes-Bozo, L, 2016. Characterization and remediation of contamination: the influences of mining and other human activities. Environ. Sci. Pollut. Res., 23: 5997-6001. https://doi.org/10.1007/s11356-016-6388-8

Hooda, P., 2010. Trace Elements in Soils, first ed. John Wiley and Sons Ltd, Chichester, West Sussex.

Hothorn, T., Bretz, F., Westfall, P., 2008. Simultaneous Inference in General Parametric Models. Biom. J., 50(3): 346-363. https://doi.org/10.1002/bimj.200810425

Illera, V., Garrido, F., Serrano, S., García-González, M.T., 2004. Immobilization of the heavy metals Cd, Cu and Pb in an acid soil amended with gypsum- and lime-rich industrial by-products. Eur. J. Soil Sci., 55: 135–145. https://doi.org/10.1046/j.1365-2389.2003.00583.x

Jiménez-Moraza, C., Iglesias, N., Palencia, I., 2006. Application of sugar foam to a pyrite-polluted soil. Minerals Engineering, 19(5): 399-406. https://doi.org/10.1016/j.mineng.2005.10.011

Kabata-Pendias, A., Pendias, H., 2000. Trace Elements in Soils and Plants, third ed. CRC Press, Boca Raton.

Kabata-Pendias, A., 2011. Trace Elements in Soils and Plants, fourth ed. CRC Press, Boca Raton.

Kumpiene, J., Antelo, J., Brännvall, E., Carabante, I., Ekc, K., Komárek, M., Söderbergc, C., Wårell, L., 2019. In situ chemical stabilization of trace element-polluted soil – Field demonstrations and barriers to transition from laboratory to the field – A review. Appl. Geochem., 100: 335-351. https://doi.org/10.1016/j.apgeochem.2018.12.003

Madejón, P., Murillo, J.M., Marañón, T., Cabrera, F., López, R., 2002. Bioaccumulation of As, Cd, Cu, Fe and Pb in wild grasses affected by the Aznalcóllar mine spill (SW Spain). Sci. Total Environ., 290: 105-120. https://doi.org/10.1016/s0048-9697(01)01070-1

Madejón, E., Pérez-de-Mora, A., Felipe, E., Burgos, P., Cabrera, F., 2006. Soil amendments reduce trace element solubility in a polluted soil and allow regrowth of natural vegetation. Environ. Pollut., 139: 40-52. https://doi.org/10.1016/j.envpol.2005.04.034

Madejón, P., Domínguez, M.T., Madejón, E., Cabrera, F., Marañón, T., Murillo-Carpio, J.M., 2018. Soil-plant relationships and contamination by trace elements: A review of twenty years of experimentation and monitoring after the Aznalcóllar (SW Spain) mine accident. Sci. Total Environ., 625: 50-63. https://doi.org/10.1016/j.scitotenv.2017.12.277

MAPA, 1994. Métodos oficiales de análisis. Tomo III: Métodos oficiales de análisis de suelos y aguas para el riego. Ministerio de Agricultura, Pesca y Alimentación. Servicio de Publicaciones, Madrid, pp. 205-285.

Martín-Peinado, F.J., Romero-Freire, A., García-Fernández I., Sierra-Aragón, M., Ortiz-Bernad, I., Simón-Torres, M., 2015. Long-term contamination in a recovered area affected by a mining spill. Sci. Total Environ., 514: 219-223. https://doi.org/10.1016/j.scitotenv.2015.01.102

McGrath, S.P., Chaudri, A.M., Giller, K.E., 1995. Long-term effects of metals in sewage sludge on soils, microorganisms and plants. J. Ind. Microbiol., 14: 94-104. https://doi.org/10.1007/BF01569890

Morales, M., 2017. Sciplot: Scientific Graphing Functions for Factorial Designs. Available at : https://cran.r-project.org/web/packages/sciplot/index.html

Muyessar, T., Linsheng Y., 2016. Trace Elements Contamination and Human Health Risk Assessment in Drinking Water from the Agricultural and Pastoral Areas of Bay County, Xinjiang, China. Int. J. Environ. Res. Public Health, 13(10): 938. https://doi.org/10.3390/ijerph13100938

Nakamaru, Y.M., Martín-Peinado, F.J., 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

Nirola, R., Megharaj, M., Beecham, S., Aryal, R., Thavamani, P., Vankateswarlu, K., Saint, C., 2016. Remediation of metalliferous mines, revegetation challenges and emerging prospects in semi-arid and arid conditions. Environ. Sci. Pollut. Res., 23(20): 20131-20150. https://doi.org/10.1007/s11356-016-7372-z

Nworie, O.E., Qin, J., Lin, C., 2019. Trace Element Uptake by Herbaceous Plants from the Soils at a Multiple Trace Element-Polluted Site. Toxics, 7(1): 3. https://doi.org/10.3390/toxics7010003

O´Neill, P., 1995. Arsenic, in: Alloway, B.J., (Ed.) Heavy metals in soils, second ed. Blackie Academic and Professional, Glasgow, UK.

Page, K., Harbottle, M.J., Cleall, P.J., Hutchings, T.R., 2014. Heavy metal leaching and environmental risk from the use of compost-like output as an energy crop growth substrate. Sci. Total Environ., 487: 260-271. https://doi.org/10.1016/j.scitotenv.2014.04.021

Payá-Pérez, A., Rodríguez-Eugenio, N., 2018. Status of local soil contamination in Europe: Revision of the indicator 'Progress in the management Polluted Sites in Europe'. EUR 29124 EN, Publications Office of the European Union, Luxembourg.

Pérez-de-Mora, A., Madejón, E., Burgos, P., Cabrera, F., 2006. Trace element availability and plant growth in a mine-spill-polluted soil under assisted natural remediation: II. Plants. Sci. Total Environ., 363: 38- 45. https://doi.org/10.1016/j.scitotenv.2005.10.016

Pilon-Smits, E., 2005. Phytoremediation. Annu. Rev. Plant Biol., 56: 15-39. http://doi.org/10.1146/annurev.arplant.56.032604.144214

Querol, X., Alastuey, A., Moreno, N., Alvarez-Ayuso, E., García-Sánchez, A., Cama, J., Simón, M., 2006. Immobilization of heavy metals in polluted soils by the addition of zeolitic material synthesized from coal fly ash. Chemosphere, 62(2): 171-180. https://doi.org/10.1016/j.chemosphere.2005.05.029

Quevauviller, P., Lachica, M., Barahona, E., Gomez, A., Rauret, G., Ure, A., Muntau, H., 1998. Certified reference material for the quality control of EDTA-and DTPA-extractable trace metal contents in calcareous soil (CRM 600). Fresen. J. Anal. Chem., 360 (5): 505-511. https://doi.org/10.1007/s002160050750

R Core Team, 2017. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. Available at: https://www.r-project.org.

Rehman, M.Z., Rizwan, M., Ali, S., Sabir, M., Sohail, M.I., 2017. Contrasting Effects of Organic and Inorganic Amendments on Reducing Lead Toxicity in Wheat. Bull. Environ. Contam. Toxicol, 99(5): 642-647. https://doi.org/10.1007/s00128-017-2177-4

Rodríguez-Eugenio, N., McLaughlin, M., Pennock, D., 2018. Soil Pollution: a hidden reality. Rome, FAO, 142.

Sah, R.N., Miller, R.O., 1992. Spontaneous reaction for acid dissolution of biological tissue in closed vessels. Anal. Chem., 64: 230-233. https://doi.org/10.1021/ac00026a026

Sekabira, K., Oryem-Origa, H., Mutumba, G., Kakudidi, E., Basamba, T. A., 2011. Heavy metal phytoremediation by *Commelina benghalensis* (L) and *Cynodon dactylon* (L) growing in Urban stream sediments. Int. J. Plant Physiol. Biochem., 3(8): 133-142.

Shainberg, I., Sumner, M.E., Miller, W.P., Farina, M.P.W., Pavan, M.A., Fey M.Y., 1989. Use of Gypsum on Soils: A Review. In: Stewart, B.A., (Eds), Advances in Soil Science. Advances in Soil Science. Springer, New York, NY, v9. https://doi.org/10.1007/978-1-4612-3532-3_1

Sherene, T., 2010. Mobility and transport of heavy metals in polluted soil environment. Biol. Forum, 2(2): 112-121.

Simón, M., Diez, M., González, V., García, I., Martín, F., De Haro, S., 2010. Use of liming in the remediation of soils polluted by sulphide oxidation: A leaching-column study. J. Hazard. Mater., 180(1-3): 241-246. https://doi.org/10.1016/j.jhazmat.2010.04.020

Sposito, G., Lund, L.J., Chang, A.C., 1982. Trace metal chemistry in arid-zone field soils amended with sewage sludge. I: fractionation of Ni, Cu, Zn, Cd and Pb in solid phases. Soil Sci. Soc. Am. J., 46: 260-264. https://doi.org/10.2136/sssaj1982.03615995004600020009x

Therneau, T., 2015. A Package for Survival Analysis in S. R Package Version 2.44-1.1. Available at: https://CRAN.R-project.org/package=survival

Tiller, K.G., Merry, R.H., 1981. Copper pollution of agricultural soils, in: Loneragan, J. F., Robson, A. D., Graham, R. D., (Eds.), Copper in Soils and Plants. Academic Press, Australia, pp. 119.

Toma, M.P.T., Sumner, M.E., Weeks, G., Saigusa, M., 1999. Long-term Effects of Gypsum on Crop Yield and Subsoil Chemical Properties. Soil Sci. Soc. Am. J., 63: 891-895. https://doi.org/10.2136/sssaj1999.634891x

Undersander, U., Hall, H.M., Vassalotti, P., Cosgrove, D., 2011. Alfalfa germination and growth. National alfalfa and forage alliance (NAFA), University of Wisconsin-Extension, Cooperative Extension, Wisconsin.

U.S. Geological Survey, 2019. Mineral commodity summaries 2019: U.S. Geological Survey, 74-75. https://doi.org/10.3133/70202434

Vangronsveld, J., Cunningham, S.D., 1998. Metal-contaminated soils. In situ inactivation and phytorestoration. R.G. Landes Company, Georgetown, TX.

Wallace, A., Wallace, G.A., 1995. Gypsum is almost a universal soil amendment, Soil amendments and amendment technologies, Wallace Laboratories, El Segundo.

Wickham, H., 2011. The Split-Apply-Combine Strategy for Data Analysis. R Package Version 1.8.4. J. Stat. Softw., 40(1): 1-29. https://doi.org/10.18637/jss.v040.i01

Wickham, H., 2016. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York.

Zuur, A.F., Ieno, E.N., Elphick, C.S., 2010. A protocol for data exploration to avoid common statistical problems. Methods Ecol. Evol, 1: 3-14. https://doi.org/10.1111/j.2041-210X.2009.00001.x

APPENDICES

Appendix 2.1. Mean values (±SD where applicable) of PHEs (Cu, Zn, Cd, Sb, As and Pb) accumulated in plants (*Cynodon dactylon* on top and *Medicago sativa* at the bottom). Soil treatments: T1, 90% C_0 + 10% G; T2, 80% C_0 + 20% G; T3, 50% C_0 + 50% G. C_0: polluted soil. G: Gypsum mining spoil. N: number of composite mixed samples.

PHEs in *Cynodon dactylon*'s samples per soil treatment					
PHE	**Soil treatments**			**Normal levels** (mg kg^{-1} dry foliage) [a]	**Phytotoxic levels** (mg kg^{-1}dry foliage) [a]
	T1 (N=1)	**T2** (N=3)	**T3** (N=4)		
Cu	100.01	94.09 ± 14.05	62.08 ± 11.94	*5-30*	*20-100*
Zn	262.18	214.63 ± 32.96	189.52 ± 4.95	*27-150*	*100-400*
Cd	1.99	1.83 ± 0.38	1.80 ± 0.25	*0.05-0.2*	*5-30*
Sb	5.49	5.56 ± 0.70	4.56 ± 0.32	*7-50* [b]	*150* [b]
As	113.62	99.57 ± 17.27	69.94 ± 31.08	*1-1.7*	*5-20*
Pb	181.3	167.78 ± 31.37	93.81 ± 27.04	*5-10* [b]	*30-300* [b]
PHEs in *Medicago sativa*'s samples per soil treatment					
PHE	**Soil treatments**			**Normal levels** (mg kg^{-1}dry foliage) [a]	**Phytotoxic levels** (mg kg^{-1}dry foliage) [a]
	T1 (N=1)	**T2** (N=1)	**T3** (N=1)		
Cu	136.83	84.87	79.93	*5-30*	*20-100*
Zn	518.8	258.7	223.76	*27-150*	*100-400*
Cd	8.26	3.33	3.11	*0.05-0.2*	*5-30*
Sb	22.65	13.53	6.12	*7-50* [b]	*150* [b]
As	114.93	71.38	47.52	*1-1.7*	*5-20*
Pb	200.65	118.3	81.63	*5-10* [b]	*30-300* [b]

[a] Kabata-Pendias, 2011.

Appendix 2.2. Mean percentage (±SD where applicable) of PHEs (Cu, Zn, Cd, Sb, As and Pb) accumulated in roots and shoots (*Cynodon dactylon* on top and *Medicago sativa* at the bottom). Soil treatments: T1, 90% C_0 + 10% G; T2, 80% C_0 + 20% G; T3, 50% C_0 + 50% G. C_0: polluted soil. G: Gypsum mining spoil. N: total number of composite mixed samples.

PHE accumulation in *Cynodon dactylon*'s tissues												
PHE / **Soil treatment**	**Cu**		**Zn**		**Cd**		**Sb**		**As**		**Pb**	
	shoot	**root**	**shoot**	**root**	**shoot**	**root**	**shoot**	**root**	**shoot**	**root**	**shoot**	**root**
T1 (N=1)	17.65	82.35	42.19	57.81	42.66	57.34	8.57	91.43	2.92	97.08	1.69	98.31
T2 (N=3)	16.27 ±1.83	83.73 ± 1.83	41.63 ±5.20	58.37 ±5.20	41.81 ±5.78	58.19 ±5.78	8.69 ±4.38	91.31 ±4.38	2.29 ±0.39	97.71 ±0.39	1.10 ±0.18	98.90 ±0.18
T3 (N=4)	19.87 ±6.52	80.13 ± 6.52	53.93 ±9.90	46.07 ±9.90	51.58 ±12.78	48.42 ±12.78	9.15 ±5.31	90.85 ±5.31	4.73 ±2.60	95.27 ±2.60	3.00 ±1.66	97.00 ±1.66
PHE accumulation in *Medicago sativa*'s tissues												
PHE / **Soil treatment**	**Cu**		**Zn**		**Cd**		**Sb**		**As**		**Pb**	
	shoot	**root**	**shoot**	**root**	**shoot**	**root**	**shoot**	**root**	**shoot**	**root**	**shoot**	**root**
T1 (N=1)	34.60	65.40	46.75	53.25	24.40	75.60	43.53	56.47	30.90	69.10	28.41	71.59
T2 (N=1)	32.32	67.68	50.18	49.82	24.37	75.63	38.46	61.54	30.03	69.97	25.25	74.75
T3 (N=1)	18.76	81.24	39.41	60.59	12.24	87.76	26.12	73.88	13.97	86.03	12.76	87.24

Appendix 2.3. Element content (mean± SD) in natural soils (Nat), in polluted soils (C_0) and in of gypsum mining spoil (G). Cu, Zn, Cd, Sb, As and Pb: potentially harmful elements (PHEs); Letter "T" before PHEs, Fe, Ca, S, Al and P refers to total content; "S": soluble fraction; "B": bioavailable fraction. In all cases, units are mg kg^{-1}. N= number of samples used for the analyses. [1]NA= Not Available. [2]<LOD = Under limit of detection. Different letters (rows) represent statistically significant differences ($p < 0.05$) for the *post-hoc* Tukey tests performed after the GLMs.

	Elements	**Nat** (N=18)	**C_0** (N=5)	**G** (N=5)
Total forms (mg kg^{-1})	**TFe**	NA[1]	43032.69 ± 6414.78	9501.07 ± 713.12
	TCa	NA[1]	7669.26 ± 1139.16	186153.44 ± 4288.77
	TS	NA[1]	18836.98 ± 2879.15	126094.49 ± 4552.09
	TAl	NA[1]	6243.83 ± 1804.20	2529.57 ± 532.98
	TP	NA[1]	372.44 ± 64.04	<LOD[2]
	TCu	50.29 ± 22.25 b	103.50 ± 4.25 c	10.10 ± 0.84 a
	TZn	81.08 ± 12.99 b	220.20 ± 11.45 c	18.07 ± 2.06 a
	TCd	<LOD[2] a	0.77 ± 0.03 c	0.04 ± $4.50E^{-3}$ b
	TSb	<LOD[2] a	68.37 ± 4.56 c	2.62 ± 1.18 b
	TAs	17.35 ± 7.63 b	198.53 ± 11.57 c	3.50 ± 0.41 a
	TPb	46.69 ± 31.31 b	240.14 ± 17.71 c	4.39 ± 0.73 a
Soluble fraction (mg kg^{-1})	**SCu**	0.21 ± 0.23 a	123.72 ± 7.12 b	0.10 ± 0.02 a
	SZn	0.30 ± 0.16 a	185.81 ± 16.84 b	0.20 ± 0.05 a
	SCd	0.00 ± 0.00 a	0.54 ± 0.04 b	$4.00E^{-3}$ ± $1.00E^{-3}$ a
	SSb	0.02 ± 0.02 a	0.06 ± 0.01 b	0.12 ± 0.02 c
	SAs	0.03 ± 0.02 a	0.12 ± 0.01 b	0.03 ± 0.02 a
	SPb	0.51 ± 0.52 b	0.04 ± 0.01 ab	$3.00E^{-3}$ ± $3.00E^{-3}$ a
Bioavailable fraction (mg kg^{-1})	**BCu**	6.69 ± 6.91 b	40.06 ± 1.36 c	0.85 ± 0.08 a
	BZn	7.23 ± 5.07 b	166.87 ± 4.69 c	1.35 ± 0.22 a
	BCd	0.10 ± 0.04 b	0.62 ± 0.02 c	0.01 ± $3.00E^{-3}$ a
	BSb	0.10 ± 0.08 a	0.54 ± 0.06 b	0.03 ± 0.03 a
	BAs	0.13 ± 0.08 b	3.90 ± 0.18 c	0.01 ± 0.01 a
	BPb	9.33 ± 9.28 b	0.55 ± 0.05 a	0.62 ± 0.05 a

Appendix 2.4. Chemical properties (mean± SD) of polluted soils (C_0) and of gypsum mining spoil (G= mineral amendment). EC: electrical conductivity; CEC: cation exchange capacity. $CaCO_3$: calcium carbonate; Na^+, K^+, Ca^{2+}, Mg^{2+}: exchangeable cations. N= number of samples used for the analyses. [1]NA= Not Available. [2]12 samples (from Ballesteros, 2018).

Properties		C_0 (N=5)	G (N=5)
pH		3.5 ± 0.1	7.5 ± 0.2
EC (dS m^{-1})		2.76 ± 0.01	2.90 ± 0.04
Organic carbon (%)		0,79 ± 0.07	0.04 ± 0.03[2]
$CaCO_3$ (%)		1,37 ± 0.02	27.25 ± 3.52[2]
Gypsum (%)		NA[1]	47.96 ± 28.22[2]
CEC (cmol kg^{-1})		9.63 ± 0.97	8.48 ±1.52
Exchangeable bases (cmol kg^{-1})	**K^+**	0.03 ± 0.01	0.38 ± 0.05
	Na^+	0.01 ± 3.00E^{-3}	0.03 ± 4.00E^{-3}
	Mg^{2+}	3.80 ± 0.20	0.88 ± 0.13
	Ca^{2+}	5.80 ± 1.14	7.18 ± 1.54

Appendix 2.5. Chemical properties and components (mean±SD) of treated and non-treated soil samples (C_f, $T1_f$, $T2_f$, $T3_f$) at the end of the experiment. EC (dS m^{-1}): electrical conductivity; CEC (cmol kg^{-1}): cation exchange capacity. Na^+, K^+, Ca^{2+}, Mg^{2+} (cmol kg^{-1}): exchange cations. Letter "T" before P, Ca, S, Fe and Al refers to total concentration (in mg kg^{-1}). Treatments: C, non-treated soil (100% C_0); T1, 90% C_0 + 10% G; T2, 80% C_0 + 20% G; T3, 50% C_0 + 50% G. C_0: polluted soil. G: Gypsum mining spoil. Different letters (rows) represent statistically significant differences ($p < 0.05$) between experimental soils for the *post hoc* Tukey tests performed after the GLMs for each property and component.

Soil samples (*Cynodon dactylon*)				
Properties	**Treatments**			
	C_f (N=5)	**$T1_f$** (N=5)	**$T2_f$** (N=5)	**$T3_f$** (N=5)
pH	3.77 ± 0.11 a	5.43 ± 0.31 b	6.41 ± 0.20 c	6.71 ± 0.15 c
CE	2.25 ± 0.13 a	2.39 ± 0.08 b	2.30 ± 0.08 ab	2.33 ± 0.10 b
K^+	0.02 ± 0.01 a	0.02 ± 0.01 a	0.07 ± 0.01 b	0.03 ± 0.01 a
Na^+	0.04 ± 0.02 a	0.05 ± 0.01 ab	0.06 ± 0.004 bc	0.08 ± 0.01 c
Mg^{2+}	0.48 ± 0.11 a	2.18 ± 0.92 b	1.79 ± 0.77 b	1.62 ± 0.87 ab
Ca^{2+}	8.69 ± 0.86 c	6.76 ± 1.01 b	6.02 ± 0.81 ab	5.18 ± 1.14 a
CEC	9.42 ± 1.18 c	9.01 ± 0.53 bc	7.94 ± 0.29 ab	6.90 ± 0.46 a
TP	403.07 ± 123.86 a	415.85 ± 61.16 a	482.90 ± 62.05 a	428.72 ± 91.03 a
TCa	6229.37 ± 715.39 a	13071.93 ± 1777.14 b	21997.23 ± 1775.72 c	35157.79 ± 8125.99 d
TS	14558.94 ± 2223.25 a	18618.73 ± 1949.29 b	21843.33 ± 1519.88 c	25713.16 ± 1006.83 d
TFe	50412.96 ± 6854.40 b	47984.79 ± 4062.27 ab	45990.99 ± 2892.77 ab	41228.32 ± 4999.07 a
TAl	7748.08 ± 2193.80 a	7377.61 ± 1193.12 a	7869.69 ± 323.20 a	7450.84 ± 885.05 a
Soil samples (*Medicago sativa*)				
Properties	**Treatments**			
	C_f (N=5)	**$T1_f$** (N=5)	**$T2_f$** (N=5)	**$T3_f$** (N=5)
pH	3.75 ± 0.16 a	5.74 ± 0.27 b	6.39 ± 0.12 c	6.87 ± 0.10 d
CE	2.22 ± 0.09 a	2.34 ± 0.10 b	2.39 ± 0.04 b	2.36 ± 0.05 b
K^+	0.02 ± 0.01 a	0.06 ± 0.01 b	0.06 ± 0.01 b	0.14 ±0.03 c
Na^+	0.03 ± 0.01 a	0.08 ± 0.01 b	0.08 ± 0.01 b	0.09 ± 0.02 b
Mg^{2+}	0.79 ± 0.31 a	1.66 ± 0.47 b	2.48 ± 0.49 c	2.26 ± 0.35 bc
Ca^{2+}	9.16 ± 2.36 b	7.86 ± 1.56 ab	6.58 ± 1.26 ab	5.92 ± 0.92 a
CEC	10.33 ± 1.73 b	9.66 ± 1.26 ab	9.20 ± 1.15 ab	8.15 ± 0.76 a
TP	407.84 ± 61.90 b	416.61 ± 99.85 b	460.18 ± 22.16 b	291.31 ± 23.15 a
TCa	6776.12 ± 900.90 a	14691.51 ± 1872.04 b	21538.53 ± 1348.38 c	52562.49 ± 15836.67 d
TS	15894.84 ± 1890.62 a	18086.68 ± 1694.97 ab	21520.94 ± 1572.12 b	31886.94 ± 6184.92 c
TFe	52287.88 ± 3700.09 c	47223.68 ±4463.61 bc	44965.53 ± 4084.53 b	36390.33 ± 5031.67 a
TAl	8711.95 ± 844.81 a	7762.13 ± 1258.45 a	7420.23 ± 792.22 a	6408.54 ± 1996.16 a

Appendix 2.6. Ratio (mean±SD) of soluble and total (S/T) PHEs per species (*Cynodon dactylon* and *Medicago sativa*) in non-treated soil samples before (C_0) and after the experiment (C_f) and in treated soil samples at the end of the experiment ($T1_f$, $T2_f$, $T3_f$). Treatments: C, non-treated soil (100% C_0); T1, 90% C_0 + 10% G; T2, 80% C_0 + 20% G; T3, 50% C_0 + 50% G**.** C_0: polluted soil. G: Gypsum mining spoil. Different letters (column) represent statistically significant differences ($p < 0.05$) for the *post hoc* Tukey tests performed after the GLMs.

			Cynodon dactylon			
Treatment	**S/T Cu**	**S/T Zn**	**S/T Cd**	**S/T Sb**	**S/T As**	**S/T Pb**
C_0	1,20± 0,05 c	0,84± 0,06 c	0,70± 0,03 c	1.00E^{-3} ± 1.00E^{-4} b	6.00E^{-4}± 1.00E^{-4} cd	2.00E^{-4}± 0.00 b
C_f	0.04± 0.03 b	0.23± 0.12 b	0.68± 0.50 c	4.00E^{-4}± 1.00E^{-4} a	3.00E^{-4}± 1.00E^{-4} ab	9.00E^{-6}± 1.00E^{-5} a
$T1_f$	1.70E^{-3} ± 2.00E^{-4} a	8.00E^{-4} ± 3.00E^{-4} a	0.02± 0.01 b	1.60E^{-3}± 2.00E^{-4} b	3.00E^{-4} ± 0.00 a	3.00E^{-7}± 3.00E^{-7} a
$T2_f$	1.70E^{-3} ± 1.00E^{-4} a	4.00E^{-4} ± 1.00E^{-4} a	0.01± 5.00E^{-4} a	2.60E^{-3}± 1.00E^{-4} c	4.00E^{-4} ± 0.00 bc	1.00E^{-7}± 1.00E^{-6} a
$T3_f$	1.50E^{-3} ± 3.00E^{-4} a	4.00E^{-4} ± 2.00E^{-4} a	4.10E^{-3} ± 1.30E^{-3} a	3.00E^{-3}± 4.00E^{-4} d	5.00E^{-4} ± 1.00E^{-4} c	2.00E^{-6}± 2.00E^{-6} a
			Medicago sativa			
Treatment	**S/T Cu**	**S/T Zn**	**S/T Cd**	**S/T Sb**	**S/T As**	**S/T Pb**
C_0	1,20± 0,05 c	0,84± 0,06 d	0,70± 0,03 e	1.00E^{-3}± 1.00E^{-4} b	6.00E^{-4}± 1.00E^{-4} d	2.00E^{-4}± 0.00 b
C_f	0.03± 0.01 b	0.08± 0.03 c	0.21± 0.05 d	4.00E^{-4}± 1.00E^{-4} a	2.00E^{-4}± 0.00 b	1.00E^{-5}± 1.00E^{-5} a
$T1_f$	1.30E^{-3}± 1.00E^{-4} a	1.40E^{-3}± 6.00E^{-4} b	0.02± 4.60E^{-3} c	1.30E^{-3}± 3.00E^{-4} b	1.00E^{-4}± 0.00 a	1.00E^{-5}± 0.00 a
$T2_f$	1.60E^{-3}± 1.00E^{-4} a	4.00E^{-4}± 1.00E^{-4} a	6.00E^{-3}± 9.00E^{-4} b	3.00E^{-3}± 2.00E^{-4} c	3.00E^{-4}± 0.00 b	1.00E^{-5}± 1.00E^{-5} a
$T3_f$	1.50E^{-3}± 5.00E^{-4} a	3.00E^{-4}± 1.00E^{-4} a	4.00E^{-3}± 1.10E^{-3} a	4.10E^{-3}± 7.00E^{-4} d	4.00E^{-4}± 1.00E^{-4} c	1.00E^{-6}± 1.00E^{-6} a

Appendix 2.7. Ratio (mean±SD) of bioavailable and total (B/T) PHEs per species (*Cynodon dactylon* and *Medicago sativa*) in non-treated soil samples before (C_0) and after the experiment (C_f) and in treated soil samples at the end of the experiment ($T1_f$, $T2_f$, $T3_f$). Treatments: C, non-treated soil (100% C_0); T1, 90% C_0 + 10% G; T2, 80% C_0 + 20% G; T3, 50% C_0 + 50% G. C_0: polluted soil. G: Gypsum mining spoil. Different letters (column) represent statistically significant differences ($p < 0.05$) for the *post hoc* Tukey tests performed after the GLMs.

			Cynodon dactylon			
Treatment	**B/T Cu**	**B/T Zn**	**B/T Cd**	**B/T Sb**	**B/T As**	**B/T Pb**
C_0	0,39± 0,02 a	0,76 ± 0,04 c	0,81 ± 0,04 a	8.00E^{-3} ± 1.00E^{-3} b	0,020 ± 2.00E^{-3} cd	2.00E^{-3} ± 0,00 a
C_f	0.39 ± 0.04 a	0.18 ± 0.05 a	0.77 ± 0.11 a	8.00E^{-3} ± 3.00E^{-3} b	0.01 ± 2.00E^{-3} bc	2.00E^{-3} ± 0.00 a
$T1_f$	0.38 ± 0.05 a	0.49 ± 0.11 c	1.09 ± 0.13 b	3.00E^{-3} ± 1.00E^{-3} a	8.00E^{-3} ± 2.00E^{-3} bc	1.00E^{-3} ± 1.00E^{-3} a
$T2_f$	0.42 ± 0.07 a	0.30 ± 0.06 b	1.05 ± 0.18 b	0.007 ± 0.002 b	0.01 ± 2.00E^{-3} c	2.00E^{-3} ± 1.00E^{-3} a
$T3_f$	0.37 ± 0.04 a	0.23 ± 0.03 ab	1.00 ± 0.07 b	0.009 ± 0.001 b	5.00E^{-3} ± 1.00E^{-3} a	2.00E^{-3} ± 0.00 a
			Medicago sativa			
Treatment	**B/T Cu**	**B/T Zn**	**B/T Cd**	**B/T Sb**	**B/T As**	**B/T Pb**
C_0	0,39 ± 0,02 b	0,76 ± 0,04 c	0,81 ± 0,04 c	8.00E^{-3} ± 1.00E^{-3} bc	0,02 ± 2.00E^{-3} d	2.00E^{-3} ± 0,00 a
C_f	0.37 ± 0.03 ab	0.17 ± 0.09 ab	0.54 ± 0.10 a	6.00E^{-3} ± 1.00E^{-3} b	8.00E^{-3} ± 1.00E^{-3} b	2.00E^{-3} ± 1.00E^{-3} a
$T1_f$	0.37 ± 0.06 b	0.25 ± 0.03 b	0.73 ± 0.08 b	4.00E^{-3} ± 1.00E^{-3} a	8.00E^{-3} ± 2.00E^{-3} b	2.00E^{-3} ± 0.00 a
$T2_f$	0.45 ± 0.03 c	0.26 ± 0.04 b	0.86 ± 0.08 c	9.00E^{-3} ± 1.00E^{-3} c	1.20E^{-2} ± 1.00E^{-3} c	3.00E^{-3} ± 1.00E^{-3} b
$T3_f$	0.31 ± 0.04 a	0.14 ± 0.02 a	0.64 ± 0.05 ab	0.01 ± 2.00E^{-3} c	5. 00E^{-3} ± 0.00 a	4.00E^{-3} ± 1.00E^{-3} b

- CHAPTER 3 -

REMEDIATION OF SOILS AFFECTED BY RESIDUAL POLLUTION THROUGH THE USE OF ORGANIC AND INORGANIC AMENDMENTS

ABSTRACT

Soils are a key part of ecosystems and human society. Nevertheless, anthropogenic activities such as mining, may seriously degrade them, causing the spread of Potentially Harmful Elements (PHEs) throughout the ecosystem and posing a toxicity risk for living organisms. The recovery of soils polluted with PHEs require Assisted Natural Remediation (ANR), what, in most cases, entails the improvement of soil properties to enable the growth of vegetation. This improvement is usually made through the addition of harmless waste materials as soil amendments, which can be an eco-effective solution for a dual environmental issue, in line with zero waste strategy. This study is developed in soils with high concentrations of PHEs (mainly As and Pb) located at the Guadiamar Green Corridor, natural area affected by the Aznalcóllar mining spill. In these soils, the residual pollution is heterogeneously distributed, and some areas are still acidic and deprived of vegetation. We have tested under field conditions the potential role of gypsum mining spoil, marble sludge and vermicompost in recovering soils affected by residual pollution. The effectiveness of soil treatments in reducing PHE mobility and bioavailability was assessed through the analysis of PHE fractions and a toxicity bioassay with *Lactuca sativa* L. Results showed that marble sludge was the most effective treatment by significantly increasing pH and reducing PHE toxicity risk. Gypsum mining spoil also presented promising results, although less positive than marble sludge. The addition of vermicompost significantly increased the organic carbon content in the amendments, essential for vegetation recovery, and did not significantly increased Pb or As availability. According to our findings, the amendments tested in these experiments could be potentially used to remediate soils polluted with PHEs, especially marble sludge and its combination with vermicompost, although further monitoring would be advisable to guarantee the positive evolution of unvegetated soils in the area.

Keywords: Toxicity risk; soil remediation; gypsum mining spoil; marble sludge; vermicompost; PHE immobilization

1. INTRODUCTION

Soil is a key part of ecosystems (Lombi et al. 1998). It not only provides resources, but also essential services (FAO, 2015). As a result of its unsustainable and intense exploitation through human activities, soil is becoming degraded, being soil pollution a major environmental concern worldwide (Liu, et al., 2018; Sierra-Aragón et al., 2019).

Potentially harmful elements (PHEs) include heavy metals and metalloids naturally found in soils. Their principal hazard lies in the fact that they cannot be degraded and tend to accumulate in soils, generating a potential risk of pollution for the environment (Khan et al., 2000) and, when exceeding a specific threshold, for living organisms (Kabata-Pendias, 2011; Bini and Bech, 2014; Simon, 2014). In terms of food chain pollution and health risk, As and Pb are among the most dangerous PHEs (Simon, 2014), because they may have very harmful effects on biota at low concentrations (Rahman and Singh, 2019).

Soil pollution can be managed through *ex situ* and/or *in situ* techniques (Liu et al. 2018). For big extensions of polluted soils, *ex-situ* treatments are not feasible for economical, logistical and environmental reasons (Illera et al., 2004; Liu et al., 2018). However, in these scenarios, *in-situ* techniques are gaining more and more recognition as an eco-efficient solution (Adriano et al., 2004; Aguilar et al., 2004c). In humid climates, natural remediation (NR) is usually effective and vegetation recovers after little or no human intervention; however, in drier climates, Assisted Natural Remediation (ANR), based in the improvement of soil properties by the use of amendments, is necessary to rehabilitate these ecosystems, where natural remediation is too slow (Adriano et al., 2004).

One of the most important case studies worldwide about soil pollution and remediation is the Aznalcóllar mine spill held in 1998 (Grimalt and Macpherson, 1999). After the accident, a coordinated large-scale rehabilitation plan was executed. First, the cleaning and removal of the toxic tailings and strongly polluted soils was accomplished. Then, soil amendments were applied to reduced PHE mobility and availability to the ecosystem. Moreover, native plant species promoted the stabilization of PHEs in soils. Finally, a public natural area (“Protected Landscape”) called the Guadiamar Green Corridor (GGC) (CMA, 2003), was implemented in order to facilitate the rehabilitation of the affected areas over time. Twenty years after the accident, PHE content in soils still surpass regulatory threshold values in some areas, what implies that residual pollution is still present in around 7% of the original affected area (Martín-Peinado et al., 2015). The rapid clean-up operations and the use of heavy machinery provoked the irregular distribution of the residual pollution, with highly polluted and unvegetated spots surrounded by less polluted and vegetated areas (Simón et al., 2008; Martín-Peinado et al., 2015). Since the absence of vegetation in the most polluted soils seems to be strongly related to the high concentrations of PHEs (Sierra-Aragón, 2019), these spots could be a threat for the surrounding areas (García-Carmona et al., 2019a) and, therefore, rehabilitate them is especially important and urgent. In order to preserve the positive evolution of the less polluted and vegetated areas, *in-situ* measures such as the addition of soil amendments, should be the preferential choice.

Organic and inorganic soil amendments (i.e. compost, lime, iron oxides) play a key role in the recovery of soil properties, enhancing soil natural mechanisms to reduce PHE availability and toxicity, and helping reactivate soil biological functions (Adriano et al., 2004; Aguilar et al., 2004c; Fernández-Caliani and Barba-Brioso, 2010). The improvement of soil properties and functions would facilitate the recolonization of the barren soils by plant species from the surrounding areas (Sierra-Aragón et al., 2019), and thus the rehabilitation of the area.

The demand of large quantities of natural resources and the overall land degradation are a global concern (Cowie et al., 2017). In a changing and unsustainable world, circular economy is lately seen as a plausible solution. Moreover, considering that the primary sector is the most wasteful (Velenturf et al., 2019), reusing waste materials from mining and agro-food industries to remediate degraded soils would not only reduce waste production and disposal, but remediate soil physicochemical properties at the same time (Xiong et al., 2015). As a result, the revalorization of industrial wastes through their use as soil amendments is currently a rising trend (González-Núñez et al., 2015).

As the effectiveness of *in-situ* techniques depends on the properties of the amendment used, on the specific PHEs involved, and on the synergistic or antagonistic relation between PHEs in multielement pollution, the effectiveness of new wastes should be tested prior to their use (Rodríguez-Jordá et al., 2010a). 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, the addition of calcium-rich and organic matter-rich amendments are among the most effective ones, through the correction of soil acidic pH, the increase of nutrient availability and the immobilization of certain PHEs (Palmer, 1978; Bernal et al., 2007; Pérez-de-Mora et al., 2007). Accordingly, for this study we have used gypsum mining spoil, marble sludge and vermicompost as amendments of unvegetated and highly polluted soils.

Gypsum mining spoil is the part discarded during gypsum processing because 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 need to be sustainably managed. Gypsum mining spoil is a low-cost waste 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 and reduce soil crusting, to provide essential nutrients, and to decrease PHE toxicity (Sharma et al., 1974; Kabata-Pendias and Pendias, 2000; Amezketa et al., 2005; Franzen et al., 2006; Gadepalle et al., 2007). Despite its potential as soil amendment, there are no references so far for the use of gypsum mining spoil in soil remediation. Nevertheless, some authors have already tested other gypsum byproducts (i.e. red gypsum and phosphogypsum) to amend soils affected by PHEs with good results, such as increasing soil acidic pH, reducing soil crust and Cu, Cd, As and Pb leachability, and enhancing plant rooting (Sumner et al., 1990; Garrido et al., 2003; Illera et al., 2004; Rodríguez-Jordá et al., 2010a, 2010b, 2012; Dinake and Kelebemang, 2019). As well, Rodríguez-Jordá et al. (2010a; 2012) detected that the combination of red gypsum with organic amendments reduced Pb, Zn and Cu leachability and neutralized soil pH.

Marble sludge is a waste material that derives from cutting and polishing marble stones. Considering that marble is the largest produced natural stone in the world, large amounts of this low-cost waste material are annually produced (Rayed et al., 2019), potentially producing negative effects on the ecosystem if incorrectly discarded (Al-Hamaiedh, 2010; El-Sayed et al., 2018). Marble sludge is very rich in calcium carbonate, which plays a key role in buffering soil pH and PHE immobilization in soils (Fernández-Caliani and Barba-Brioso, 2010). On top of that, it promotes water retention and tends to decrease the electrical conductivity of soils (Sánchez et al., 2010; González et al., 2012). This amendment has been previously used and tested by other authors, with promising results for pH increase and PHE toxicity reduction in soils affected by mining activities (Pérez-Sirvent et al., 2007; Fernández-Caliani and Barba-Brioso, 2010; Del-Moral-Torres et al., 2010; González et al., 2012, 2013, 2017).

Vermicompost is an organic amendment that, in our case, derives from composting horse manure, boosted by *Eisenia Andrei*. The use of earthworms optimizes the composting process, enhancing the remediation potential of the final product (Huang et al., 2016). Large quantities of horse manure are yearly produced (Kenny et al., 2019). Its use 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 PHE toxicity, what will promote the recovery of vegetation (Pérez-Esteban et al., 2012). Nevertheless, some authors have described the controversial role of organic matter in the restoration of soils polluted with PHEs (Gadepalle et al., 2007); for instance, Sierra-Aragón et al. (2019) observed a rise in Pb bioavailability and As bioavailability and solubility after vermicompost application in moderately vegetated areas, so its application should be studied with caution.

Some authors have pointed out that the combination of organic and calcium-rich amendments is also a good and common technique to rise pH, immobilize PHEs, improve soil conditions, facilitate revegetation and restore soil ecological functions (Clemente et al., 2003; Zornoza et al., 2012).

Apart from amendment application, other physicochemical techniques were tested as a reference point. Tillage is an inexpensive technique that would break soil crust and dilute pollutants by loosening the soil (Kochem Mallmann et al., 2014; Busari et al., 2015), and mixing vegetated and unvegetated affected soils could reduce PHE availability and provide unvegetated soils with essential microbiota and a seed bank (García-Carmona et al., 2017).

In view of the above, we have carried out an *in-situ* experiment treating PHE polluted soils mainly with inorganic and organic amendments. The principal objective of this works lies on assessing the effectiveness of gypsum mining spoil, marble sludge and their combination with vermicompost, in the assisted natural remediation of residually polluted soils in the Guadiamar Green Corridor (SW Spain), in order to provide valuable information about new remediation strategies that could also be extrapolated to similar scenarios all over the world.

2. MATERIAL AND METHODS

2.1. Study area

The study area is located in the Guadiamar Green Corridor (GGC) (CMA, 2003), at the closest sector to the Aznalcóllar pyrite mine (SW Spain) and beside the Agrio river floodplain. In geological terms, it is placed next to the Iberian Pyrite Belt (IPB), which has been intensely exploited since the 3rd Millenium BC (Nocete et al., 2014). The bedrock consists in Neogene-Quaternary sediments (Alastuey et al., 1999), with alluvial deposits made of silty-sand materials, gravels dominated by quartzite, shales and schists, and a basement consisting of thick Miocene blue marls (Manzano et al., 2000; Salvany et al., 2001). According to the FAO classification (IUSS, 2015), the dominant soil types in this sector are eutric Fluvisols (soils close to the river channel) and eutric Regosols (soils not influenced by periodic floodings). The Guadiamar river floodplain was mainly dedicated to agriculture (Madejón et al., 2003), but also had riverbank vegetation characterized by ash, poplar, elm and willow trees. The climate is typically Mediterranean, with hot dry summers, cold wet winters, and variable rainfall during temperate autumns and springs (Simón et al., 2008). In 1998, one of the largest mining spills in the world occurred in the Aznalcóllar pyrite mine (SW Spain) (Simón et al., 1999; Sierra-Aragón et al., 2019). Despite the implementation of intense rehabilitation activities, more than twenty years after the accident, PHE concentrations in soils are higher than pre-accident values and, in some areas heterogeneously distributed, these concentrations also exceed the regulatory levels, producing negative effects on the environment (Simón et al., 2008). Within the residually polluted area, the so-called “affected area” (AA) hereafter, we can distinguish between vegetated (V-AA) and unvegetated (UV-AA) areas.

The UV-AA areas were localized and described in previous works (Martín-Peinado et al., 2015) using satellite images, and preliminary characterized by García-Carmona et al. (2019a). The UV-AA areas mainly appeared in the sector of the GGC closest to the mine (first 18 km downstream the tailing pond), occupying up to 7% of the AA in the form of heterogeneously distributed spots with a surface ranging from 1 to 200 m^2 (Martín-Peinado et al., 2015).

2.2. Experimental design

For this study, we selected 6 unvegetated areas (UV-AA) to establish 6 experimental plots (Figure 3.1). As external controls, we also selected 6 neighboring vegetated affected areas (V-AA) and an unaffected area (UA) within the GGC but where the spill did never reach.

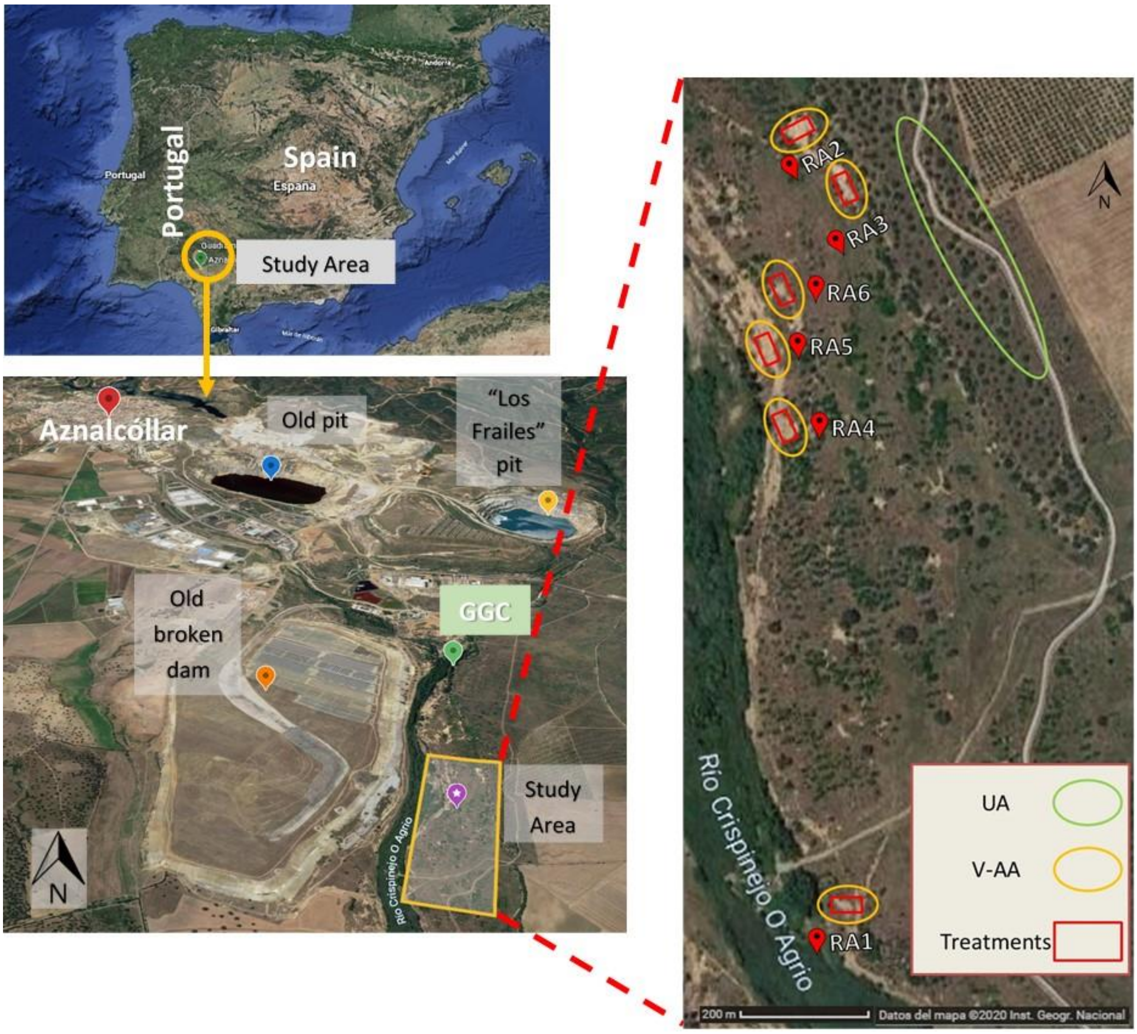


Figure 3.1. Study area. GGC: Guadiamar Green Corridor. UA: Unaffected Area. RA1-RA6: Affected Areas selected for the study. V-AA: Vegetated Affected Areas. UV-AA: Unvegetated Affected Areas.

Experimental plots (36 m^2) consisted in 9 subplots (4 m^2 each), where eight different treatments were applied, and a polluted control was defined (UV-AA) (Figure 3.2). Treatments consisted in: i) Simple treatments: 1. G: Addition of 5 kg m^{-2} of gypsum mining spoil; 2. M: Addition of 5 kg m^{-2} of marble sludge; 3. T: crust breaking through tillage; 4. V: mixture of V-AA and UV-AA (50% w/w) in the uppermost 10 cm of the soil; and ii) Mixed treatments (the same treatments mixed with vermicompost "v"): 5. Gv: G + v; 6. Mv: M + v; 7. Tv: T + v; 8. Vv: V + v. 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 V 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.

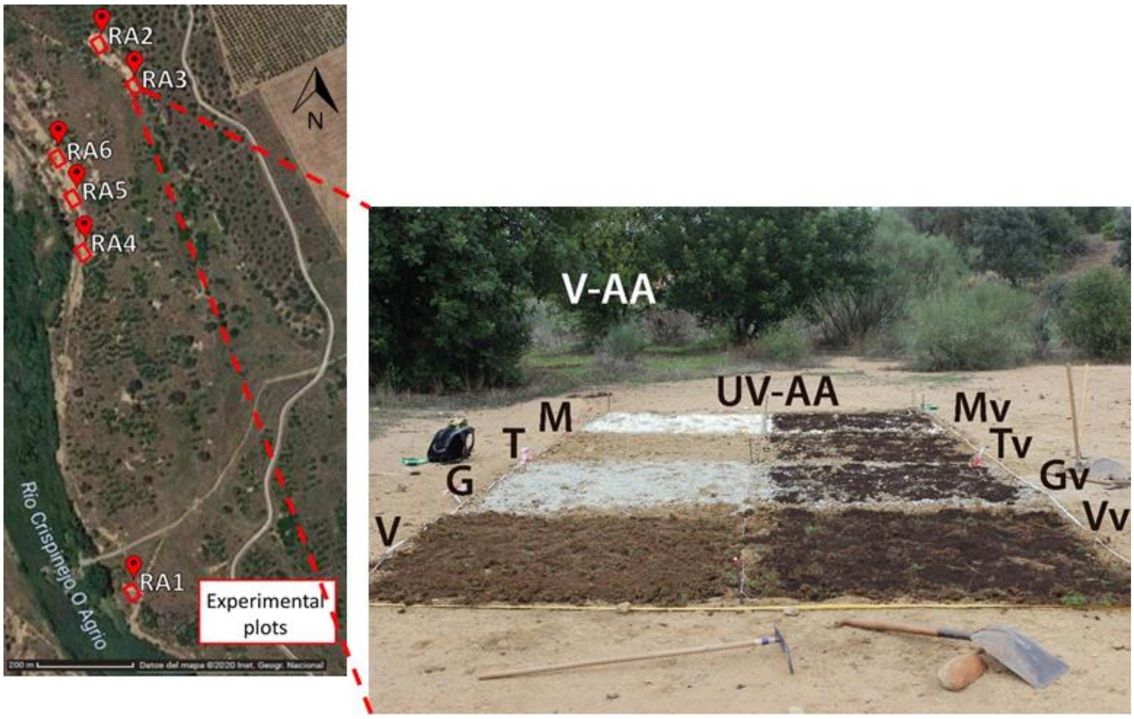


Figure 3.2. Experimental plot with soil treatments: **G**: Addition of gypsum mining spoil; **M**: Addition of marble sludge; **T**: Tillage; **V**: mixture of UV-AA and V-AA; **Gv**: G plus vermicompost (v); **Mv**: M + v; **Tv**: T + v; **Vv**: V + v. **UV-AA**: Unvegetated Affected Area. **V-AA**: Vegetated Affected Area.

In relation to the amendment source, gypsum mining spoil derives from a gypsum quarry in Escúzar (Granada, Spain) and contains 48% of gypsum and 27% of $CaCO_3$; marble sludge derives from a marble quarry in Macael (Almería, Spain) and contains 99% of $CaCO_3$; and vermicompost was produced by Lombricor S.C.A, and has the ecological certificate. The properties of the amendments are showed in Appendix 3.1.

Three soil samples per treatment (from the uppermost 10 cm) were collected at each experimental plot every six months for 2 years (T0: right after the preparation of the experimental plots; T6: after the first 6 months; T12 after the first year of experiment; T18 after a year and a half after the start) in order to analyse the main soil properties and PHE content, and the same was done for the neighbouring vegetated soils (V-AA). Additionally, eighteen soil samples were collected in the unaffected surrounding area (UA) to lay out the PHE background and main properties of natural soils.

2.3. Laboratory analysis

Afterwards, we analysed the main physicochemical properties (pH, electrical conductivity -EC-, organic carbon -OC-, calcium carbonates -$CaCO_3$-), according to standard methods (MAPA, 1994), and PHE (Cu, Zn, As and Pb) content in all soil types (unvegetated: UV-AA, vegetated: V-AA and unaffected: UA). Soil samples were sieved (2mm), finely ground and digested in strong acids (HNO_3:HF, 3:1) in a microwave oven XP1500Plus (Mars®) to analyse the total concentrations (T) of PHEs. Then, water-soluble (S) and bioavailable (B) fractions were extracted as described by Sposito et al. (1982) and Quevauviller et al. (1998), respectively. In all cases, PHE concentrations in soil samples were measured by inductively coupled plasma-mass spectrometry (ICP-MS) in a PE SCIEX ELAN-5000 spectrometer. The accuracy of the method was confirmed by analysing the Standard Reference Material “CRM025-050”; the average recoveries for Cu, Zn, As and Pb ranged between 78% and 100% of the certified reference values, being inside either the confidence interval (Cu, Zn and Pb) or the Prediction interval (As).

The PHEs selected for this study were Cu, Zn, As and Pb as it has been reported that after intense rehabilitation, soils are still affected by relatively high concentrations of these elements (Martín-Peinado et al., 2015). Galán et al. (2002) suggested Cd and Sb were not a great concern, considering that their total concentrations were under the regional thresholds, whereas As exceeded intervention values (Aguilar et al., 1999), requiring reclamation, and the total concentrations of Cu, Zn and Pb indicated that further investigation in some areas would be advisable. Moreover, according to the results in Chapter 2, our plants accumulated Cu, Zn, As and Pb above phytotoxic levels (Kabata-Pendias, 2011), while Cd and Sb were below. Consequently, Cd and Sb were not considered for this study.

In this work, we apply the term "soluble" to the fraction of pollutants that are soluble in water, or in other words, to the pollutant concentrations that are easily mobile (short-term) from the soil to the plant or to groundwater through leaching. Similarly, we apply the term "bioavailable" to refer to the fraction of pollutants that are extracted by EDTA and that could be considered a potentially available fraction for plants (Beckett, 1989), as plants could absorb them from the soil in the long-term evolution.

In order to compare the relative mobility and bioavailability of PHEs in the different soils types (UA, UV-AA and V-AA) and treatments, solubility and bioavailability ratios were calculated for each PHE as the relation between soluble (or bioavailable) and total concentration of each element. Higher values indicate that the solubility/bioavailability of the element is higher in relation to total concentrations, posing a higher risk. Therefore, low values are desirable.

Finally, a toxicity bioassay was conducted using *Lactuca sativa* L. to assess the effectiveness of each remediation technique. In Petri dishes containing 5 ml of 1:5 soil:water extract, 15 seeds of *L. sativa* were placed and incubated at 20 ± 1 º C during 120 h following OECD (2003) and US EPA (1996) recommendations. Then, *L. sativa* germination (germ) and root elongation (LsE) were measured. Results were expressed as root elongation in the sample in relation to water unpolluted controls; the values obtained ranged from 0 to 100, where 0 indicates no elongation/germination (maximum toxicity) and 100 indicates same elongation/germination (no toxicity) compared to the water control. Values above 100 indicate overstimulation of the elongation in relation to the control (hormesis) and were considered as no toxic response (value 100).

2.4. Data analysis

All statistical analyses were performed using R version 3.4.2 (R Core Team, 2017). By means of "lme4" package (Bates et al., 2015), we applied linear mixed models (LMMs) to assess the effectiveness of soil treatments in terms of soil properties (pH, EC, $CaCO_3$, OC), PHE immobilization in soils, and *Lactuca sativa* germination and root elongation in toxicity tests. We assumed gaussian error and identity-link function, and the logarithmical transformation of variables (or arcsine square root transformation for proportions and percentages) was used for $CaCO_3$, OC, germination, bioavailable and soluble fractions, and bioavailable and soluble ratios to meet normality assumption. Soil treatment was included as fixed factor, and residual areas and sampling period as random factors. To fit LMMs, "lme4" package was used (Bates et al., 2015). Then, model suitability was assessed by graphical exploration of the residuals (Zuur et al. 2010), and pairwise comparisons between soil treatments (including unaffected (UA), unvegetated (UV-AA) and vegetated (V-AA) affected soils) were performed for all the variables with Tukey's *post-hoc* tests using "multcomp" package (Hothorn et al., 2008).

We calculated the variance explained by LMMs using "r.squaredGLMM" function of "MuMIn" library (Barton, 2016).

A non-metric multidimensional scaling (NMDS) ordination analysis was performed by means of metaMDS function in the vegan package (Oksanen et al. 2013), in order to investigate the influence of soil properties and solubility ratios on PHE toxicity over *Lactuca sativa* (bioassays). The Bray-Curtis index was chosen as a dissimilarity measure between sites.

Graphs and confidence intervals were obtained with R "ggplot2" v3.1.1 (Wickham, 2016) and "sciplot" v1.1-1 (Morales, 2017). Mean values, standard errors and standard deviations were calculated by using ddply function (R "plyr" package, v1.8.4; Wickham, 2011).

3. RESULTS AND DISCUSSION

3.1. Soil properties

Unaffected soils in the area (UA) were sampled close to the plots affected by residual pollution, but out of the area covered by the spill (Figure 3.1). These soils are characterized by a slightly acid pH (mean 6.34 ± 0.09), no calcium carbonate ($CaCO_3$) content, and very low electrical conductivity (EC: mean <0.1 dS m^{-1}). The presence of natural vegetation over time in UA has produced the accumulation of organic carbon (OC) in the uppermost part of these soils, with values higher than 3%. The spill and the subsequent rehabilitation of the affected area (AA) strongly changed the main properties and constituents of soils (Figure 3.3), with significant differences, not only comparing the unaffected (UA) and affected (AA) areas, but also between the affected areas where vegetation grows (V-AA) and where it does not (UV-AA). In this sense, UV-AA soils presented a very strongly acid pH (mean 3.43 ± 0.05) and very high EC values (mean >3.2 dS m^{-1}), indicating the oxidative effects of the pollution associated to the tailing oxidation process (Simón et al., 2001) and limiting vegetation growth (García-Carmona et al., 2019a). Although the presence of $CaCO_3$ was detected in these soils (mean values close to 1%), indicating that liming was applied in this area, the precipitation of metals around $CaCO_3$ particles produced the isolation of this element into the soil matrix and the strong acidification of the environment (Simón et al., 2005; Aguilar et al., 2007). The absence of vegetation in these polluted areas (UV-AA) led to the low OC content (mean 0.50 ± 0.03%). Otherwise, V-AA soils presented slightly acid pH (mean 6.34 ± 0.33) and low EC values (mean <0.8 dS m^{-1}), similar to those values measured in UA. The $CaCO_3$ content was significantly higher (mean > 2 %) than in UV-AA, indicating the effectiveness of the liming treatment over time and the improvement of soil properties, which promoted vegetation growth and thus, the significant increase observed in the OC content (mean values close to 2%), that is already approaching to the values of the unaffected soils (UA).

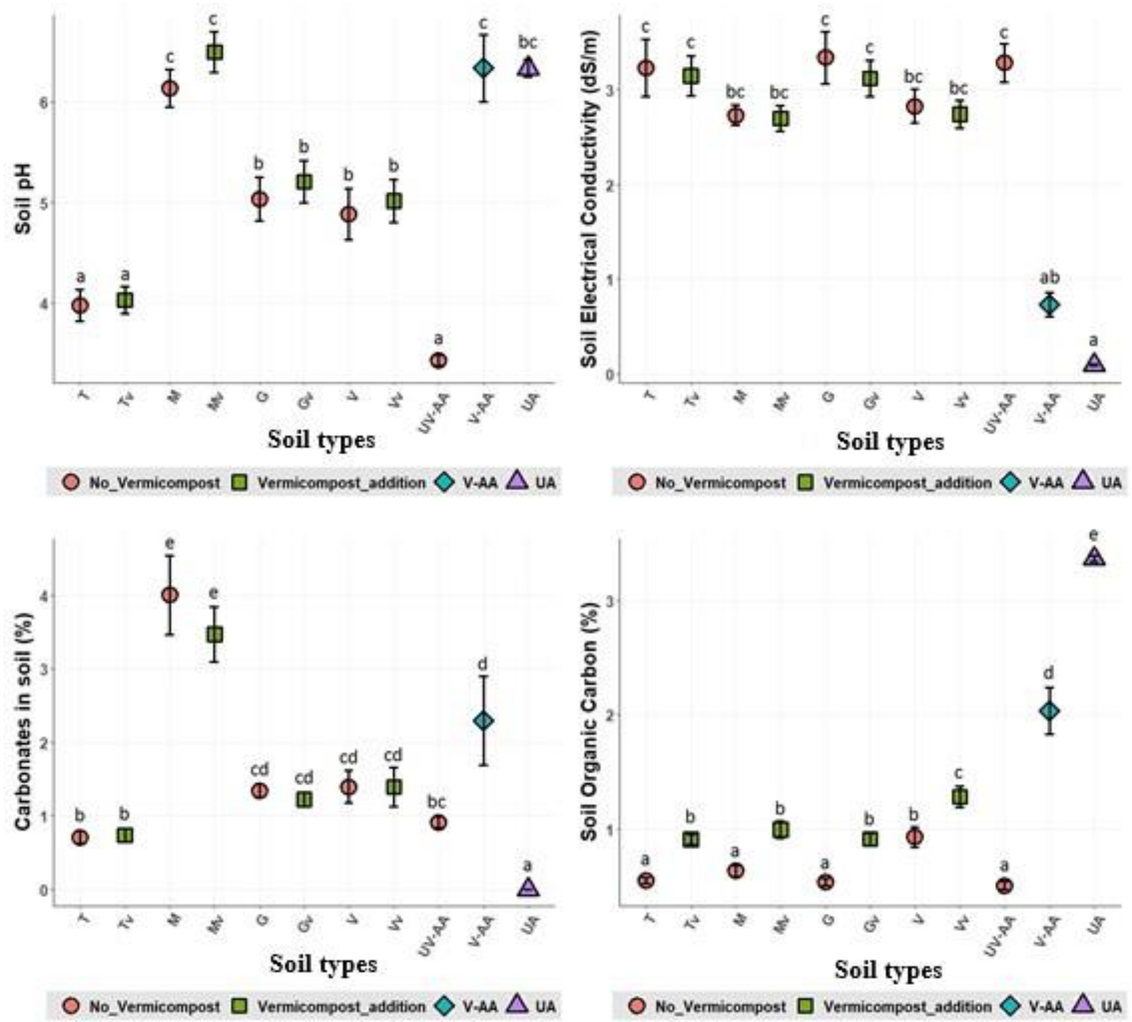


Figure 3.3. Main soil properties and constituents (mean±SE). Soil types: i) **UA**: Unaffected soils; ii) **UV-AA**: Unvegetated Affected soils; iii) **V-AA**: Vegetated Affected soils; and iv) Soil treatments: **G**: Addition of gypsum mining spoil; **M**: Addition of marble sludge; **T**: Tillage; **V**: mixture of UV-AA and V-AA; **Gv**: G plus vermicompost (v); **Mv**: M + v; **Tv**: T + v; **Vv**: V + v. Different letters above symbols represent statistically significant differences ($p < 0.05$) for the *post-hoc* Tukey tests performed after the LMMs.

3.2. Residual pollution in soils

The geochemical background concentrations of potentially harmful elements (PHEs) were measured in the unaffected soils in the area (UA). The mean concentrations (± SE) of Pb, As, Zn and Cu in these soils were 61.27 ± 10.62, 19.23 ± 2.14, 87.04 ± 3.12 and 37.37 ± 5.05 mg kg^{-1}, respectively, and are in the range of the background values measured in the area (Simón et al., 1999). The unvegetated affected area (UV-AA) presented significantly higher concentrations of all the elements (Figure 3.4), with mean values (± SE) of 818.46 ± 57.60 for Pb, 478.52 ± 28.03 for As, 293.48 ± 32.95 for Zn and 175.84 ± 14.87 mg kg^{-1} for Cu, respectively. The vegetated affected area (V-AA) also presented higher concentrations of potentially harmful elements in relation to the background concentrations, with mean values (± SE) of 293.26 ± 36.28, 169.91 ± 25.88, 429.76 ± 43.74 and 150.49 ± 21.88 mg kg^{-1}, for Pb, As, Zn and Cu, respectively, although the high standard deviation of these concentrations produced that the increase was significant only in the case of Zn and As. The comparison of these values with the current regulatory thresholds set by the Regional Government of Andalusia (BOJA, 2015) [275 mg kg^{-1} for Pb, 36 mg kg^{-1} for As, 10000 mg kg^{-1} for Zn, and 595 mg kg^{-1} for Cu], indicated that these levels are exceeded in the case of Pb and As, both in UV-AA (3- and 13-fold, respectively) and in V-AA (in the case of As, 5- fold). In this sense, Pb and As seem to have been strongly retained in soils by iron oxides, which were present in elevated quantities in the affected areas (Aguilar et al., 2004a; Aguilar et al., 2007); tailings contained high concentrations of iron, and after the clean-up operations, large quantities of iron-rich amendments were also added to the affected soils, resulting in high quantities of total iron in UV-AA soils (73620.15 ± 3088.34 mg kg^{-1}). In the vegetated affected soils (V-AA), the concentrations of total iron have been reduced by 33%, approaching to total concentrations in the unaffected soils (UA, 37117.42 ± 1726.10 mg kg^{-1}).

According to these results, and in a scenario where a long aging process have increased the complexity of PHE availability (Romero-Freire et al., 2016), the assessment of potential mobility and bioavailability is needed, and the implementation of *in-situ* rehabilitation measures is completely justified. Applying bioassays and comparing UV-AA soils before and after the addition of amendments will unveil the remediation potential of each amendment in terms of enhancing soil properties and of limiting PHE availability, and will allow us to evaluate the risk of dispersion of these pollutants and their potential toxicity to the surrounding areas.

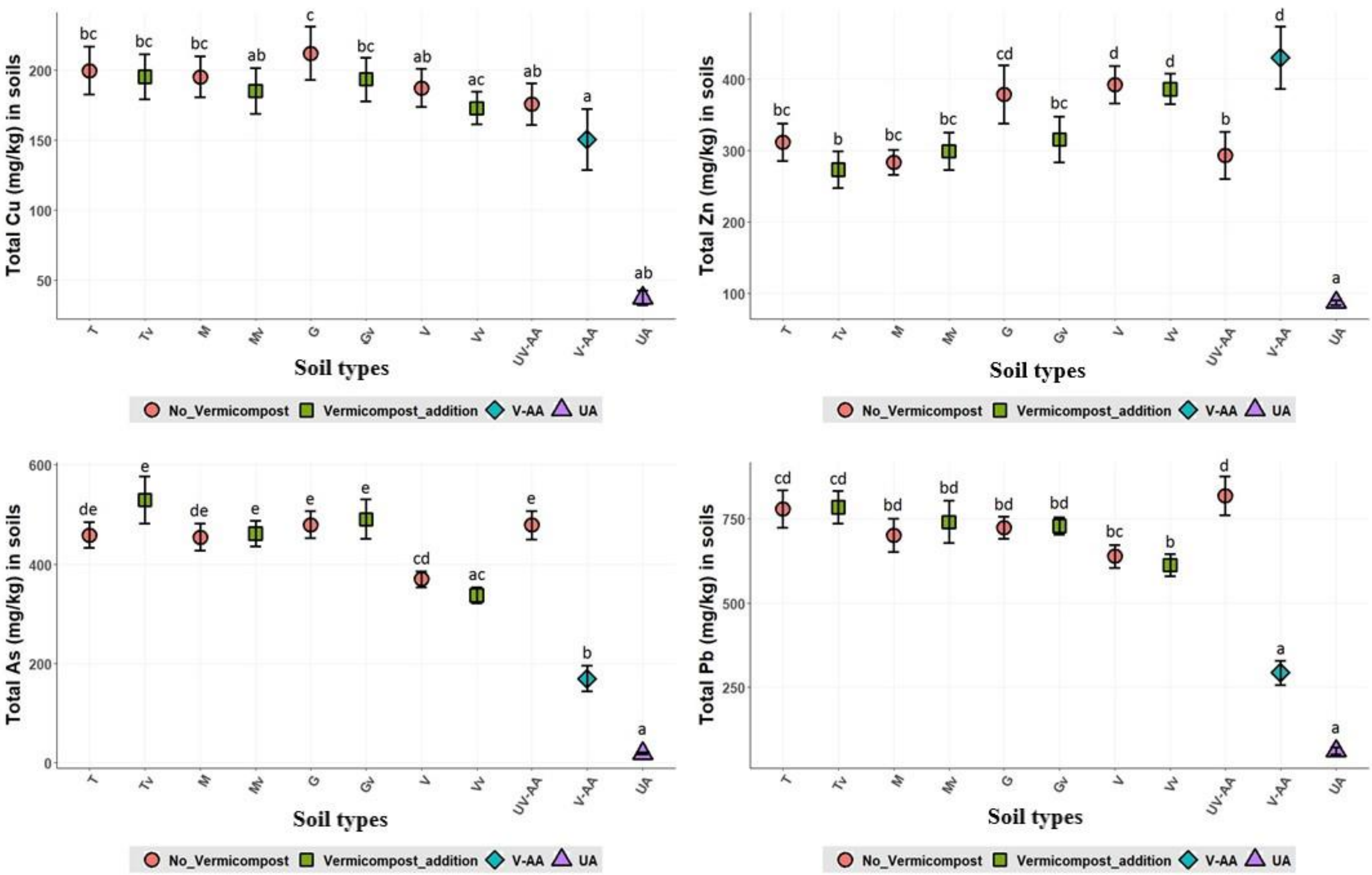


Figure 3.4. Total concentration (mean±SE) of main pollutants (Cu, Zn, As and Pb) in soils. Soil types: i): **UA**: Unaffected soils; ii) **UV-AA**: Unvegetated Affected soils; iii) **V-AA**: Vegetated Affected soils; and iv) Soil treatments: **G**: Addition of gypsum mining spoil; **M**: Addition of marble sludge; **T**: Tillage; **V**: mixture of UV-AA and V-AA; **Gv**: G plus vermicompost (v); **Mv**: M + v; **Tv**: T + v; **Vv**: V + v. Different letters above symbols represent statistically significant differences ($p < 0.05$) for the *post-hoc* Tukey tests performed after the LMMs.

3.3. Effect of amendments on soil properties and PHEs

The effectiveness of the application of certain organic and/or inorganic amendments in the remediation of these soils have been previously reported by other authors (Adriano et al., 2004; Aguilar et al., 2004c; Madejón et al., 2018). In our case, the application of gypsum mining spoil (G) improved some soil properties (Figure 3.3). For instance, the calcium carbonate content and pH significantly increased in relation to UV-AA, tending to approximate the values measured in V-AA, as a result of the high amount of $CaCO_3$ in the gypsum-rich amendment (27.25 ± 3.52 %). The EC did not show significant differences in relation to UV-AA because gypsum in soil releases available salts that are exchangeable with PHEs (Wallace and

Wallace, 1995; Casas-Castro and Casas-Barba, 1999). The OC content did not show a significant increase because gypsum mining spoil contains negligible quantities of organic matter, what resulted in a very low OC input to the soil (0.04 ± 0.03 %). Comparing with the other treatments, the gypsum mining spoil amendment (G) improved pH and calcium carbonate content significantly more than treatment T and raised these variables to similar values to those observed in treatment V. Treatment M was the one that most strongly increased the $CaCO_3$ content (marble sludge contains 99% $CaCO_3$), thus promoting the raise of pH (Martín et al., 2003; Aguilar et al., 2007) to similar values to those of the soils from both the unaffected (UA) and vegetated affected (V-AA) areas, but it neither reduced EC significantly (marginal differences), nor increased OC content. The application of vermicompost (v), which contained 23 % of OC, to all the treatments produced significant changes in the OC content, but not in the other soil properties analysed. The rate at which OC increased after vermicompost application in Tv, Mv and Gv treatments was higher (between 56-69%) than in Vv (39%). This lower relative increase in OC content in Vv treatment (addition of soils from the vegetated affected area, V-AA) may be directly related to a higher biological activity that produced a higher mineralization rate of the organic matter included with the vermicompost; meanwhile, in the other treatments, as the biological activity was strongly limited before the addition of vermicompost, the rate at which organic matter was mineralized after adding this amendment was lower than in Vv (García-Carmona et al., 2017).

The effects of treatments on the reduction of the total concentrations of Pb and As, the least mobile elements (Galán et al., 2002; Simón et al., 2008; Yang et al., 2012), were only significant in V treatment (Figure 3.4), as a result of a dilution effect. This dilution effect was also assessed during the implementation of the remediation plan, and was indeed used as a treatment itself to reduce the PHE total concentrations reached in the uppermost part of the soil in some areas after the clean-up operations, below the regulatory thresholds (Aguilar et al., 2004b). The case of total Cu and Zn, elements with higher mobility (Galán et al., 2002; Kraus and Wiegand, 2006; Simón et al., 2008), was the opposite; since the concentration of these elements in V-AA were either similar (Cu: 150.49 ± 21.88 mg kg^{-1}) or higher (Zn: 429.76 ± 43.74 mg kg^{-1}) than in UV-AA (Cu: 175.84 ± 14.87 and Zn: 293.49 ± 32.95, in mg kg^{-1}) (Figure 3.4), their mixture in V treatment resulted in a significant increase of total Zn (392.51 ± 26.03 mg kg^{-1}). In the treated soils where amendments were applied (G and M), the dose was not high enough as to produce a significant reduction of PHE total concentrations, and the same was true for vermicompost addition.

3.4. Mobility of PHEs in soils and treatments

The values of soluble forms were analysed and represented as the ratio between soluble and total concentrations (Figure 3.5), because both the solubility in water of the main pollutants and their total concentrations strongly changed between soil types: unaffected area (UA), vegetated (V-AA) and unvegetated (UV-AA) affected areas. The content of PHE soluble forms can be found in Appendix 3.2.

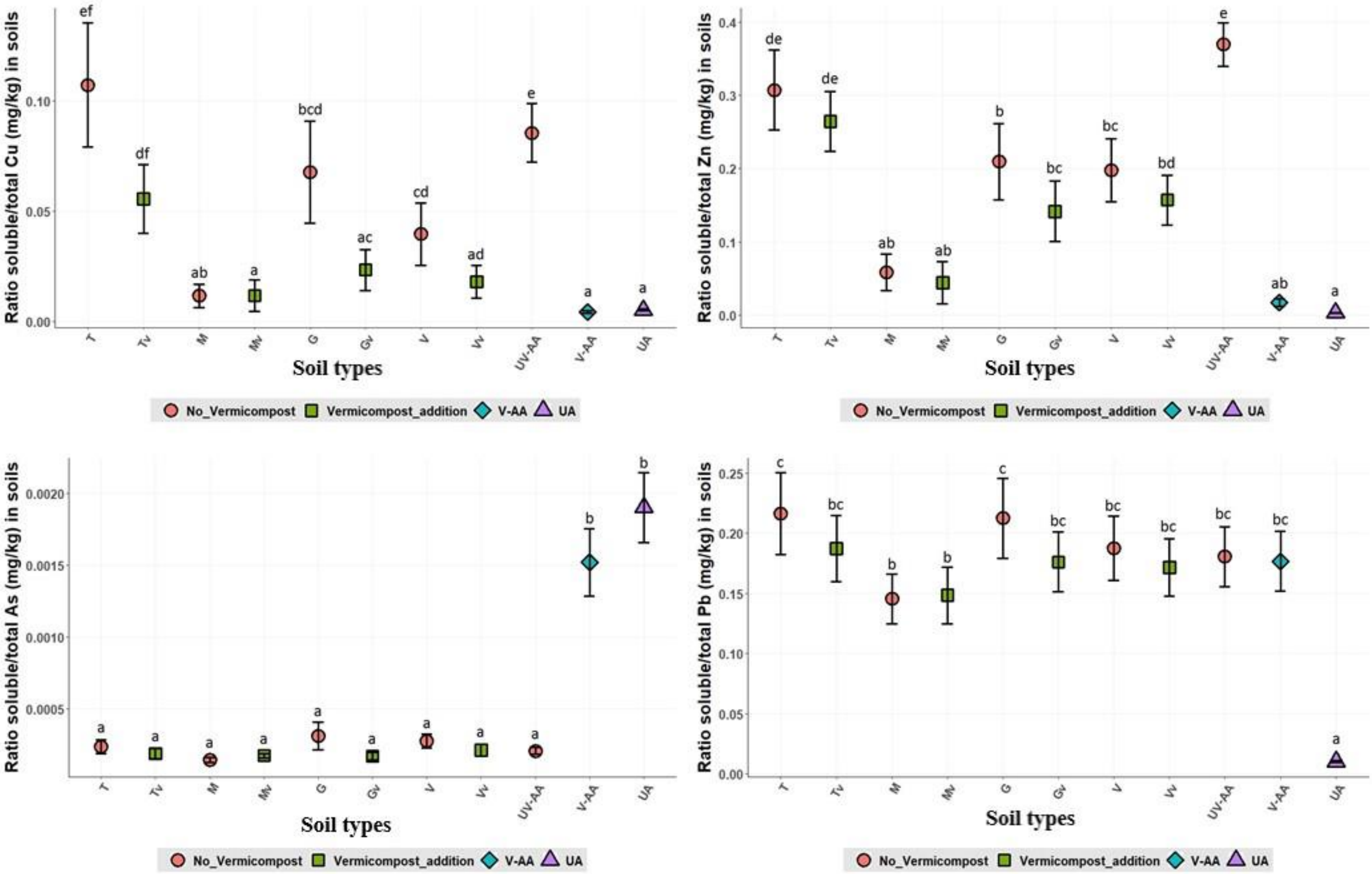


Figure 3.5. Ratio (mean±SE) between soluble and total concentrations of main pollutants (Cu, Zn, As and Pb) in soils. Soil types: i) **UA**: Unaffected soils; ii) **UV-AA**: Unvegetated Affected soils; iii) **V-AA**: Vegetated Affected soils; and iv) Soil treatments: **G**: Addition of gypsum mining spoil; **M**: Addition of marble sludge; **T**: Tillage; **V**: mixture of UV-AA and V-AA; **Gv**: G plus vermicompost (v); **Mv**: M + v; **Tv**: T + v; **Vv**: V + v. Different letters above symbols represent statistically significant differences ($p < 0.05$) for the *post-hoc* Tukey tests performed after the LMMs.

The solubility ratio of Cu and Zn in UA and V-AA soils (Cu: 4.79 E^{-3} ± 4.50 E^{-4} and Zn: 0.01 ± 7.00 E^{-3}, on average) did not present significant differences, pointing out that the rehabilitation plan implemented in the GGC after the accident, along with the role of vegetation, have effectively reduced the solubility of these elements in V-AA (Simón et al., 2010). Conversely, a sharp increase in Cu and Zn solubility was detected in UV-AA (0.09 ± 0.01 and 0.35 ± 0.03, respectively); the increase in soluble forms of these elements is strongly related to soil properties, being the acid pH in these soils the main responsible (Simón et al., 2005). Pb presented a different behaviour, with the lowest solubility ratio in UA (0.01 ± 9.00 E^{-4}), and a very high increase both in V-AA (0.18 ± 0.02) and UV-AA (0.18 ± 0.02), with no significant differences between them. The presence of residual pollution in the affected area (both in vegetated and in unvegetated soils) indicates a clear risk of dispersion of Pb soluble forms; in this way, the increase in Pb solubility was previously reported in acidic conditions (McLean and Bledsoe, 1992), like in UV-AA, and also in neutral soils in the area, like in V-AA, probably as a result of the interference of the calcium carbonate applied as part of the rehabilitation plan, which would have reacted with iron oxides, releasing Pb from sorption sites (Martín-Peinado et al., 2014). Moreover, the formation of complexes between Pb and organic matter in V-AA soils may have also contributed to the high Pb solubility under these soil conditions (Sauvé et al., 1998). Consequently, Pb solubility should be monitored over time in more detail to avoid its rise in the areas affected by residual pollution. Arsenic presented the highest solubility ratios both in UA (1.90 E^{-3} ± 2.44 E^{-4}) and V-AA (1.52 E^{-3} ± 2.34 E^{-4}), despite the concentrations of soluble forms in UA were the lowest (0.03 mg kg^{-1}) and significantly different to V-AA (0.17 mg kg^{-1}). The solubility of As significantly decreased in UV-AA (2.09 E^{-4} ± 2.55^{-5}), indicating a different behaviour of this element compared to the other pollutants. In this way, the adsorption of As in soils under acidic conditions is very high due to the presence of iron oxides (Simón et al., 2005), but when the pH approximates neutrality (like in V-AA and UA), an increase in bioaccessibility has also been reported (McLean and Bledsoe, 1992; Yang et al., 2012; Fleming et al., 2013).

Treatments strongly modified the ratio between soluble forms and total concentrations for some elements when compared to UA, V-AA and UV-AA soils (Figure 3.5). For instance, the most effective treatment in terms of reducing Cu and Zn solubility was the application of marble sludge (M) (0.01 ± 0.01 and 0.06 ± 0.02 mg kg^{-1}, respectively), and the least effective was tillage (T), whose solubility was similar to that in UV-AA soils. The role of

$CaCO_3$ has previously been proved to reduce Cu and Zn solubility, due to the great adsorption capacity of carbonates (Simón et al., 2010; García-Carmona et al., 2019b). The other simple treatments (G and V) presented an intermediate effect on Cu and Zn solubility reduction, without significant differences between them. The addition of vermicompost (v) to simple treatments decreased Cu and Zn soluble forms in Tv, Gv and Vv, although these differences were only significant for Cu. The influence of organic matter on the reduction of Cu mobility has previously been reported (McLaren et al., 1981) and is related to the specific binding of Cu by organic matter. The application of treatments did not significantly change Pb solubility compared to V-AA and UV-AA, although marginal differences in solubility reduction were detected when marble sludge was applied (0.15 ± 0.02 mg kg^{-1}); in this sense, Simón et al. (2005) observed that $CaCO_3$ can promote the precipitation of Pb soluble forms in soils with pH> 3.8. In the case of As, treatments did not significantly change its solubility compared to the UV-AA, maintaining it low in all the cases and with no significant differences. Nevertheless, considering that the solubility ratio of As in V-AA and UA soils was significantly higher, the mobility of this element should be monitored over time, especially in those treatments that contains $CaCO_3$ and organic matter (García-Carmona et al., 2017) such as Mv and Gv (marble sludge and gypsum mining spoil with vermicompost, respectively), to prevent rise in As mobility over time. Finally, the addition of vermicompost did not produce any significant reduction in terms of As solubility ratio, although marginally reduced it for Pb in Tv and Gv.

3.5. Bioavailability of pollutants in soils and treatments

Bioavailable forms were analysed and represented as the ratio between bioavailable and total concentrations (Figure 3.6), because both the availability of the main pollutants for living organisms and their total concentrations strongly changed between soil types, especially between unaffected (UA) and unvegetated affected (UV-AA) areas. The content of PHE bioavailable forms can be found in Appendix 3.3.

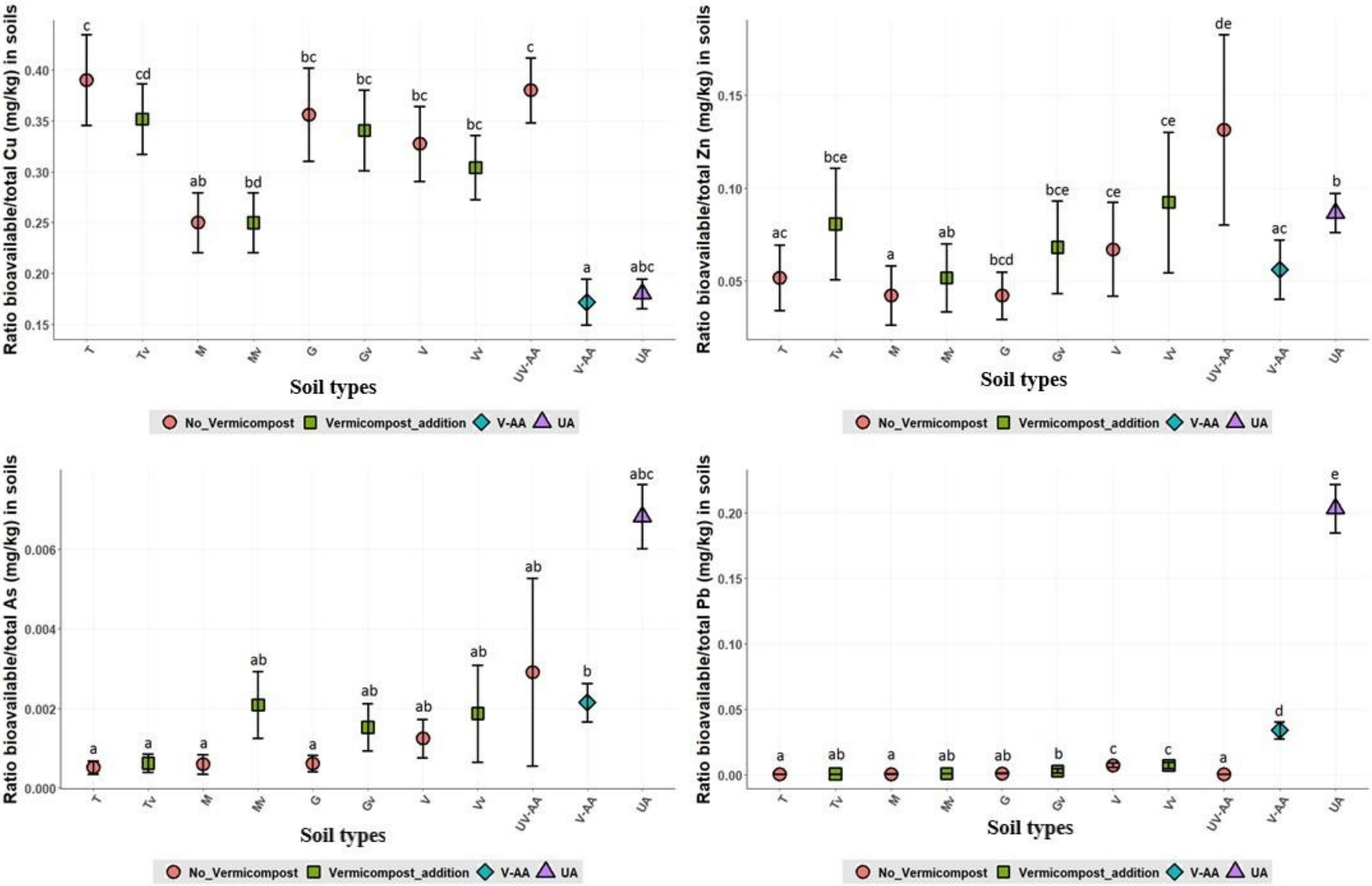


Figure 3.6. Ratio (mean±SE) between bioavailable and total concentrations of main pollutants (Cu, Zn, As and Pb) in soils. Soil types: i) **UA**: Unaffected soils; ii) **UV-AA**: Unvegetated Affected soils; iii) **V-AA**: Vegetated Affected soils; and iv) Soil treatments: **G**: Addition of gypsum mining spoil; **M**: Addition of marble sludge; **T**: Tillage; **V**: mixture of UV-AA and V-AA; **Gv**: G plus vermicompost (v); **Mv**: M + v; **Tv**: T + v; **Vv**: V + v. Different letters above symbols represent statistically significant differences ($p < 0.05$) for the *post-hoc* Tukey tests performed after the LMMs.

The lowest values of Cu bioavailability ratios were measured in the soils from the unaffected (UA, 0.18 ± 0.01) and vegetated affected (V-AA, 0.17 ± 0.02) areas, whereas a sharp increase was observed in the soils from the unvegetated affected areas (UV-AA, 0.38 ± 0.03), where residual pollution is still high. These results indicate that the clean-up operations, followed by the addition of amendments and afforestation significantly reduced Cu bioavailable forms in V-AA, probably due to the significant pH increase (García-Carmona et al., 2017; Madejón et al., 2018), while the poor effectiveness of remediation measures in the unvegetated soils (UV-AA) maintained the acid pH and thus a high ratio of bioavailable Cu. Regarding Zn bioavailability ratio, differences between soil types were just marginal due to the high standard deviation; even though, the ratio (mean ± SE) was highest in UV-AA (0.13 ± 0.05), followed by UA (0.09 ± 0.01), and lowest in V-AA (0.06 ± 0.01), what implies that the risk of Zn bioavailability in the affected areas has been drastically reduced, especially in the vegetated ones (V-AA). In this sense, it has been described that the elements with high mobility such as Cu and Zn, infiltrated to deeper layers or were displaced to the surrounding less polluted soils as a result of runoff processes (Kraus and Wiegand, 2006; Martín-Peinado et al., 2015). Conversely, the trend of the least mobile elements such as Pb and As is different; Pb and As bioavailability ratios in the affected areas (V-AA and UV-AA) were lower than in UA (Pb: 0.20 ± 0.02 and As: 6.8 E^{-3} ± 8.01 E^{-4}), with significant (for Pb) and marginal (for As) differences, suggesting that, despite the high total concentrations of these two elements in V-AA and UV-AA, they seem to have been immobilized in soils by iron oxides (Aguilar et al., 2004a; Aguilar et al., 2007). In the case of Pb, there were also significant differences between vegetated (0.03 ± 6.59 E^{-3}) and unvegetated soils (5.02 E^{-4}± 1.04 E^{-4}), what could be related to differences in the solubility of the precipitates formed in each soil type; in UV-AA soils, Pb may have been immobilized by Fe-hydroxysulphates and co-precipitated in coatings isolating $CaCO_3$ particles (Simón et al., 2010; García-Carmona et al., 2019a); meanwhile, in V-AA, co-precipitation with carbonates should be dominant (Simón et al., 2005), and the presence of vegetation may have promoted the formation of organo-Pb complexes that increased Pb availability when the pH raised above 6.5 (Sauvé et al., 1998). It has been described that pH and OC may increase the availability of Pb and As (Sierra-Aragón et al., 2019); hence, Pb and As bioavailability is controversial, and there could be a potential environmental risk in the medium-long term if these areas are revegetated without the proper immobilization of Pb and As. Consequently, the application of amendments to reduce the bioavailability of these elements is necessary and requires monitoring over time.

According to the effects of treatments (Figure 3.6), Cu bioavailability ratio was reduced by the treatments with gypsum mining spoil (G, 0.36 ± 0.05) and with V-AA soil mixture (V, 0.33 ± 0.04), with marginal differences, and by the treatment with marble sludge (M, 0.25 ± 0.03), with significant differences and approaching the values in V-AA and UA, probably due to the presence of $CaCO_3$ and thus pH raise (García-Carmona et al., 2017; Madejón et al., 2018). The M, G and T treatments significantly reduced Zn bioavailability ratio (0.04 ± 3.17 E^{-3} on average), towards similar values to those in V-AA. The addition of vermicompost (v) did not produce any significant change in the Cu or Zn bioavailability in relation to the simple treatments. Pb bioavailability in any treated soil was lower than in UA and in V-AA, especially where G, M and T treatments were applied (5.94 E^{-4} ± 1.44 E^{-4} on average). Nonetheless, the addition of soil from V-AA areas (treatment V), where Pb was more bioavailable, significantly increased the bioavailability ratio (7.34 E^{-3} ± 1.82 E^{-3}). Although the addition of vermicompost (v) to these treatments (Gv, Mv and Tv) increased Pb bioavailability above the ratio in UV-AA (marginal differences), it was still lower than that in the unaffected soil (UA). Finally, the standard deviation of UV-AA soil samples in terms of As bioavailability ratio was high, masking all the possible significant effects that treatments could have had on the reduction of As bioavailable forms; for instance, G, M and T treatments tended to have the lowest values (5.81 E^{-4} ± 3.16 E^{-5}). Nevertheless, focusing on As bioavailable concentration (Appendix 3.3) in the soils treated with gypsum mining spoil (G), this value was high (at the same level as T and UV-AA, 4.06 ± 0.09 mg kg^{-1} on average) probably due to the interference of sulphates with As adsorption by iron oxides at acid pH (Hering et al., 1997; Meng et al., 2000). The addition of vermicompost (v) to M and G treatments (Mv and Gv) tended to increase the ratio (marginal differences), probably because $CaCO_3$ (from marble sludge and gypsum mining spoil) and organic matter (from vermicompost) may be forming co-precipitates with Fe (García-Carmona et al., 2019a), releasing As from sorption sites.

These results suggest that monitoring is needed when calcium, sulphate- and/or organic matter- rich amendments are used to restore polluted soils when Pb and As are present, to avoid an increase of bioavailability over time.

3.6. Toxicity bioassay

Regarding the potential toxicity of PHEs in a scenario where vegetation would spontaneously recolonize unvegetated soils after the application of amendments, a toxicity bioassay would help evaluate the potential negative effects of PHEs on living organisms (Romero-Freire et al., 2016). Hence, we conducted a short-term toxicity bioassay with *Lactuca sativa* L. based on seed germination and root elongation. According to our results (Figure 3.7), there was no PHE toxicity in the unaffected area (UA) (100% germination and root elongation). Despite the presence of relatively high concentrations of PHEs in the vegetated affected areas (V-AA), *L. sativa* germination and root elongation were very high (>90% and > 95% respectively) and similar to those in the unaffected area (UA), with no significant differences between them, indicating no phytotoxicity in the vegetated affected areas. Conversely, the germination of *L. sativa* in the water extract from UV-AA soils was significantly lower (<40%), and the same happened when comparing the root elongation of *L. sativa* (<20%) with the control (distilled water), suggesting the existence of PHE toxicity in UV-AA soils, as observed by other authors in previous studies in the area (Romero-Freire et al., 2015; García-Carmona et al., 2017; Pastor-Jáuregui et al. 2020).

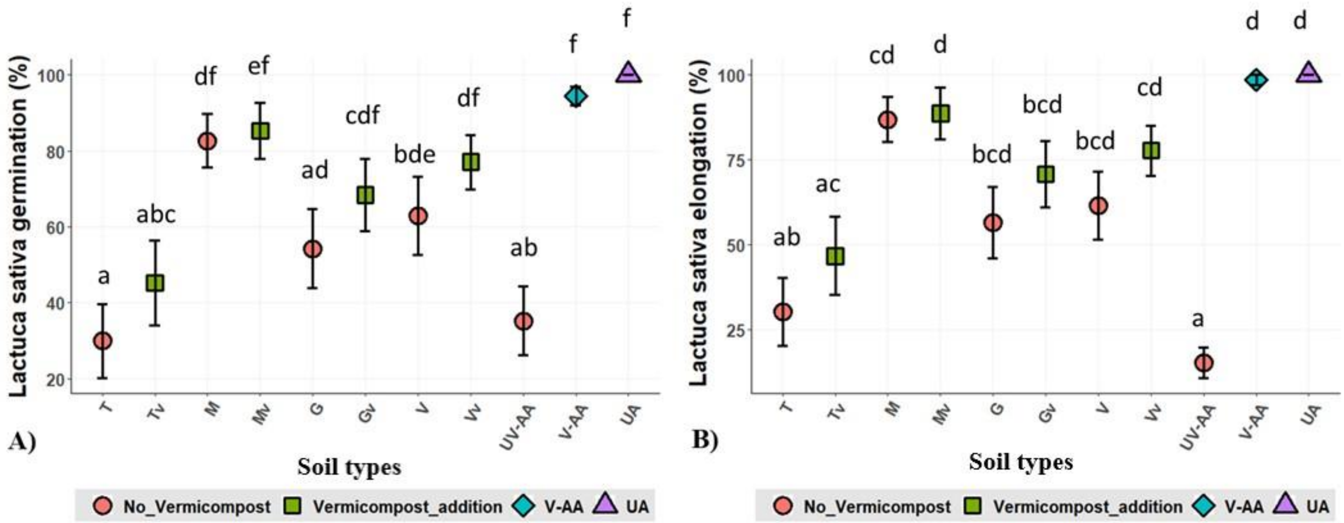


Figure 3.7. Toxicity bioassays with *Lactuca sativa*: A) germination; B) root elongation. Soil types: i) **UA**: Unaffected soils; ii) **UV-AA**: Unvegetated Affected soils; iii) **V-AA**: Vegetated Affected soils; and iv) Soil treatments: **G**: Addition of gypsum mining spoil; **M**: Addition of marble sludge; **T**: Tillage; **V**: mixture of UV-AA and V-AA; **Gv**: G plus vermicompost (v); **Mv**: M + v; **Tv**: T + v; **Vv**: V + v. Different letters above symbols represent statistically significant differences ($p < 0.05$) for the *post-hoc* Tukey tests performed after the LMMs.

In order to evaluate the relative influence of soil properties and PHE soluble and total concentrations on organisms (bioassays), a Non-Metric Multidimensional Scaling (NMDS) ordination analysis was performed (R^2= 0.99, stress= 0.055) (Figure 3.8).

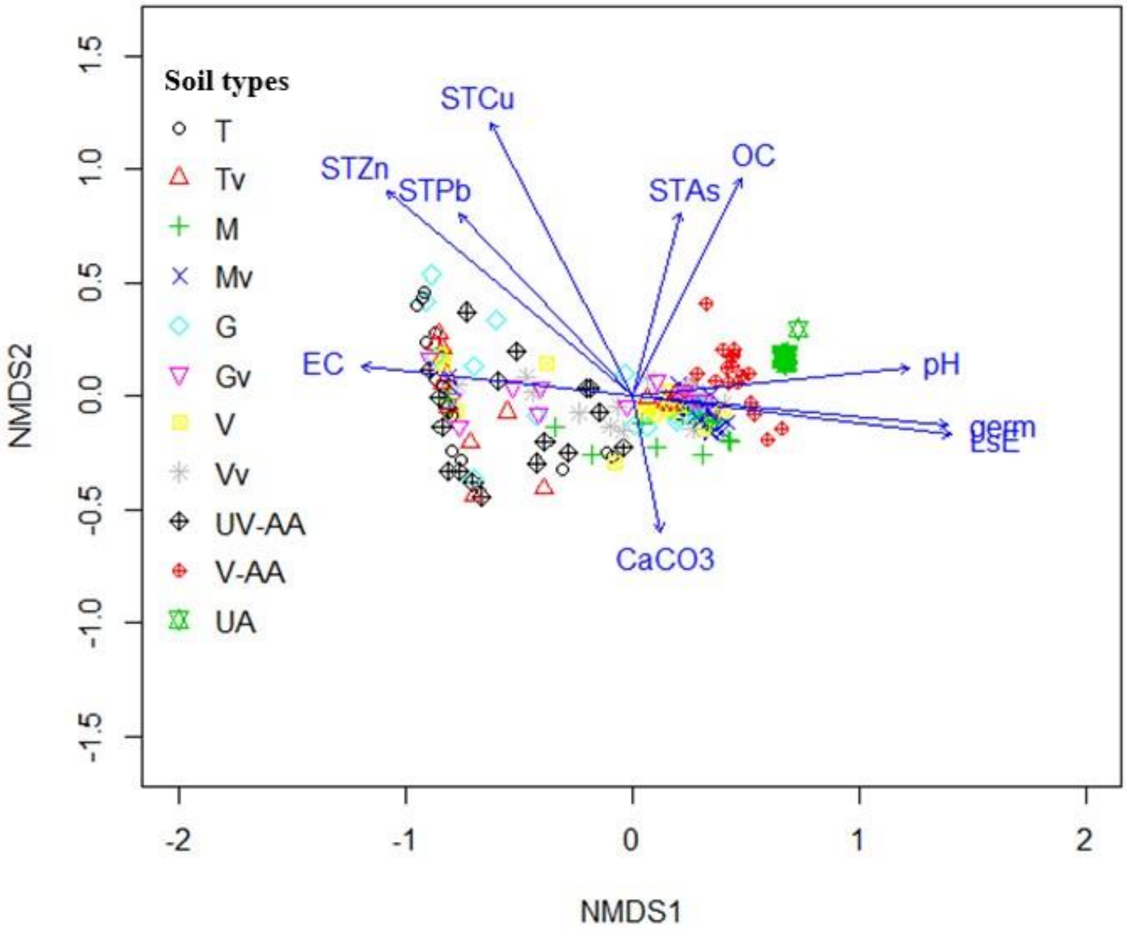


Figure 3.8. Non-Metric Multidimensional Scaling (NMDS) ordination diagrams showing the relative influence of Zn, Cu, Pb and As solubility ratios (STZn, STCu, STPb, STAs, respectively) and soil properties: EC, OC, pH, $CaCO_3$, on *Lactuca sativa* germination (germ) and root elongation (LsE) according to soil types: i) **UA**: Unaffected soils; ii) **UV-AA**: Unvegetated Affected soils; iii) **V-AA**: Vegetated Affected soils; and iv) Soil treatments: **G**: Addition of gypsum mining spoil; **M**: Addition of marble sludge; **T**: Tillage; **V**: mixture of UV-AA and V-AA; **Gv**: G plus vermicompost (v); **Mv**: M + v; **Tv**: T + v; **Vv**: V + v.

In figure 3.8, factors are represented by arrows, whose lengths reflect their relative weight. In this sense, we observe that *L. sativa* germination and root elongation are highly related to pH and inversely related to the electrical conductivity (EC), probably due to nutritional imbalances (Ünlükara et al., 2008). The solubility ratio of Cu, Zn and Pb are strongly correlated among each other, and inversely related to $CaCO_3$ content, which could have promoted Cu, Zn and Pb precipitation through pH rise (Martín et al., 2003; Aguilar et al., 2004a). Moreover, the solubility of these elements is inversely related to *L. sativa* germination and root elongation. Altogether suggests a potential synergistic relation among these elements (Kutrowska et al., 2017). The solubility ratio of As was inversely related to $CaCO_3$, but directly related to OC; it has been described that organic matter increases pH in acidic environments, what may transform iron oxy-hydroxides into more crystalline forms, promoting As desorption (Pedersen et al., 2006; Sierra-Aragón et al., 2019). Moreover, a higher availability of phosphorus may have interfered with As for sorption sites (Costa et al., 2012).

In the unaffected (UA) and vegetated affected (V-AA) areas, the most important factor influencing *L. sativa* germination and root elongation was mainly soil pH, and secondarily OC content, suggesting that soil properties are positive for plant development and that PHE toxicity was low. Nevertheless, in the case of the unvegetated affected areas (UV-AA), *L. sativa* germination and root elongation were mainly dominated by EC, and secondarily by Zn, Cu and Pb solubility ratios, indicating that these factors are the main responsible of phytotoxicity in this area.

Focusing on the effect of soil treatments, remediation measures have reduced PHE toxicity in UV-AA soils in different rates. According to bioassays (Figures 3.7 and 3.8), gypsum mining spoil decreased toxicity (germination and elongation rates >50%) in line with the response in V treatment (addition of V-AA soils), but just with marginal differences with UV-AA; however, marble sludge (M) was the most effective treatment, presenting germination and elongation rates (>80% for both) close to those in V-AA and UA, and with just marginal differences. Both gypsum mining spoil and marble sludge are calcium-rich amendments, however, marble sludge increased soil pH significantly more than gypsum mining spoil, probably due to the higher $CaCO_3$ content in the former. As a result, we observed (Figure 3.8) that the influence in Cu, Zn and Pb toxicity is higher on the soils treated with gypsum mining spoil (G) (and in treatment V). In this sense, Romero-Freire et al. (2016) reported that Cu and Zn solubility are strongly related to soluble salts, mainly sulphates. The electrical conductivity in treatment G was high, what negatively affected *L. sativa* germination and root elongation (Figure 3.8); in this respect, Van Lierop and Mackenzie (1975) observed that lettuce yield in gypsum-amended soils were the lowest, probably due to a sulphate ion toxicity, and Zoca and Penn (2017) reported that even if gypsum improves soil chemical properties, this may not be translated into increased yields. The least effective treatment was crust breaking through tillage (T), with germination and elongation rates (<35% in both cases) similar to those recorded for UV-AA; in this treatment there was no PHE immobilization by amendments or PHE solubility reduction, just a dilution effect. Finally, the addition of vermicompost enhanced the properties of simple treatments, with marginal differences in all the cases, probably due to the capacity of organic matter to bind PHEs such as Cu, Zn (Sierra-Aragón et al., 2019).

Assisted Natural Remediation through amendment application could improve soil properties (increase in OC, pH, etc.), what may produce changes in PHE mobility that should be considered over time. Our results along with previous studies, focus on the need of monitoring the evolution of PHEs in the Guadiamar Green Corridor, since these elements can be remobilized both from acid soils (Kraus and Wiegand, 2006), and neutral-alkaline soils (Sierra-Aragón et al., 2019) making them more available for living organisms.

4. CONCLUSIONS

Total concentrations of As and Pb in some areas of the Guadiamar Green Corridor surpassed toxicity regulatory threshold and, thus, these areas need to be considered as potentially polluted. In this sense, potential mobility of these elements was assessed. Results indicated that the solubility and bioavailability of As were low in the polluted areas, probably due to the effect of iron oxides, which strongly adsorbed this element. In the case of Pb, its bioavailability was also low, probably because of the co-precipitation of this element with iron-hydroxysulphates; however, Pb solubility was high in these soils, probably due to the competence with $CaCO_3$ for sorption sites in the iron oxides.

Soil treatments with inorganic and organic amendments presented different results in relation to the reduction of PHE mobility and toxicity. Although none of the treatments managed to reduced Pb solubility under background concentrations, marble sludge was the most effective treatment, managing to increase pH towards neutrality as a result of the significant increase of calcium carbonate content in soils, and to reduce PHE solubility, bioavailability and thus, toxicity. Gypsum mining spoil was less effective, mainly because pH increased was less pronounced and the electrical conductivity was still high, resulting in a lesser reduction of PHE availability and toxicity. Regarding the other soil treatments, tillage did not improve soil conditions, whereas the mixture of unvegetated and vegetated soils reduced total concentrations of Pb and As, and Cu and Zn solubility. Despite the controversial role of organic matter in PHE immobilization, the addition of vermicompost to simple treatments did not increase PHE solubility or bioavailability, except in the case of As, which tended to be more bioavailable when combined with marble sludge and gypsum mining spoil.

Toxicity assays indicated that marble sludge was the most promising treatment and that the addition of vermicompost to the unvegetated affected soils treated with gypsum mining spoil, tillage or with the mixture of vegetated soils could improve their capacity to reduce PHE toxicity.

In spite of the positive results obtained for marble sludge and gypsum mining spoil, and due to the controversial behavior of Pb and As, there could be a potential environmental risk in the medium-long term if these areas are revegetated without the proper immobilization of these two elements. Thus, monitoring is highly required, especially in those areas treated with amendments that contain $CaCO_3$, sulphates and/or organic matter, such as marble sludge and gypsum mining spoil with vermicompost.

The main challenge lies on finding the right dose and combination of amendments in order to assist the natural remediation of these affected soils. Several studies have highlighted the need to retreat the affected areas in order to maintain the effect of amendments and to prevent new acidification processes, which could remobilize PHEs in the soils. According to our findings, retreatment could be advisable to guarantee the positive evolution of unvegetated soils in the area. All the lessons learnt in this study provide valuable information about new rehabilitation strategies that could be transposed to similar scenarios all over the world.

5. ACKNOWLEDGEMENTS

The authors would like to thank KNAUF for the financial support. We also would like to thank Francisco Javier Martínez Garzón, Yasuo Mitsui Nakamaru and Mariano Simón for their valuable help setting up the experimental plots.

This work was supported by the Research Project RTI 2018-094327-B-I00 (Ministry of Science, Innovation and Universities).

Helena García Robles was financed by Tatiana-Pérez-de-Guzmán-el-Bueno Foundation PhD grant Programme 2016.

6. REFERENCES

Adriano, D.C., Wenzel, W., Vangronsvel, J., Bolan, N., 2004. Role of assisted natural remediation in environmental cleanup. Geoderma, 122 (2): 121-142. https://doi.org/10.1016/j.geoderma.2004.01.003

Aguilar, J., Dorronsoro, C., Galán, E., Gómez, L., 1999. Criterios y estándares para declarar un suelo como contaminado en Andalucía. Metodología, toma de muestras y análisis para su investigación, in: Investigación y Desarrollo Ambiental en Andalucía, ed Universidad de Sevilla. Seville, Spain. pp 45-59.

Aguilar, J., Dorronsoro, C., Fernández, E., Fernández, J., García, I., Martín, F., Simón, M., 2004a. Remediation of Pb-contaminated soils in the Guadiamar River Basin (SW Spain). Water Air Soil Poll., 151: 323-333. https://doi.org/10.1023/b:wate.0000009909.31857.aa

Aguilar, J., Dorronsoro, C., Fernández, E., Fernández, J., García, I., Martín, F., Simón, M., 2004b. Soil pollution by a pyrite mine spill in Spain: evolution in time. Environ. Pollut., 132: 395-401. https://doi.org/10.1016/j.envpol.2004.05.028

Aguilar, J., Bouza, P., Dorronsoro, C., Fernández, E., Fernández, J., García, I., Martín, F., Simón, M., 2004c. Application of remediation techniques for immobilization of metals in soils contaminated by a pyrite tailing spill in Spain. Soil Use Manage., 20: 451-453. https://doi.org/10.1111/j.1475-2743.2004.tb00396.x

Aguilar, J., Dorronsoro, C., Fernández, E., Fernández, J., García, I., Martín, F., Sierra, M., Simón, M., 2007. Remediation of As-contaminated soils in the Guadiamar River Basin (SW Spain). Water Air Soil Poll., 180: 109-118. https://doi.org/10.1007/s11270-006-9254-3

Al-Hamaiedh, H., 2010. Reuse of marble sludge slime in ceramic industry. Jordan J. Civ. Eng., 4 (3): 264-271.

Alastuey, A., García-Sánchez, A., López, F., Querol, X., 1999. Evolution of pyrite mud weathering and mobility of heavy metals in the Guadiamar valley after the Aznalcóllar spill, south-west Spain. Sci. Total Environ., 642: 41-55. https://doi.org/10.1016/S0048-9697(99)00375-7

Amezketa, E., Aragues, R., Bazol, R., 2005. Efficiency of sulfuric acid, mined gypsum and two gypsum byproducts in soil crusting prevention and sodic soil reclamation. Agron. J., 97: 983-989. https://doi.org/10.2134/agronj2004.0236

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

Barton, K., 2016. Multi-model inference. R package version 1.15.6. Available at: https://cran.r-project.org/web/packages/MuMIn/index.html

Bates, D., Maechler, M., Bolker, B., Walker, S., 2015. Fitting linear mixed-effects models using lme4. J. Stat. Softw., 67(1): 1-48. https://doi.org/10.18637/jss.v067.i01

Beckett P.H.T., 1989. The use of extractants in studies on trace metals in soils, sewage, sludge, and sludges-treated soils. Adv. Soil S., 9: 143-176.

Bernal, M.P., Clemente, R., Walker, D.J., 2007. The role of organic amendments in the bioremediation of heavy metal-polluted soils, in: Gore, R.W., (Ed.), Environmental Research at the Leading Edge, Nova Science Publishers, Inc., New York, EEUU.

Bini, C., Bech, J., 2014. PHEs, environment and human health: Potentially harmful elements in the environment and the impact on human health. Springer Dordrecht Heidelberg New York London. https://doi.org/10.1007/978-94-017-8965-3

BOJA 2015. Decreto 18/2015, de 27 de enero, por el que se aprueba el reglamento que regula el régimen aplicable a los suelos contaminados. Junta de Andalucía.

Busari, M.A., Kukal, S.S., Kaur, A., Bhatt, R., Dulazi, A.A., 2015. Conservation tillage impacts on soil, crop and the environment. Int. Soil Water Conserv. Res., 3: 119-129. https://doi.org/10.1016/j.iswcr.2015.05.002

Cabrera, F., Clemente, L., Cordón, R., Hurtado, M.D., López, R., Madejón, P., Marañón, T., Moreno, F., Murillo, J.M., Nagel, I., 2005. Effect of remediation on trace metal pollution in soils of Guadiamar river valley, in: Del Valls, T.A., Blasco, J. (Eds.), Integrated Assessment and Management of the Ecosystems Affected by the Aznalcóllar Mining Spill (SW, Spain). Cátedra UNESCO/Unitwin, Cádiz, pp. 33-40.

Cabrera, F., Ariza, J.L., Madejón, P., Madejón, E., Murillo, J.M., 2008. Mercury and other trace elements in soils affected by the mine tailing spill in Aznalcóllar (SW Spain). Sci. Total Environ., 390: 311-322. https://doi.org/10.1016/j.scitotenv.2007.10.002

Casas-Castro, A., Casas-Barba, E., 1999. El análisis de suelo-agua-planta y su aplicación en la nutrición de los cultivos hortícolas en la zona del sureste peninsular. Caja Rural de Almería.

Clemente, R., Walker, D.J., Roig, A., Bernal, M.P., 2003. Heavy metal bioavailability in a soil affected by mineral sulphides contamination following the mine spillage at Aznalcóllar (Spain). Biodegradation, 14: 199-205. https://doi.org/10.1023/A:1024288505979

CMA (Consejería de Medio Ambiente), 2003. Ciencia y restauración del río Guadiamar. PICOVER 1998-2002. Junta de Andalucía, Sevilla, Spain.

Costa, E.T.S., Guilherme, L.R.G., Lopes, G., Lima, J.M., Curi, N., 2012. Competitive sorption of arsenate and phosphate on aluminium mining by-product. Water Air Soil Pollut., 223: 5433-5444. https://doi.org/10.1007/s11270-012-1291-5

Cowie, A.L., Orr, B.J., Castillo Sánchez, V.M., Chasek, P., Crossman, N.D., Erlewein, A., Louwagie, G., Maron, M., Metternicht, G.I., Minelli, S., Tengberg, A.E., Walter, S., Welton, S., 2018. Land in balance: The scientific conceptual framework for implementing land degradation neutrality. Environ. Sci. Policy, 79: 25-35. https://doi.org/10.1016/j.envsci.2017.10.011

Del-Moral-Torres, F., González, V., García, I., Sánchez, J.A., de-Haro, S., Simón, M., 2010. Effect of organic and inorganic residues on the solubility of trace elements and phytotoxicity of mining waste. Fresen. Environ. Bull., 19: 2071-2076.

Dinake, P., Kelebemang, R., 2019. Critical assessment of mechanistic pathways for chemical remediation techniques applied to Pb impacted soils at shooting ranges -A review. Environ. Pollutants Bioavailab., 31 (1): 282-305. https://doi.org/10.1080/26395940.2019.1676170

El-Sayed, H.A., Farag, A.B., Kandeel, A.M., Younes, A.A., Yousef, M.M., 2018. Characteristics of the marble processing powder waste at Shaq El-Thoaban industrial area, Egypt, and its suitability for cement manufacture. HBRC J., 14 (2): 171-179. https://doi.org/10.1016/j.hbrcj.2016.06.002

Escavy, J.I., Herrero, M.J., Arribas, M.E., 2012. Gypsum resources of Spain: Temporal and spatial distribution. Ore Geol. Rev., 49: 72-84. https://doi.org/10.1016/j.oregeorev.2012.09.001

FAO (Food and Agriculture Organization), 2015. Revised world soil charter. http://www.fao.org/3/a-i4965e.pdf. (Accessed April 2019).

Fernández-Caliani, J.C., Barba-Brioso, C., 2010. Metal immobilization in hazardous contaminated mine soils after marble slurry waste application. A field assessment at the Tharsis mining district (Spain). J. Hazard. Mater., 181: 817-826. https://doi.org/10.1016/j.jhazmat.2010.05.087

Fleming, M., Tai, Y., Zhuang, P., McBride, M.B., 2013. Extractability and bioavailability of Pb and as in historically contaminated orchard soil: effects of comport amendments. Environ. Pollut., 177: 90-97. https://doi.org/10.1016/j.envpol.2013.02.013

Franzen, D., Rehm, G., Gerwing, J., 2006. Effectiveness of gypsum in the North-Central Region of the U.S. NDSU Extension Service, North Dakota.

Gadepalle, V.P., Ouki, S.K., Van Herwijnen, R., Hutchings, T., 2007. Immobilization of heavy metals in soil using natural and waste materials for vegetation establishment on contaminated sites. Soil Sediment. Contam., 16: 233-251. https://doi.org/10.1080/15320380601169441

Galán, E., González, I., Fernández-Caliani, J.C., 2002. Residual pollution load of soils impacted by the Aznalcóllar (Spain) mining spill after clean-up operations. Sci. Total Environ., 286: 167-179. https://doi.org/10.1016/S0048-9697(01)00974-3

García-Carmona, M., Romero-Freire, A., Sierra Aragón, M., Martínez Garzón, F.J., Martín Peinado, F.J., 2017. Evaluation of remediation techniques in soils affected by residual contamination with heavy metals and arsenic. Journal Environ. Manage., 191: 228-236. http://doi.org/10.1016/j.jenvman.2016.12.041

García-Carmona, M., García-Robles, H., Turpín Torrano, C., Fernández Ondoño, E., Lorite Moreno, J., Sierra Aragón, M., Martín Peinado, F.J., 2019a. Residual pollution and vegetation distribution in amended soils 20 years after a pyrite mine tailings spill (Aznalcóllar, Spain). Sci. Total Environ., 650: 933-940. https://doi.org/10.1016/j.scitotenv.2018.09.092

García-Carmona, M., Romero-Freire, A. Sierra Aragón, M., Martín Peinado, F.J., 2019b. Effectiveness of ecotoxicological tests in relation to physicochemical properties of Zn and Cu polluted Mediterranean soils. Geoderma, 338: 259-268. https://doi.org/10.1016/j.geoderma.2018.12.016

Garrido, F., Illera, V., Vizcayno, C., García-González, M.T., 2003. Evaluation of industrial by-products as soil acidity amendments: chemical and mineralogical implications. Eur. J. Soil Sci., 54: 411-422. https://doi.org/10.1046/j.1365-2389.2003.00522.x

González, V., García, I., Del Moral, F., Simón, M., 2012. Effectiveness of amendments on the spread and phytotoxicity of contaminants in metal-arsenic polluted soil. J. Hazard. Mater., 205-206: 72-80. https://doi.org/10.1016/j.jhazmat.2011.12.011

González, V., Simón, M., García, I., Sánchez, J.A., del Moral, F., 2013. Effectiveness of amendments to restore metal-arsenic-polluted soil functions using *Lactuca sativa* L. bioassays. J. Soils Sediments, 13: 1213-1222. https://doi.org/10.1007/s11368-013-0701-7

González, V., Salinas, J., García, I., del Moral, F., Simón, M., 2017. Using marble sludge and phytoextraction to remediate metal(loid) polluted soils. J. Geochem. Explor., 174: 29-34. http://doi.org/10.1016/j.gexplo.2016.03.008

González-Núñez, R., Alba, M.D., Vidal, M., Rigol, A., 2015. Viability of adding gypsum and calcite for remediation of metal-contaminated soil: laboratory and pilot plant scales. Int. J. Environ. Sci. Technol., 12: 2697-2710. https://doi.org/10.1007/s13762-014-0671-3

Grimalt, J.O., Macpherson, E. (Eds.), 1999. The environmental impact of the mine tailing accident in Aznalcóllar. Sci. Total Environ., 242 (special volume): 1-337.

Hering, J.G., Chen, P.Y., Wilkie, J.A., Elimelech, M., 1997. Arsenic removal from drinking water during coagulation. J. Environ. Eng., 123: 800-807. https://doi.org/10.1061/(asce)0733-9372(1997)123:8(800)

Hothorn, T., Bretz, F., Westfall, P., 2008. Simultaneous inference in general parametric models. Biom. J., 50 (3): 346-363. https://doi.org/10.1002/bimj.200810425

Huang, M., Zhu, Y., Li, Z., Huang, B., Luo, N., Liu, C., Zeng, G., 2016. Compost as a soil amendment to remediate heavy metal-contaminated agricultural soil: mechanisms, efficacy, problems, and strategies. Water Air Soil Poll., 227: 359. https://doi.org/10.1007/s11270-016-3068-8

Illera, V., Garrido, F., Serrano, S., García-González, M.T., 2004. Immobilization of the heavy metals Cd, Cu and Pb in an acid soil amended with gypsum- and lime-rich industrial by-products. Eur. J. Soil Sci., 55: 135-145. https://doi.org/10.1046/j.1365-2389.2003.00583.x

IUSS Working Group WRB, 2015. World Reference Base for Soil Resources 2014, update 2015. International soil classification system for naming soils and creating legends for soil maps. World Soil Resources Reports No. 106. FAO, Rome.

Kabata-Pendias, A., Pendias, H., 2000. Trace elements in soils and plants, third ed. CRC Press, Boca Raton.

Kabata-Pendias, A., 2011. Trace elements in soils and plants, fourth ed. CRC Press, Boca Raton.

Kenny, L.B., Westendorf, M., Williams, C.A., 2019. Chapter 14 - Managing manure, erosion, and water quality in and around horse pastures, in: Sharpe, P., (Ed), Horse Pasture Management. Academic Press, pp. 245-259. https://doi.org/10.1016/B978-0-12-812919-7.00014-7

Khan, A.G., Kuek, C., Chaudhry, T.M., Khoo, C.S., Hayes, W.J., 2000. Role of plants, mycorrhizae and phytochelators in heavy metal contaminated land remediation. Chemosphere, 41 (1-2): 197-207. https://doi.org/10.1016/S0045-6535(99)00412-9

Kraus, U., Wiegand, J., 2006. Long-term effects of the Aznalcóllar mine spill-heavy metal content and mobility in soils and sediments of the Guadiamar river valley (SW Spain). Sci. Total Environ., 367 (2): 855-871. https://doi.org/10.1016/j.scitotenv.2005.12.027

Kochem Mallmann, F.J., dos Santos Rheinheimer, D., Ceretta, C.A., Cella, C., Gomes Minella, J.P., Lamana Guma, R., Filipovic, V., van Oort, F., Šimunek, J., 2014. Soil tillage to reduce surface metal contamination - model development and simulations of zinc and copper concentration profiles in a pig slurry-amended soil. Agr. Ecosyst. Environ., 196: 59-68. https://doi.org/10.1016/j.agee.2014.06.024

Kutrowska, A., Małecka, A., Piechalak, A., Masiakowski, W., Hanć, A., Barałkiewicz, D., Andrzejewska, B., Zbierska, J., Tomaszewsk, B., 2017. Effects of binary metal combinations on zinc, copper, cadmium and lead uptake and distribution in *Brassica juncea*. J. Trace Elem. Med. Biol., 44: 32-39. https://doi.org/10.1016/j.jtemb.2017.05.007

Liu, L., Li, W., Song, W., Guo, M., 2018. Remediation techniques for heavy metal-contaminated soils: Principles and applicability. Sci. Total Environ., 633: 206-219. https://doi.org/10.1016/j.scitotenv.2018.03.161

Lombi, E., Wenzel, W.W., Adriano, D.C., 1998. Soil contamination, risk reduction and remediation. Land Contam. Reclamat., 6 (4): 183-197.

Madejón, P., Murillo, J.M., Marañón, T., Cabrera, F., Soriano, M.A., 2003. Trace element and nutrient accumulation in sunflower plants two years after the Aznalcóllar mine spill. Sci. Total Environ., 307: 239-257. https://doi.org/10.1016/S0048-9697(02)00609-5

Madejón, P., Domínguez, M.T., Madejón, E., Cabrera, F., Marañón, T., Murillo-Carpio, J.M., 2018. Soil-plant relationships and contamination by trace elements: A review of twenty years of experimentation and monitoring after the Aznalcóllar (SW Spain) mine accident. Sci. Total Environ., 625: 50-63. https://doi.org/10.1016/j.scitotenv.2017.12.277

Manzano, M., Custodio, E., Ayora, C., Navarrete, P., 2000. Contamination of the Guadiamar river aquifer after the Aznalcóllar mine accident, SW Spain. Boletín Geológico y Minero, 111 (6): 93-106.

MAPA, 1994. Métodos oficiales de análisis. Tomo III: Métodos oficiales de análisis de suelos y aguas para el riego. Ministerio de Agricultura, Pesca y Alimentación. Servicio de Publicaciones, Madrid, pp. 205-285.

Martín, F.J., Simón, M., García, I., Bouza, P., Dorronsoro, C., Aguilar, J., 2003. Efecto del encalado en suelos contaminados por lodos piríticos. Edafología, 101(2): 177-183.

Martín-Peinado, F.J., Simón, M., García, I., Romero-Freire, A., González, V., 2014. Pollution of Pb in soils affected by pyrite tailings: Influence of soil properties, in: Hernández-Soriano, M.C., (Ed.), Environmental Risk Assessment of Soil Contamination. Chapter 27. Tech Open, pp. 785-798. https://doi.org/10.5772/57270

Martín-Peinado, F.J., Romero-Freire, A., García-Fernández I., Sierra-Aragón, M., Ortiz-Bernad, I., Simón-Torres, M., 2015. Long-term contamination in a recovered area affected by a mining spill. Sci. Total Environ., 514: 219-223. https://doi.org/10.1016/j.scitotenv.2015.01.102

McLaren, R.G., Swift, R.S., Williams, J.G., 1981. The adsorption of copper by soil materials at low equilibrium solution concentrations. J. Soil Sci., 32: 247-256. https://doi.org/10.1111/j.1365-2389.1981.tb01704.x

McLean, J.E., Bledsoe, B.E., 1992. Behavior of metals in soils. EPA Ground Water Issue. EP 1.43/2: M 56. (EPA/540/S-018 October 1992).

Meng, X., Bang, S., Korfiatis, G.P., 2000. Effects of silicate, sulfate, and carbonate on arsenic removal by ferric chloride. Water Res., 34 (4): 1255-1261. https://doi.org/10.1016/S0043-1354(99)00272-9

Morales, M., 2017. Sciplot: Scientific graphing functions for factorial designs. Available at: https://cran.r-project.org/web/packages/sciplot/index.html

Nakamaru, Y.M., Martín-Peinado, F.J., 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

Nocete, F., Sáez, R., Bayona, M.R., Nieto, J.M., Peramo, A., López, P., Gil-Ibarguchi, J.I., Inácio, N., García, S., Rodríguez, J., 2014. Gold in the southwest of the Iberian Peninsula during the 3rd millennium BC. J. Archaeol. Sci., 41: 691-704. https://doi.org/10.1016/j.jas.2013.10.006

OECD (Organization for Economic Cooperation and Development), 2003. Test No. 208: Terrestrial plant test: Seedling emergence and seedling growth test, in: Guideline for the testing of Chemicals, OECD Publishing, Paris, France, p. 208. https://doi.org/10.1787/9789264070066-en

Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Szoecs, E., Wagner, H., 2013. Package 'vegan'. Community ecology package, version 2. Available at: http://CRAN.R-project.org/package=vegan

Palmer, M.E., 1978. Acidity and nutrient availability in colliery spoil, in: Goodman, G.T., Chadwick, M.J., (Eds.)., Environmental management of minerals wastes. Kluwer Academic Publishers, p. 378.

Pastor-Jáuregui, R., Paniagua-López, M., Martínez-Garzón, J., Martín-Peinado, F., Sierra-Aragón, M., 2020. Evolution of the residual pollution in soils after bioremediation treatments. Appl. Sci., 10: 1006. https://doi.org/10.3390/app10031006

Pedersen, H.D., Postma, D., Jakobsen, R., 2006. Release of arsenic associated with the reduction and transformation of iron oxides. Geochem. Cosmochim. Acta, 70: 4116-4129. https://doi.org/10.1016/j.gca.2006.06.1370

Pérez-Esteban, J., Escolástico, C., Masaguer, A., Moliner, A., 2012. Effects of sheep and horse manure and pine bark amendments on metal distribution and chemical properties of contaminated mine soils. Eur. J. Soil Sci., 63: 733-742. https://doi.org/10.1111/j.1365-2389.2012.01468.x

Pérez-de-Mora, A., Burgos, P., Cabrera, F., Madejón, E., 2007. "In situ" amendments and revegetation reduce trace element leaching in a contaminated soil. Water Air Soil Poll., 185: 209-222. https://doi.org/10.1007/s11270-007-9443-8

Pérez-Sirvent, C., García-Lorenzo, M.L., Martínez-Sánchez, M.J., Navarro, M.C., Marimón, J., Bech, J., 2007. Metal-contaminated soil remediation by using sludges of the marble industry: Toxicological evaluation. Environ. Int., 33: 502-504. https://doi.org/10.1016/j.envint.2006.11.003

Quevauviller, P., Lachica, M., Barahona, E., Gomez, A., Rauret, G., Ure, A., Muntau, H., 1998. Certified reference material for the quality control of EDTA-and DTPA-extractable trace metal contents in calcareous soil (CRM 600). Fresen. J. Anal. Chem., 360 (5): 505-511. https://doi.org/10.1007/s002160050750

R Core Team, 2017. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available at: https://www.r-project.org.

Rahman, Z., Singh V.P., 2019. The relative impact of toxic heavy metals (THMs) (arsenic (As), cadmium (Cd), chromium (Cr)(VI), mercury (Hg), and lead (Pb)) on the total environment: an overview. Environ Monit Assess., 191(7): 419. https://doi.org/10.1007/s10661-019-7528-7

Rayed, A., Omrane, B., Chokri, S., Mohamed-Amine, K., Abdeliazim-Mustafa M., 2019. Effects of incorporation of marble powder obtained by recycling waste sludge and limestone powder on rheology, compressive strength, and durability of self-compacting concrete. Adv. Mater. Sci. Eng., Volume 2019. https://doi.org/10.1155/2019/4609353

Rodríguez-Jordá, M.P., Garrido, F., García-González, M.T., 2010a. Potential use of gypsum and lime rich industrial by-products for induced reduction of Pb, Zn and Ni leachability in an acid soil. J. Hazard. Mater., 175: 762-769. https://doi.org/10.1016/j.jhazmat.2009.10.074

Rodríguez-Jordá, M.P., Garrido, F., García-González, M.T., 2010b. Assessment of the use of industrial by-products for induced reduction of As, and Se potential leachability in an acid soil. J. Hazard. Mater., 175: 328-335. https://doi.org/10.1016/j.jhazmat.2009.10.007

Rodríguez-Jordá, M.P., Garrido, F., García-González, M.T., 2012. Effect of the addition of industrial by-products on Cu, Zn, Pb and As leachability in a mine sediment. J. Hazard. Mater., 213-214: 46-54. https://doi.org/10.1016/j.jhazmat.2012.01.049

Romero-Freire, A., Martín Peinado, F., van Gestel, C.A.M., 2015. Effect of soil properties on the toxicity of Pb: assessment of the appropriateness of guideline values. J. Hazard Mater., 289: 46-53. https://doi.org/10.1016/j.jhazmat.2015.02.034

Romero-Freire, A., García Fernández, I., Simón Torres, M., Martínez Garzón, F.J., Martín Peinado, F.J., 2016. Long-term toxicity assessment of soils in a recovered area affected by a mining spill. Environ. Pollut., 208: 553-561. https://doi.org/10.1016/j.envpol.2015.10.029

Salvany, J.M., Mediavilla, C., Mantecón, R., Manzano, M., 2001. Geología del Valle del Guadiamar y áreas colindantes. Boletín Geológico y Minero. Vol. Especial, 57-68.

Sánchez, J.A., García, I., del Moral, F., de Haro, S., González, V., Simón, M., Martín, F., Iriarte, A., 2010. Utilización de lodos de corte y pulido del mármol en la recuperación de escombreras de mármol. Rev. Cienc. Agrar., 33 (1): 182-190.

Sauvé, S., McBride, M., Hendershot, W., 1998. Soil Solution Speciation of Lead (II): Effects of organic matter and pH. Soil Sci. Soc. Am. J., 62: 618-621. https://doi.org/10.2136/sssaj1998.03615995006200030010x

Sharma, A.K., Fehrenbacher, J.B., B.A., 1974. Effect of gypsum, soil disturbance and tile spacing on the amelioration of Huey silt loam, a nitric soil in Illinois. Soil Sci. Soc. Am. Pro., 38: 628-632. https://doi.org/10.2136/sssaj1974.03615995003800040029x

Sierra-Aragón, M., Mitsui-Nakamaru, Y., García-Carmona, M., Martínez-Garzón, F.J., Martín-Peinado, F.J., 2019. The role of organic amendment in soils affected by residual pollution of potentially harmful elements. Chemosphere, 237: 124549. https://doi.org/10.1016/j.chemosphere.2019.124549

Simon, L., 2014. Potentially harmful elements in agricultural soils, in: Bini, C., Bech, J., (Eds), PHEs, Environment and Human Health. Potentially harmful elements in the environment and the impact on human health, Chapter 3. Springer Science and Business Media, Dordrecht. https://doi.org/10.1007/978-94-017-8965-3_3

Simón, M., Ortiz, I., García, I., Fernández, E., Fernández, J., Dorronsoro, C., Aguilar, J., 1999. Pollution of soils by the toxic spill of a pyrite mine (Aznalcóllar, Spain). Sci. Total Environ., 242: 105-115. https://doi.org/10.1016/S0048-9697(99)00378-2

Simón, M., Martín, F., Ortiz, I., García, I., Fernández, J., Fernández, E. Dorronsoro, C., Aguilar, J., 2001. Soil pollution by oxidation of tailings from toxic spill of a pyrite mine. Sci. Total Environ., 279: 63-74. https://doi.org/10.1016/S0048-9697(01)00726-4

Simón, M., Martín, F., García, I., Bouza, P., Dorronsoro, C., Aguilar, J., 2005. Interaction of limestone grains and acidic solutions from the oxidation of pyrite tailings. Environ. Pollut., 135: 65-72. https://doi.org/10.1016/j.envpol.2004.10.013

Simón, M., García, I., Martín, F., Díez, M., del Moral, F., Sánchez, J.A., 2008. Remediation measures and displacement of pollutants in soils affected by the spill of a pyrite mine. Sci. Total Environ., 407: 23-39. https://doi.org/10.1016/j.scitotenv.2008.07.040

Simón, M., Diez, M., González, V., García, I., Martín, F., de Haro, S., 2010. Use of liming in the remediation of soils polluted by sulphide oxidation: A leaching-column study. J. Hazard. Mater., 180: 241-246. https://doi.org/10.1016/j.jhazmat.2010.04.020

Sposito, G., Lund, L.J., Chang, A.C., 1982. Trace metal chemistry in arid-zone field soils amended with sewage sludge. I: fractionation of Ni, Cu, Zn, Cd and Pb in solid phases. Soil Sci. Soc. Am. J., 46: 260-264. https://doi.org/10.2136/sssaj1982.03615995004600020009x

Sumner, M.E., Radcliffe, D.E., McCray, M., Carter, E., Clark, R.L., 1990. Gypsum as an ameliorant for subsoil hardpans. Soil Technol., 3: 253-258. https://doi.org/10.1016/0933-3630(90)90005-N

Ünlükara A., Cemek, B., Karaman, S., Erşahin, S., 2008. Response of lettuce (*Lactuca sativa* var. *crispa*) to salinity of irrigation water. New Zeal. J. Crop. Hort., 36: 263-271. https://doi.org/10.1080/01140670809510243

US EPA (US Environmental Protection Agency), 1996. Ecological effects test guidelines. Seed germination/root elongation toxicity test. OPPTS 850.4200. National Service Center for Environmental Publications: Washington, DC, USA, p. 8.

Van Lierop, W.M., Mackenzie, A.F., 1975. Effects of calcium carbonate and sulphate on the growth of lettuce and radish in some organic soils of Southwestern Quebec. Can. J. Soil Sci., 55: 205-212.

Velenturf, A.P.M., Purnell, P., Macaskie, L.E., Mayes, W.M., Sapsford, D.J., 2019. Chapter 1: A new perspective on a global circular economy, in: Macaskie, L.E., Sapsford, D.J., Mayes, W.M., (Eds.), Resource recovery from wastes: Towards a circular economy, Royal Society of Chemistry, 2019, pp. 1-2. https://doi.org/10.1039/9781788016353-00001

Wallace, A., Wallace, G.A., 1995. Gypsum is almost a universal soil amendment. Soil amendments and amendment technologies, Wallace Laboratories, El Segundo.

Wickham, H., 2011. The split-apply-combine strategy for data analysis. R Package Version 1.8.4. J. Stat. Softw., 40 (1): 1-29. https://doi.org/10.18637/jss.v040.i01

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

Xiong, J., Madejón, P., Madejón, E. Cabrera, F., 2015. Assisted natural remediation of a trace element-contaminated acid soil: An eight-year field study. Pedosphere, 25 (2): 250-262. https://doi.org/10.1016/S1002-0160(15)60010-8

Yang, X., Hou, Q., Yang, Z., Zhang, X., Hou, Y., 2012. Solid-solution partitioning of arsenic (As) in the paddy soil profiles in Chengdu Plain, Southwest China. Geosci. Front., 3 (6): 901-909. https://doi.org/10.1016/j.gsf.2012.03.006

Zoca, S.M., Penn, C., 2017. Chapter one-an important tool with no instruction manual: a review of gypsum use in agriculture. Adv. Agron., 144: 1-44. https://doi.org/10.1016/bs.agron.2017.03.001

Zornoza, R., Faz, A., Carmona, D.M., Kabas, S., Martínez-Martínez, S., Acosta, J.A., 2012. Plant cover and soil biochemical properties in a mine tailing pond five years after application of marble wastes and organic amendments. Pedosphere, 22(1): 22-32. https://doi.org/10.1016/S1002-0160(11)60188-4

Zuur, A.F., Ieno, E.N., Elphick, C.S., 2010. A protocol for data exploration to avoid common statistical problems. Methods Ecol. Evol., 1: 3-14. https://doi.org/10.1111/j.2041-210X.2009.00001.x

APPENDICES

Appendix 3.1. Chemical properties and element content in organic and inorganic amendments. Chemical properties: pH; EC: electrical conductivity; OC: organic carbon; $CaCO_3$: calcium carbonates. Potentially Harmful Elements (PHEs): Cu, Zn, As and Pb. Letter "T" before Fe, Ca, S, Al, P and PHEs refers to total concentrations. NA= Not Available.

Properties		Vermicompost [1] (mean±SD)	Marble sludge [2]	Gypsum mining spoil [3] (mean±SD)
pH		7.88 ± 0.12	9.1	7.5 ± 0.2
EC (dS m^{-1})		4.18 ± 0.25	2.03	2.90 ± 0.04
OC (%)		23.16 ± 1.87	0.06	0.04 ± 0.03
CaCO3 (%)		NA	99	27.25 ± 3.52
Total forms (mg kg^{-1})	**TFe**	NA	1100	9501.07 ± 713.12
	TCa	NA	386,900	186,153.44 ± 4,288.77
	TS	NA	NA	126,094.49 ± 4,552.09
	TAl	NA	1,900	2,529.57 ± 532.98
	TCu	44.38 ± 3.56	1.67	10.10 ± 0.84
	TZn	17.18 ± 2.58	7.05	18.07 ± 2.06
	TAs	1.81 ± 0.27	3.76	3.50 ± 0.41
	TPb	3.90 ± 0.72	1.18	4.39 ± 0.73

[1]Nakamaru and Martín-Peinado, 2017; [2]Sanchez et al., 2010; [3]Ballesteros, 2018.

Appendix 3.2. Mean values ±SE (N= 24) of soluble PHEs (Cu, Zn, As and Pb) (mg kg^{-1}) in soils. Letter "S" before PHEs refers to soluble fraction. Soil types: i) **UA**: Unaffected soils; ii) **V-AA**: Vegetated Affected soils; iii) **UV-AA**: Unvegetated Affected soils; and iv) Soil treatments: **G**: Addition of gypsum mining spoil; **M**: Addition of marble sludge; **T**: Tillage; **V**: mixture of UV-AA and V-AA; **Gv**: G plus vermicompost (v); **Mv**: M + v; **Tv**: T + v; **Vv**: V + v. Different letters (columns) represent statistically significant differences ($p < 0.05$) for the *post hoc* Tukey tests performed after the LMMs.

Soil types	SCu	SZn	SAs	SPb
T	29.01 ± 8.06 de	119.00 ± 27.93 ef	0.13 ± 0.03 ab	0.05 ± 0.01 ab
Tv	16.68 ± 5.58 cd	88.03 ± 22.24 ef	0.12 ± 0.03 ab	0.06 ± 0.02 ab
M	2.82 ± 1.31 ab	21.667 ± 9.25 ab	0.07 ± 0.01 ab	0.04 ± 0.01 ab
Mv	3.66 ± 2.41 a	25.27 ± 16.91 a	0.08 ± 0.01 ab	0.03 ± 0.01 a
G	20.36 ± 7.23 bc	114.64 ± 33.35 cde	0.16 ± 0.05 ab	0.05 ± 0.01 ab
Gv	5.36 ± 2.28 abc	52.67 ± 15.86 cd	0.08 ± 0.01 ab	0.03 ± 0.01 ab
V	10.69 ± 4.42 bc	94.73 ± 26.44 de	0.10 ± 0.02 ab	0.05 ± 0.01 b
Vv	4.50 ± 2.06 abc	74.33 ± 18.33 def	0.07 ± 0.01 ab	0.03 ± 0.01 ab
UV-AA	18.46 ± 4.17 e	125.62 ± 20.84 f	0.11 ± 0.02 ab	0.05 ± 0.01 b
V-AA	0.73 ± 0.16 ab	7.07 ± 1.91 bc	0.17 ± 0.03 b	0.26 ± 0.09 c
UA	0.21 ± 0.04 a	0.28 ± 0.03 a	0.03 ± 4.12 E-3 a	0.71 ± 0.16 abc

Appendix 3.3. Mean values ±SE (N= 24) of bioavailable PHEs (Cu, Zn, As and Pb) (mg kg^{-1}) in soils. Letter "B" before PHEs refers to bioavailable fraction. Soil types: i) **UA**: Unaffected soils; ii) **V-AA**: Vegetated Affected soils; iii) **UV-AA**: Unvegetated Affected soils; and iv) Soil treatments: **G**: Addition of gypsum mining spoil; **M**: Addition of marble sludge; **T**: Tillage; **V**: mixture of UV-AA and V-AA; **Gv**: G plus vermicompost (v); **Mv**: M + v; **Tv**: T + v; **Vv**: V + v. Different letters (columns) represent statistically significant differences ($p < 0.05$) for the *post hoc* Tukey tests performed after the LMMs.

Soil types	BCu	BZn	BAs	BPb
T	57,24 ± 9,07 b	135,06 ± 23,34 de	4,11 ± 1,97 ab	0,26 ± 0,04 a
Tv	50,28 ± 8,36 b	115,11 ± 20,14 ce	3,04 ± 1,31 bc	0,35 ± 0,05 ab
M	38,52 ± 4,45 b	73,56 ± 12,14 bc	0,85 ± 0,27 a	0,33 ± 0,07 a
Mv	40,26 ± 6,70 b	87,20 ± 21,02 bcd	1,56 ± 0,44 ac	0,46 ± 0,05 ab
G	58,83 ± 10,11 b	162,77 ± 35,78 ce	4,11 ± 1,54 bc	0,70 ± 0,22 ab
Gv	45,18 ± 5,41 b	121,48 ± 21,34 ce	1,72 ± 0,29 bc	1,92 ± 1,13 b
V	47,50 ± 6,03 b	146,84 ± 25,95 e	1,61 ± 0,28 bc	3,80 ± 0,83 c
Vv	40,29 ± 4,61 b	143,97 ± 21,53 e	1,54 ± 0,43 bc	3,51 ± 0,95 c
UV-AA	44,32 ± 5,98 b	149,13 ± 23,62 e	3,96 ± 1,46 b	0,38 ± 0,08 ab
V-AA	36,89 ± 4,73 b	56,54 ± 6,52 b	1,37 ± 0,31 bc	6,25 ± 1,05 c
UA	7,19 ± 1,35 a	7,65 ± 1,08 a	0,12 ± 0,01 ab	14,43 ± 3,71 abc

- CHAPTER 4 -

ASSISTING THE SPONTANEOUS RECOVERY OF HERBACEOUS SPECIES IN POLLUTED SOILS WITH AMENDMENTS

ABSTRACT

The development of a healthy vegetation cover is commonly hampered by soil pollution, which could even inhibit seed emergence. Assisted natural remediation is essential to recover certain heavily polluted ecosystems, such as Mediterranean mining areas. To this end, the use of soil amendments, especially of industrial discarded wastes, is lately seen as a very convenient cost-effective and eco-efficient option. More than twenty years after Aznalcóllar mining spill (SW Spain), there are still highly polluted and unvegetated spots in the Guadiamar Green Corridor posing a serious risk for the ecosystem. In this study, we have used *in-situ* rehabilitation techniques, such as the use of calcium- and organic-rich amendments to enhance soil physicochemical properties and to control the toxicity of Potentially Harmful Elements (PHEs). Then, plant species cover, richness, diversity and PHE soil-plant transference were monitored in areas affected (treated and non-treated) and unaffected by the spill. In general terms, all treatments promoted plant emergence, cover and diversity, especially those with higher content in calcium carbonates and organic matter, but pollution acted as a filter for plant species. The most effective treatment for plant development was the mixture of unvegetated and vegetated affected soils with vermicompost. Nevertheless, considering the reduction of PHE toxicity, the addition of gypsum mining spoil and marble sludge were the best treatments. All things considered, the use of marble sludge with vermicompost offered the most beneficial alternative. On the contrary, tillage resulted the least effective option. Due to the promising results obtained for marble sludge, gypsum mining spoil and vermicompost in soil remediation and the recovery of vegetation, further studies should be conducted to enhance their positive effects.

Keywords: Mining spill; soil pollution; ecosystem rehabilitation; soil amendments; spontaneous plant recovery.

1. INTRODUCTION

Potentially harmful elements (PHEs), among which heavy metal(loid)s are included, are naturally present in the environment (Kabata-Pendias, 2011). Some of them are essential nutrients (i.e. Cu and Zn) for plants and animals; however, both essential and non-essential (As and Pb) PHEs may become toxic when exceeding a specific threshold (Kabata-Pendias, 2011; Bini and Bech, 2014; Simon, 2014). As PHEs cannot be degraded in soils, they persist in nature, facilitating that living organisms are more exposed to their adverse effects, what poses a potential (long-term) risk, not only for the environment, but also for the whole food web (Hooda, 2010; Sherene, 2010).

PHE toxicity in plants can limit seed emergence, rooting, growth, or cause plant death by disrupting the uptake of essential nutrients (Kabata-Pendias, 2011). However, many plant species are tolerant to above normal PHE concentrations by virtue to a wide range of physiological or behavioral strategies (Baker, 2010; Viehweger, 2014), especially those living in metalliferous sites, the so-called "metallophytes" (Baker, 2010). After a pollution event, some plant species outside metal-rich habitats, the pseudometallophytes (Baker, 2010), also present a high capacity to adapt to adverse soil conditions; moreover, they have a high biomass production, and are better competitors in soils with moderate metal toxicity (Poschenrieder et al., 2001). Therefore, tolerant plants promote the improvement of the physicochemical and biological conditions of these degraded soils by dealing with PHEs, increasing soil organic matter content and nutrient availability. (Arienzo et al., 2004; Bolan et al., 2011).

In certain botanical families, the incidence of species that (hyper)accumulate PHEs in above-ground tissues is high, as it is the case of Asteraceae, Brassicaceae, Fabaceae and Lamiaceae (Reeves et al., 2018). Although PHE (hyper)accumulation is a very useful tool as phytoremediation technique (Saxena et al., 2020), without the proper control, living organisms would be more exposed to PHE toxicity through the food web. In this sense, Chaney (1989) presented the maximum tolerable levels of dietary minerals for domestic livestock in comparison with levels in forages, and Gupta et al. (2019) described the adverse effects of PHEs on human beings.

In certain degraded areas, such as tropical forests, natural spontaneous succession have proved to properly recover the former vegetation state (Prach and Hobbs 2008; Crouzeilles et al., 2017). However, this is not the case of Mediterranean ecosystems (Nunes et al., 2016), especially in mining affected areas (Cooke and Johnson, 2002), where the harsh environmental conditions left after mining, along with prolonged hot dry summers, would slow down natural spontaneous recovery (Nirola et al., 2016; Nunes et al., 2016). Actually, the process to recover the original habitat could be very slow, especially if the disturbed site is large and there are other physical or environmental constrains such as soil compaction, slope instability, chemical imbalance or potentially harmful elements (Prach and Hobbs 2008; García-Carmona et al. 2019). In these cases, active restoration is strongly encouraged even by national environmental legal regulations (Burgos et al., 2013). For instance, in the case of the Aznalcóllar mining disaster (SW, Spain), one of the world largest mining accidents (Sierra-Aragón et al., 2019), rehabilitation actions definitely minimized the catastrophic environmental consequences (Burgos et al., 2013).

Many remediation techniques have been developed in order to restore metal-polluted soils (Iskandar and Adriano, 1997). Among them, Assisted Natural Remediation (ANR) through *in-situ* techniques indeed minimize disturbances and costs (Liu et al., 2018). On one hand, soil tillage is frequently used with the aim of breaking soil crust and promoting aeration, water infiltration, and pollutant dilution, although its positive effects are limited (Mallmann et al., 2014; Busari et al., 2015; García-Carmona et al., 2017). On the other hand, the addition of amendments is lately seen as a cost-effective and eco-efficient solution that not only handle pollutant toxicity but also enhance key biogeochemical processes (Adriano et al., 2004; Rodríguez-Jordá et al., 2012; Nirola et al., 2016). The use of organic or inorganic amendments is highly recommended, especially if these amendments become from industrial discarded wastes, for immobilizing PHEs and preventing the dispersion of pollutants in the ecosystem. Amendments also improve soil nutrient status and soil physicochemical properties. Altogether, amendments promote plant germination, re-establishment and growth (Clemente et al., 2015; Madejón et al., 2018). The revalorization of industrial and agro-food industry wastes by applying them to degraded soils are in line with zero waste strategy (Greyson, 2007).

Marble and gypsum mining are important economic activities all over the world and produce vast amount of wastes (Ahmed, 2011; Rayed et al., 2019), named marble sludge and gypsum mining spoil hereafter, respectively. As calcium-rich amendments, they have the potential to enhance certain physicochemical properties of soils, such as increasing pH, as well as to modify and control PHE availability (Franzen et al., 2006; Fernández-Caliani and Barba-Brioso, 2010). Nevertheless, as they are poor in organic matter (Sánchez et al., 2010; Ballesteros, 2018), Clemente et al. (2003) indicated the combination of organic and inorganic amendments as a suitable and recommended option.

Consequently, in this work, we hypothesize that gypsum mining spoil, marble sludge and their combination with an organic amendment, vermicompost, could potentially improve soil properties in soils affected by PHEs and strong acidification. More precisely, we aimed at testing the effect of different soil amendments on vegetation recovery, and on the reduction of PHE uptake by plants and PHE retention in roots.

2. MATERIAL AND METHODS

2.1. Study area

Our *in-situ* experiment is located within the Guadiamar Green Corridor (GGC) (CMA, 2003), at the closest sector to Aznalcóllar pyrite mine (Seville, SW Spain) and beside the Agrio river floodplain. In geological terms, it is placed next to the Iberian Pyrite Belt, which has been intensely exploited since the 3rd Millenium BC (Nocete et al., 2014). The bedrock consists in Neogene-Quaternary sediments (Alastuey et al., 1999), with alluvial deposits made of silty-sand materials, gravels dominated by quartzite, shales and schists, and a basement consisting of thick Miocene blue marls (Manzano et al., 2000; Salvany et al., 2001). According to the FAO classification (IUSS, 2015), the dominant soils in this sector are eutric Fluvisols (soils close to the river channel) and eutric Regosols (soils not influenced by periodic floodings). The Guadiamar river floodplain was mainly dedicated to agriculture (Madejón et al., 2003), but also had riverbank vegetation characterized by ash, poplar, elm and willow trees. The climate is typically Mediterranean, with hot dry summers, cold wet winters, and variable rainfall during temperate autumns and springs (Simón et al., 2008).

In 1998, the collapse of the holding pond of Aznalcóllar pyrite mine released approximately 6 hm^3 of acidic waters and toxic tailings, affecting more than 4000 Ha (Grimalt et al., 1999). Numerous rehabilitation activities were implemented in the affected area (CMA, 2003), among which the application of soil amendments and afforestation were included. Regarding afforestation, the activities accomplished focused on planting native Mediterranean trees and shrub species. As far as herbaceous vegetation is concerned, the surrounding areas acted as propagule donors (Pérez-de-Mora et al., 2011).

Despite the implementation of a varied and exhaustive range of rehabilitation activities, residual pollution persists in some sectors of the affected area (Burgos et al., 2013; Martín-Peinado et al., 2015; Domínguez et al., 2016; García-Carmona et al., 2019). Thus, the GGC is composed of an unaffected area (UA) where the spill never reached, and a residually polluted area, the so-called “affected area” (AA) hereafter. Within the affected area, in our study sector there are highly polluted and unvegetated spots (UV-AA) alternating with less polluted and vegetated areas (V-AA) (Madejón et al., 2018). The UV-AA areas were described in previous works (Martín-Peinado et al., 2015; García-Carmona et al., 2019).

Both the soils from the UA and V-AA areas are slightly acid (Appendix 4.1). According to Hardie and Doyle (2012) standards, UA soils are non-saline, whereas V-AA soils are highly saline. Total PHEs (Appendix 4.2) in UA soils had similar PHE content to that of background soils in the area (Simón et al., 1999). According to the regional thresholds (NGR) to declare a soil as potentially polluted for agricultural use in Andalusia (BOE, 2015) [Cu: 595, Zn: 10000, As: 36 and Pb: 275, in mg kg^{-1}], in our experimental area, total mean concentration of Cu and Zn were under these values, but total mean As exceeded 5-fold this threshold and total mean Pb had vales close to this NGR. Regarding the soils from the unvegetated affected area (UV-AA), they were strongly crusted, acidic, extremely saline, and had a high PHE load (Appendices 4.1 and 4.2), especially in terms of As and Pb, which also surpassed (13- and 3-fold respectively) their NGR (BOE, 2015). The solubility of Cu, Zn and Pb experience a sharp increase in UV-AA, whereas it decreased for As (Appendix 4.3). Regarding PHE bioavailability, there was a sharp increase for Cu, but it decreased for Pb and As (Appendix 4.4).

As a result, UV-AA soils present hostile conditions for biota (Becerra-Castro et al., 2012). In this vein, the area constitutes an excellent place to test the use of different amendments in order to improve soil conditions and promote the spontaneous recovery of vegetation (Martín-Peinado et al., 2015; García-Carmona et al., 2019).

2.2. Experimental design

For this study, we established 6 experimental plots (36 m^2 each) in 6 different UV-AA areas randomly selected (Figure 4.1) in the closest to the mine and most polluted sector of the GGC. In each plot, a polluted control subplot was established (UV-AA), and 8 different treatments were applied; four of them were simple treatments, and the other four were mixed treatments (Figure 4.1). Simple treatments consisted in either the application of marble sludge (M), gypsum mining spoil (G), soil from V-AA (V) or just crust breaking through tillage (T), whereas mixed treatments consisted in the combination of the latter with vermicompost (Mv, Gv, Vv and Tv).

Gypsum mining spoil derives from a gypsum quarry in Escúzar (Granada, Spain) and contains 48% of gypsum and 27% of $CaCO_3$; marble sludge derives from a marble quarry in Macael (Almería, Spain) and contains 99% of $CaCO_3$; and vermicompost was produced by Lombricor S.C.A and has the ecological certificate (CAAE, S.L.) (see Appendix 4.5 for amendments properties).

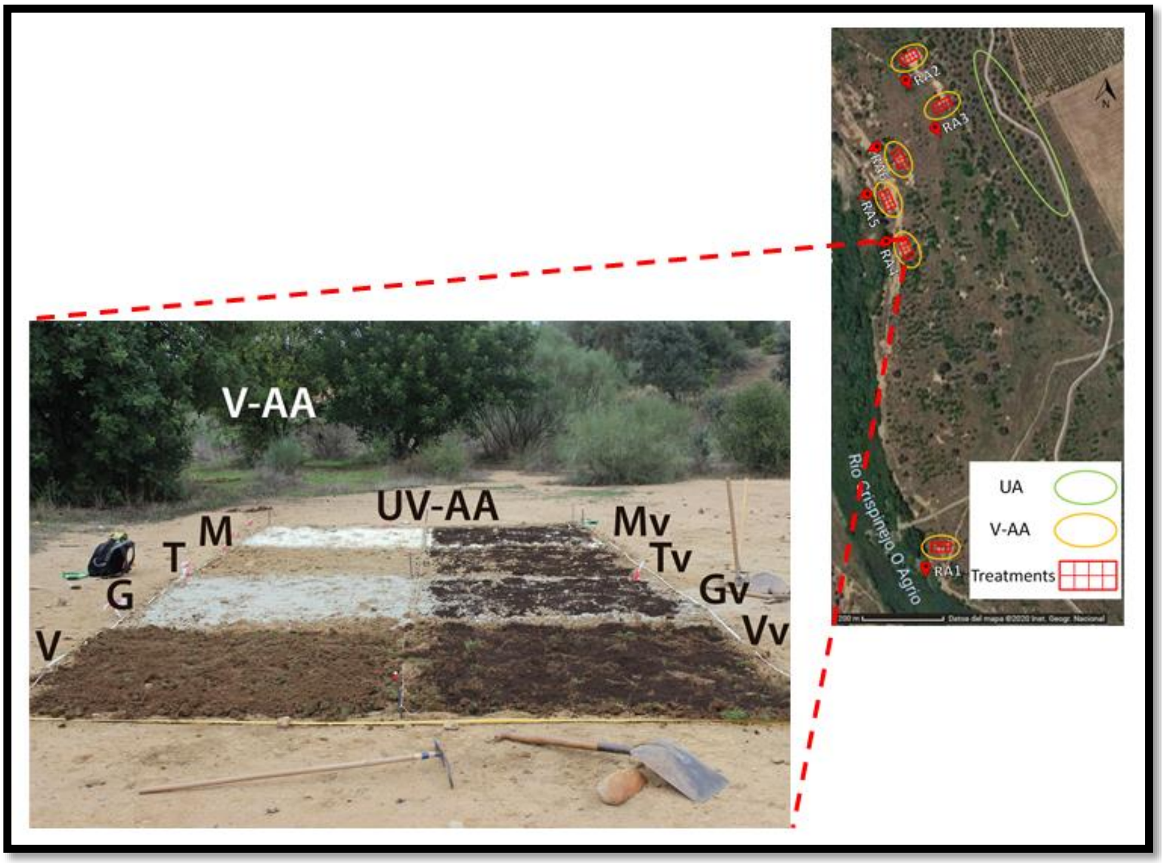


Figure 4.1. Study area on the right and design of the experimental plot on the left: **UA**: Unaffected Area; **V-AA**: Vegetated Affected Area; **UV-AA**: Unvegetated Affected Area with an experimental plot. Soil treatments: **G:** Addition of 5 kg m^{-2} of gypsum mining spoil; **M**: Addition of 5 kg m^{-2} of marble sludge; **T**: crust breaking through tillage; **V**: Removal of 300 kg of polluted soil (from the uppermost 10 cm) and refilling with 300 kg of soil from the neighboring vegetated affected area; **Gv**: G plus 5 kg m^{-2} of vermicompost (v); **Mv**: M + v; **Tv**: T + v; **Vv**: V + v.

As our experimental plots were randomly distributed and surrounded by vegetated affected areas (V-AA), after the application of soil treatments, we did not conduct seed sowing with the aim of testing the effects of soil treatments on the spontaneous recovery of herbaceous species through the improvement of soil properties and the reduction of PHE mobility (Becker and Brändel, 2007). Soil properties and PHE content of treated soils are shown in Appendices 4.1, 4.2, 4.3 and 4.4.

2.3. Vegetation sampling

Herbaceous species were monitored and sampled in the unaffected area (UA), in the unvegetated area (UV-AA), in our experimental plots (per treatment), and in the surrounding vegetated areas (V-AA) in two consecutive springs (2017 and 2018). Total and individual species cover were monitored with a 0.25 m^2 quadrat divided into 100 cells and expressed as the percentage of cells occupied by herbaceous species, both as total cover and as cover per species (Elzinga et al. 2015). We replicated these samplings four times per treatment in each experimental plot (Figure 4.2), counting with 24 pseudoreplicates in total per treatment. In each V-AA we also sampled 4 times, obtaining a total of 24 quadrats, and 24 quadrats more along the UA. Sampling sites were randomly selected, and in the case of experimental plots, avoiding subplot borders to elude ecotones.

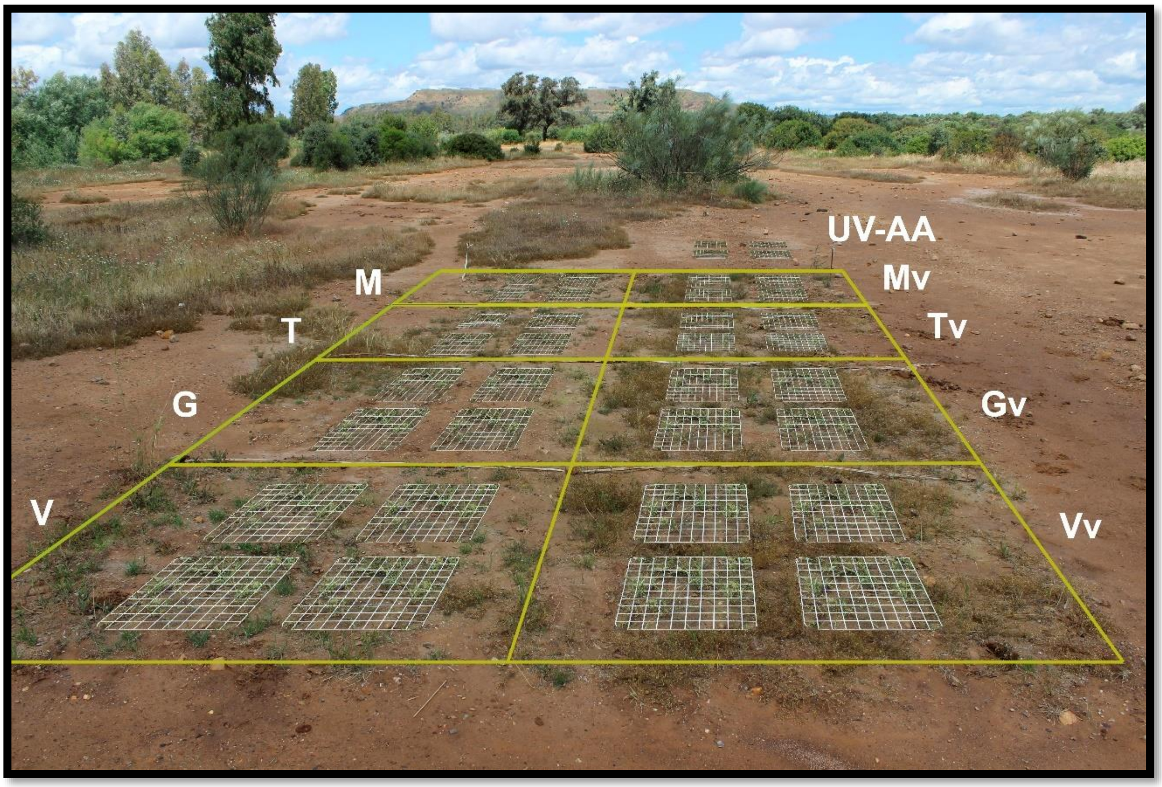


Figure 4.2. Vegetation cover sampling in an experimental plot with quadrats superimposed. **UV-AA**: Unvegetated Affected Area; **M**: Addition of marble sludge; **T**: Tillage; **G**: Addition of gypsum mining spoil; **V**: mixture of UV-AA and vegetated affected soils; **Mv**: M plus vermicompost (v); **Tv**: T + v; **Gv**: G + v; **Vv**: V + v.

Plant species richness was calculated in two ways: 1. Mean richness: average richness per treatment, and in V-AA and UA areas; 2. Total richness: total number of different species that appeared at least once per treatment, V-AA or UA areas.

The Shannon-Wiener Diversity Index (H', Shannon, 1948) was also calculated to have an idea of the herbaceous plant diversity present in each soil type. As H' values can sometimes be difficult to interpret and to compare between different areas (Magurran, 2004), the Effective Number of Species (ENS, MacArthur 1965) that characterize a community was calculated for each treatment according to Jost (2006) as:

$$ENS = e^{H'}$$

and it is understood as the number of equally-common species (Whittaker, 1972) required to give a particular value of an index, the Shannon-Wiener Index in this case (Jost, 2006).

Finally, we collected one sample of the most frequent plant species (key species hereafter) in each experimental plot (per treatment), in V-AA and in UA. In most cases, they were mixed samples formed of several individuals (same species), except where species abundance was limited to only one individual.

2.4. Plant analysis

We carefully washed all plant samples (from treatments, V-AA and UA) with distilled water and dried them in an oven (Memmert oven Model 100-800) at 70° C for 48 h. Then, we divided them per species into shoot and root, weighed them in a precision scale (GRAM PRECISION STA-310 S, ±0.001 g), and ground them. Finally, they were acidly digested (HNO_3:H_2O_2, 1:1) in a microwave XP1500Plus (Mars®) (Sah and Miller, 1992) in order to measure plant PHE content. To control the accuracy of the method, we used Standard Reference Material (ERM ® CD281); the average recoveries for the PHEs studied ranged from 77% to 110%.

In order to evaluate the capacity of plants to accumulate PHEs transferred from soils, the Bioconcentration Factor (BAF) for each PHE was calculated according to Hobbs and Streit (1986) and Van der Ent et al. (2012) as:

$$BAF = {}^{Cshoot}/_{Csoil}$$

where '*Cshoot'* is the PHE concentration in shoot (mg kg^{-1} dry weight) and '*Csoil'* the PHE bioavailable concentrations (extracted by EDTA) in soil (mg kg^{-1} dry soil). Concentrations extracted by EDTA were selected for BAF calculation, as this fraction represents PHE bioavailable forms for plants (Parra et al., 2014). High BAF values (>1) can be associated to hyperaccumulation but this is not the only criteria (Van der Ent et al., 2012). For instance, Mehes-Smith et al. (2013) reported that a plant can be classified as hyperaccumulator only if it meets four criteria: i) $[PHE]_{Shoot}$/ $[PHE]_{root}$ >1; ii) $[PHE]_{Shoot}$/ $[PHE]_{soil}$ >1; iii) $[PHE]_{polluted\ plants}$/$[PHE]_{normal\ plants}$ >10-500; iv) $[Cu]_{polluted\ plants}$ = 300, $[Zn]_{polluted\ plants}$ = 3000, $[Pb]_{polluted\ plants}$ = 1000, (in mg kg^{-1}).

Then, to assess the capacity of plants to translocate PHEs from root to shoot, the Translocator Factor (TF) for each PHE was obtained according to Van der Ent et al. (2012) as:

$$TF = {}^{Cshoot}/_{Croot}$$

where '*Cshoot'* is the PHE concentration in shoot (mg kg^{-1} dry weight) and '*Croot'* the PHE concentration in root (mg kg^{-1} dry weight). TF values > 1 are related to PHE root-to-shoot translocation (Macnair, 2003).

Finally, the quotient (Q) between PHE concentrations accumulated in plants at affected and unaffected soils (Mehes-Smith et al., 2013) was calculated, aiming at establishing a quantitative relation between the studied vegetated soil types (UA, V-AA and treatments). Values of the quotient bigger than 10-500 may indicate PHE hyperaccumulation (Mehes-Smith et al., 2013)

2.5. Data analysis

All statistical analyses were performed using R version 3.4.2 (R Core Team, 2017). We applied Linear Mixed Models (LMMs) and Generalized Linear Mixed Models (GLMMs) by means of "lme4" package (Bates et al., 2015) to assess the effectiveness of soil treatments for the following response variables: vegetation cover, plant species richness, plant diversity (H'), Effective Number of Species (ENS), PHE uptake by plants, PHE accumulation in shoots and roots, Bioaccumulation Factor (BAF), Translocation Factor (TF) and quotient between PHE uptake by affected and unaffected plants (Q). In the models, we included soil treatment as fixed factor, and residual areas, sampling period and species as random factors (in the case of cover, variable for which we had spatial pseudoreplication, the "subplot" factor was also included as random). GLMM models were fitted for species richness and ENS, assuming poisson and gamma distributions, log- and inverse-link functions, respectively. Plant cover, H', PHE uptake by plants, PHE accumulation in shoot and root, TF, BAF and Q were fitted with LMMs assuming gaussian distribution and identity-link function; in this case, the logarithmical transformation of variables (or arcsine square root transformation for percentages) was used to meet normality assumption. Model suitability in each case was assessed by graphical exploration of the residuals (Zuur et al. 2010). Then, pairwise comparisons between soil treatments were performed for all the variables with Tukey's *post-hoc* tests using "multcomp" package (Hothorn et al., 2008).

We calculated the variance explained by LMM and GLMM models using "r.squaredGLMM" function of "MuMIn" library (Barton, 2016).

Graphs and confidence intervals were obtained with R "ggplot2" v3.1.1 (Wickham, 2016) and "sciplot" v1.1-1 (Morales, 2017). Mean values and standard deviation were calculated by using ddply function (R "plyr" package, v1.8.4; Wickham, 2011).

3. RESULTS

3.1. Effect of soil treatments on plant cover, richness and diversity

According to our results, the soils from the unaffected (UA) and vegetated affected (V-AA) areas were totally covered by herbaceous vegetation (100%) (Figure 4.3). In the other extreme, vegetation was completely absent in the unvegetated affected soils (UV-AA). Regarding the experimental plots, treatments promoted plant emergence, compared to UV-AA. The addition of marble sludge (M), gypsum mining spoil (G), and tillage (T) increased plant cover (8.46 ± 0.60 %, on average) with marginal differences compared to UV-AA, and the mixture of topsoil from V-AA with UV-AA soil (V treatment) increased it (40.00 ± 0.05%) with significant differences compared to UV-AA and the other simple treatments. The addition of vermicompost (v) to simple treatments further promoted the increase of plant cover, with marginal differences for Gv (31.81 ± 0.06 %), Tv (20.27 ± 0.05 %) and Vv (47.13 ± 0.05 %), and with significant differences for Mv (31.52 ± 0.05 %). Although Vv presented the highest plant cover of all treatments, the relative increase (18%) compared with V was much lower than for the other treatments (Gv: 336%, Mv: 239% and Tv: 131%). Moreover, coverage in Mv, Gv or V was close to that in Vv, with just marginal differences.

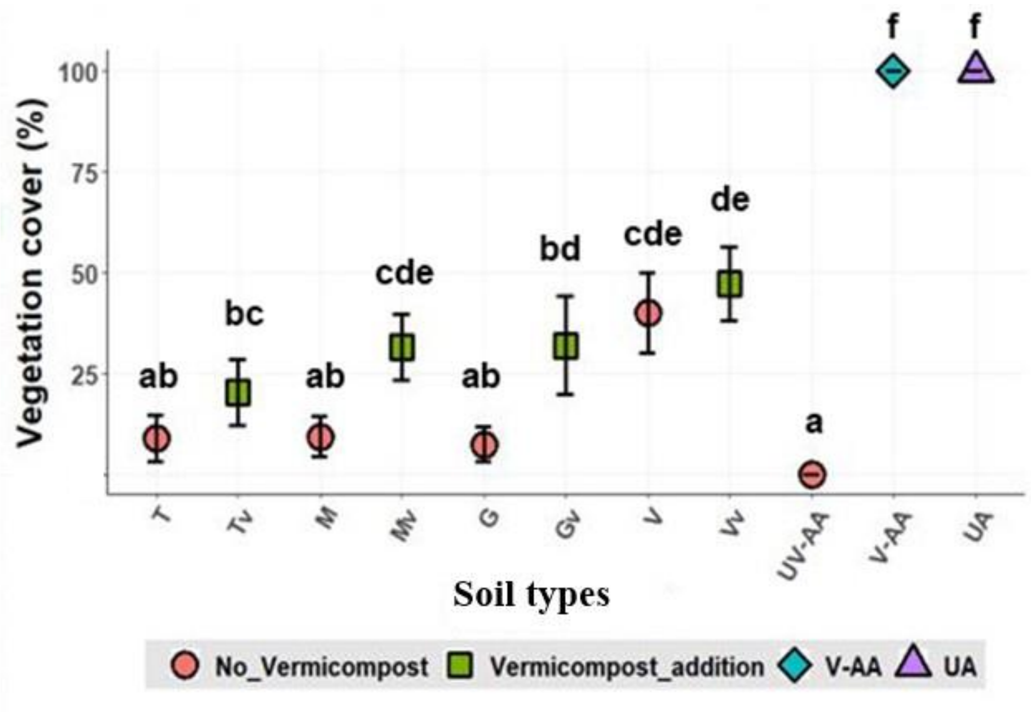


Figure 4.3. Percentage (mean ± SE) of plant cover in soils. Soil types: i) **UA:** unaffected soils; ii) **V-AA:** vegetated affected soils; iii) **UV-AA:** unvegetated affected soils; and iv) soil treatments: 1. **G**: Addition of gypsum mining spoil; 2. **M**: Addition of marble sludge; 3. **T**: Tillage; 4. **V**: mixture of UV-AA and V-AA; 5. **Gv**: G plus vermicompost (v); 6. **Mv**: M + v; 7. **Tv**: T + v; 8. **Vv**: V + v. Different letters above symbols indicate significant differences ($p<0.05$) for the *post-hoc* Tukey tests performed after the LMMs.

Concerning total richness (total number of species that appeared at least once per treatment, Appendix 4.6), in UV-AA soils, where no plant emerged, richness was 0. Nevertheless, we found at least 36 different herbaceous species in UA, 30 in V-AA and 42 within the experimental plots (Figure 4.4). Focusing on soil treatments, V treatment presented the highest richness of herbaceous species (25), whereas T, G and M treatments presented 8, 9 and 11, respectively. The addition of vermicompost (v) to simple treatments at least doubled the number of different species in Gv (21), Mv (24) and Tv (16) treatments; however, in Vv treatment, richness was just four units higher (29) than in V treatment.

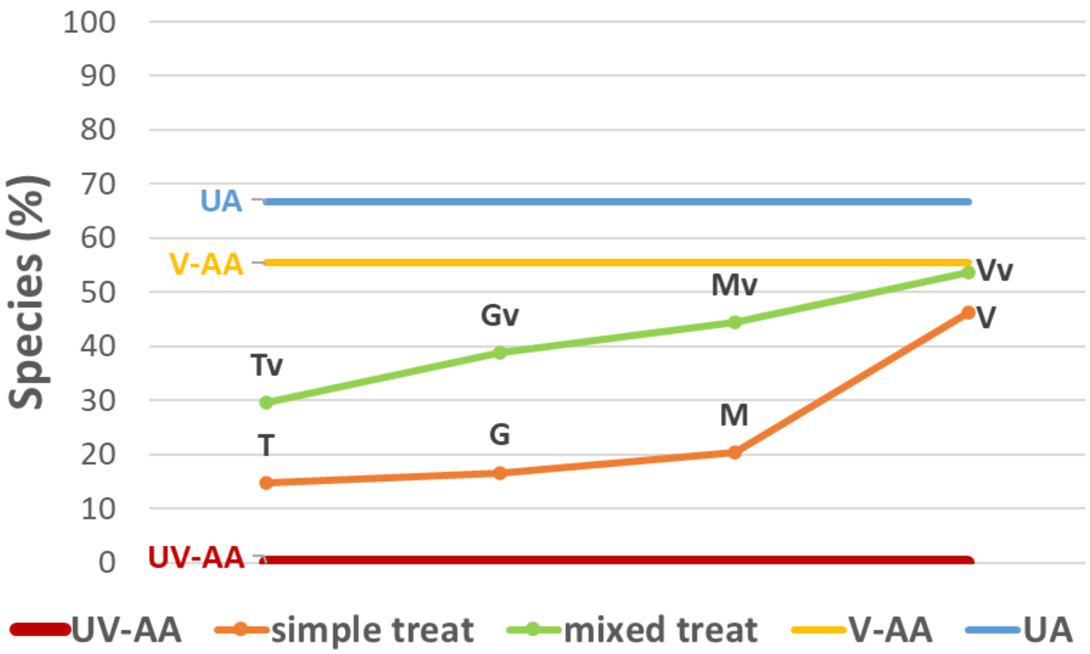


Figure 4.4. Relative percentage of different species growing in each soil type: i) **UA**: unaffected soils; ii) **V-AA**: vegetated affected soils; iii) **UV-AA**: unvegetated affected soils; and iv) soil treatments: 1. **G**: Addition of gypsum mining spoil; 2. **M**: Addition of marble sludge; 3. **T**: Tillage; 4. **V**: mixture of UV-AA and V-AA; 5. **Gv**: G plus vermicompost (v); 6. **Mv**: M + v; 7. **Tv**: T + v; 8. **Vv**: V + v.

Mean richness (average richness per treatment) followed a similar pattern to that of plant cover (Figure 4.5). V-AA and UA areas presented the highest richness (8.63 ± 0.36 and 9.90 ± 0.37, respectively), in contrast with UV-AA, where no species emerged during the experiment. Regarding the experimental plots, V treatment was the most favourable treatment (4.00 ± 0.53), whereas G, M and T treatments registered a very low mean richness (0.71 ± 0.06, on average). The addition of vermicompost (v) to simple treatments marginally increased mean richness in Gv (2.17 ± 2.56), Tv (1.48 ± 1.98) and Vv (4.69 ± 0.41) treatments, and significantly increased it in Mv treatment (2.75 ± 0.33). Although Vv treatment presented the highest richness, the relative increase (17%) compared with V treatment was lower than for the other treatments (Gv: 175%, Mv: 267% and Tv: 147%). Moreover, richness in Mv or V treatments was close to that in Vv treatment, with just marginally significant differences.

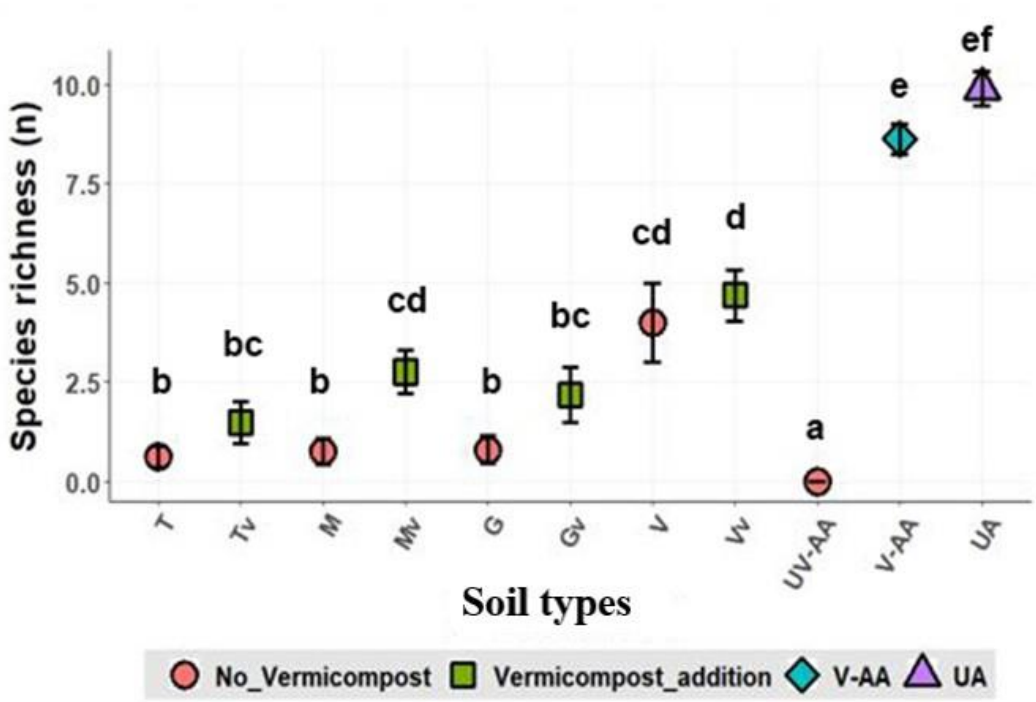


Figure 4.5. Plant species richness (mean±SE) in soils. Soil types: i) **UA:** unaffected soils; ii) **V-AA:** vegetated affected soils; iii) **UV-AA:** unvegetated affected soils; and iv) soil treatments: 1. **G**: Addition of gypsum mining spoil; 2. **M**: Addition of marble sludge; 3. **T**: Tillage; 4. **V**: mixture of UV-AA and V-AA; 5. **Gv**: G plus vermicompost (v); 6. **Mv**: M + v; 7. **Tv**: T + v; 8. **Vv**: V + v. Different letters above symbols indicate significant differences ($p<0.05$) for the *post-hoc* Tukey tests performed after the GLMMs.

Diversity Index (H', Figure 4.6a) in V-AA (2.95 ± 0.12) and UA (2.83 ± 0.15) areas was the highest, and presented significant differences with UV-AA and any soil treatment, especially compared to the unvegetated affected areas (UV-AA), where no seed emergence occurred and, thus, H' was 0, and to T and M treatments, where H' was below 0.30 (with no significant differences between them). G treatment presented marginally significant differences with the formers, with an H' lower than 0.40, and in V treatment, H' (1.06 ± 0.24) was significantly higher than in the other simple treatments. The addition of vermicompost (v) to simple treatments marginally increased H' in Gv (0.69 ± 0.22), Tv (0.44 ± 0.20) and Vv (1.36 ± 0.18) treatments, and significantly increased it in Mv treatment (1.04 ± 0.21). Although Vv presented the highest H' of all treatments, the relative increase (28%) compared with V was lower than for the other treatments (Gv: 92%, Mv: 420% and Tv: 144%). Moreover, H' in Mv or V treatment was close to that in Vv treatment, with just marginally significant differences.

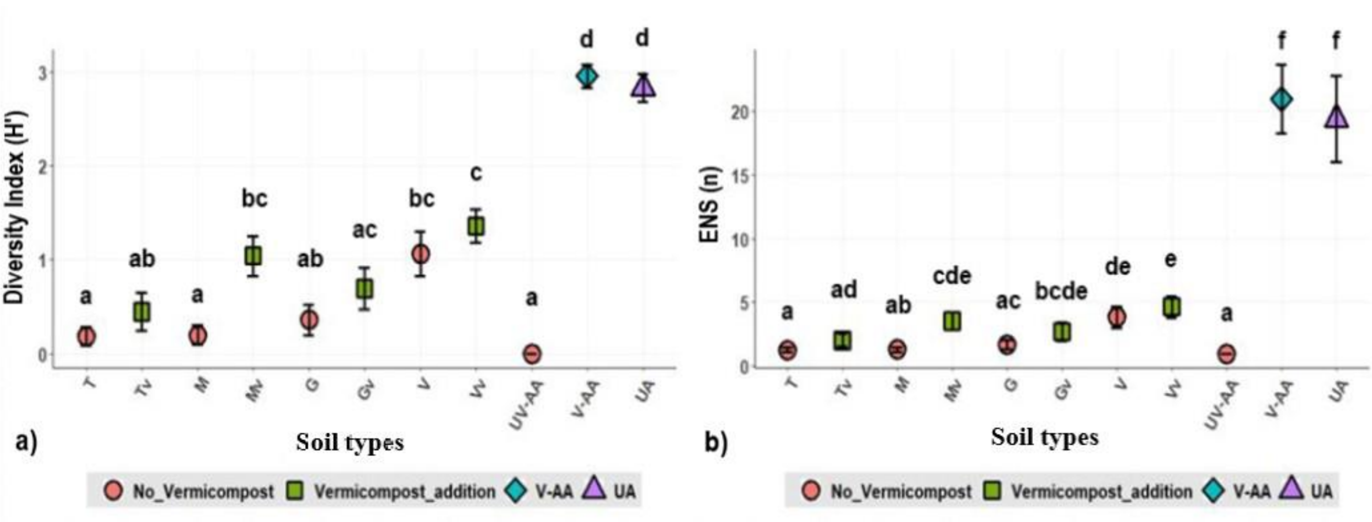


Figure 4.6. Mean (±SE) values of plant species diversity index (6a) and Effective Number of Species (ENS, 6b) in soils. Soil types: i) **UA**: unaffected soils; ii) **V-AA:** vegetated affected soils; iii) **UV-AA:** unvegetated affected soils; and iv) soil treatments: 1. **G**: Addition of gypsum mining spoil; 2. **M**: Addition of marble sludge; 3. **T**: Tillage; 4. **V**: mixture of UV-AA and V-AA; 5. **Gv**: G plus vermicompost (v); 6. **Mv**: M + v; 7. **Tv**: T + v; 8. **Vv**: V + v. ENS refers to the Effective Number of Species. Different letters above symbols indicate significant differences (p<0.05) for the *post-hoc* Tukey tests performed after the LMMs.

In terms of the Effective Number of Species (ENS, Figure 4.6b), V-AA and UA areas had 21 and 19 equally-common species, respectively. T and M treatments only had 1 equally-common species, G had 2, and V had 4. The addition of vermicompost (v) increased one unit the number of equally-common species in Gv (3) and Vv (5) treatments, and at least doubled it Tv (2) and Mv (4) treatments.

3.2. Effect of soil treatments on species composition

Regarding plant species composition, it was similar in UA and V-AA areas, whereas it rather changed among treatments (Appendix 4.6). Focusing on simple treatments, V and T treatments were the most and least diverse, respectively. The addition of vermicompost (v) to G, M, T and V treatments promoted the emergence of new different species in Gv, Mv, Tv and in Vv, although in the case of Vv, species composition was rather different to that in V treatment (Appendix 4.6).

A similar trend was observed when grouping plant species according to their botanical family (Table 4.1).

Table 4.1. List of families present per soil type: i) **UA**: unaffected soils; ii) **V-AA**: vegetated affected soils; iii) **UV-AA**: unvegetated affected soils; iv) soil treatments: 1. **G**: Addition of gypsum mining spoil; 2. **M**: Addition of marble sludge; 3. **T**: Tillage; 4. **V**: mixture of UV-AA and V-AA; 5. **Gv**: G plus vermicompost (v); 6. **Mv**: M + v; 7. **Tv**: T + v; 8. **Vv**: V + v.

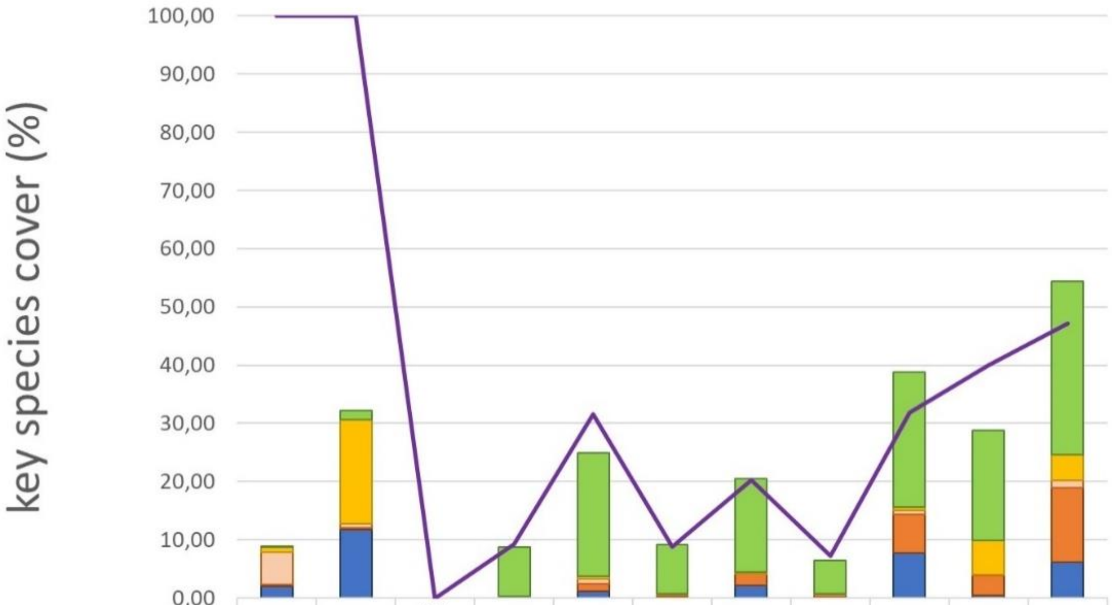
Family	UA	V-AA	UV-AA	T	Tv	M	Mv	G	Gv	V	Vv
Asteraceae	X	X		X	X	X	X	X	X	X	X
Boraginaceae	X	X									
Brassicaceae	X	X			X	X	X	X	X	X	X
Caryophyllaceae	X	X		X	X	X	X	X	X	X	X
Chenopodiaceae	X	X			X		X		X	X	
Euphorbiaceae	X	X									
Fabaceae	X	X			X	X	X		X	X	X
Geraniaceae	X	X			X	X	X		X		X
Juncaceae							X			X	
Lamiaceae	X	X									
Malvaceae							X				X
Oxalidaceae	X	X									
Plantaginaceae	X	X		X	X	X		X	X	X	X
Poaceae	X	X		X	X	X	X	X	X	X	X
Polygonaceae	X										
Primulaceae	X										X
Rosaceae	X										
Rubiaceae	X	X									
Unkonwn	X	X					X		X	X	X
TOTAL	16	13	0	4	8	7	9	5	8	8	9

Some species were only found in the UA and/or V-AA areas, such as *Astragalus sp., Echium plantagineum*, *Euphorbia sp., Galactites sp., Lupinus albus*, *Rumex bucephalophorus*, *Rubia sp*. and *Sanguisorba sp*. Conversely, *Andryala ssp., Capsella bursa-pastoris*, *Chaetopogon fasciculatus*, *Dianthus lusitanicus*, *Filago sp., Juncus sp., Gastridium ssp., Lavatera sp., Lolium sp., Malva sp., Phalaris minor*, *Plantago lanceolata*, *Poa sp., Polypogon maritimus* and *Silene gallica* only appeared in treated soils. Regarding treatments, most of the species growing in T, G or M treatments were present after vermicompost addition in Tv, Gv or Mv treatments, but that was not the case of Vv. Moreover, some species mainly appeared in soils with vermicompost, such as *Andryala sp., Hordeum spp* and *P. maritimus*; *Raphanus raphanistrum* L. was only present in treatments with marble sludge or gypsum mining spoil (M, Mv, G and Gv) and in Vv treatment;

Hirschfeldia incana (L.) Lagr.-Foss. was absent in T, G, V, and Vv treatments; *Lolium sp.* only appeared in M and Mv treatments; and *Vulpia ssp.* appeared in all the treatments except in M and Mv.

Among all the plant species present at the study area (Appendix 4.6)*, Anthemis arvensis* L., *Chrysanthemum coronarium* L.*, Lamarckia aurea* (L.) Moench, *Plantago coronopus* L., *Plantago lagopus* L., and *Spergularia rubra* (L.) J. Presl & Presl., the so-called "key species" hereafter (Appendix 4.7), were common to all the vegetated soil types (UA, V-AA and treatments). Although several species from *Vulpia*, *Bromus* and *Medicago* genera were also found in most of the treatments, we could not identify them at species level, excluding them as key species for this study.

The relative cover of each key species varied with soil properties (Figure 4.7). Hence, *L. aurea* presented very low cover (<1%) in UA, V-AA and simple treatments, but increased in mixed treatments, especially in Vv, ranging from 1.29 to 12.83%; *S. rubra* also had very low cover in UA and V-AA, especially in UA (<0.1%), while cover was higher in treatments, especially in those with higher organic carbon content, ranging from 18.83% in V treatment to 29.75% in Vv treatment; *P. coronopus* registered the highest cover in UA area (5.67%), followed by Vv treatment (1.29%), whereas it was <1 in V-AA and in the rest of treatments, although cover generally increased with the addition of vermicompost; *P. lagopus* had low cover (<1%) in all the cases, except in V-AA area (17.79%), and V and Vv treatments (5.16% on average); *A. arvensis* and *C. coronarium* presented higher cover in UA (2.04%), V-AA (11.75%) and mixed treatments (ranging from 1.19% in Mv to 7.71% in Gv), whereas cover was lower than 1% in simple treatments. In the case of *A. arvensis* and *C. coronarium*, due to their phylogenetical proximity, the distinctive morphological features of some individuals were not properly developed during plant cover samplings as to distinguish between these two species; consequently, the species cover for them was calculated jointly (Aar_Cco).



	UA	V-AA	UV-AA	M	Mv	T	Tv	G	Gv	V	Vv
Sr_cover	0,08	1,58	0,00	8,35	21,25	8,56	16,06	5,75	23,19	18,83	29,75
P.lagopus_cover	0,83	17,79	0,00	0,08	0,42	0,08	0,31	0,08	0,52	5,90	4,42
P.coronopus_cover	5,67	0,75	0,00	0,10	0,81	0,17	0,02	0,10	0,67	0,10	1,29
L.aurea_cover	0,25	0,29	0,00	0,04	1,29	0,31	1,94	0,40	6,67	3,52	12,83
Aar_Cco_cover	2,04	11,75	0,00	0,10	1,19	0,13	2,19	0,15	7,71	0,40	6,10
total_cover	99,96	100,00	0,00	9,31	31,52	8,77	20,27	7,29	31,81	40,00	47,13

Soil types

Figure 4.7. Percentage (mean) of key species cover in each soil type: i) **UA**: unaffected soils; ii) **V-AA**: vegetated affected soils; iii) **UV-AA**: unvegetated affected soils; and iv) soil treatments: 1. **G**: Addition of gypsum mining spoil; 2. **M**: Addition of marble sludge; 3. **T**: Tillage; 4. **V**: mixture of UV-AA and V-AA; 5. **Gv**: G plus vermicompost (v); 6. **Mv**: M + v; 7. **Tv**: T + v; 8. **Vv**: V + v. Aar_Cco_cover refers to the total cover of *A. arvensis* and *C. coronarium* calculated jointly.

3.3. Effect of soil treatments on PHE uptake

PHE uptake by plants showed significant differences among key species. *L. aurea* accumulated most Cu (134.83 ± 11.20 mg kg^{-1}), As (74.56 ± 6.27 mg kg^{-1}) and Pb (140.58 ± 11.72 mg kg^{-1}), followed by *S. rubra* (Cu: 86.57 ± 7.66, As: 32.87 ± 3.04 and Pb: 69.46 ± 6.57, in mg kg^{-1}) and *P. coronopus* (Cu: 66.90 ± 4.52, As: 24.19 ± 2.86 and Pb: 44.24 ± 4.42, in mg kg^{-1}), presenting significant differences between the former and *L. aurea*. The highest concentration of Zn were found in *P. coronopus* (928.19 ± 100.98 mg kg^{-1}) and *S. rubra* (709.73 ± 56.66 mg kg^{-1}), and then, in *L. aurea* (415.07 ± 32.29 mg kg^{-1}), presenting significant differences with the former. On the contrary, *A. arvensis* showed the lowest content of Cu (44.18 ± 3.20 mg kg^{-1}), Zn

(392.11 ± 33.74 mg kg^{-1}), As (17.53 ± 2.00 mg kg^{-1}) and Pb (31.82 ± 3.30 mg kg^{-1}), followed by *P. lagopus* (Cu: 46.55 ± 4.1, Zn: 453.94 ± 51.35, As: 17.59 ± 2.53, Pb: 37.20 ± 6.83, in mg kg^{-1}), with no significant differences between them.

Regardless of the species, we are especially interested in PHE uptake by plants per treatments. As no plant emerged in the unvegetated affected soils (UV-AA), there was no PHE record there, so we used PHE uptake in the unaffected (UA) and vegetated affected (V-AA) areas instead as a reference. The plants growing in UA contained significantly less PHEs (Cu: 68-82%, Zn: 46-77%, As: 91-97% and Pb: 71-93%) than the plants growing in the V-AA or the experimental plots (Figure 4.8). Considering simple treatments, the plants from M and G treatments presented similar values for Cu (Figure 4.8a) to those in V-AA soils (76.61 ± 5.77 mg kg^{-1}), and accumulated (on average) 19% and 44% less Cu than the plants from V (87.80 ± 10.12 mg kg^{-1}) and T (125.95 ± 37.89) treatments, respectively. With respect to Zn uptake (Figure 4.8b), plants in M and G treatments accumulated 37% less Zn than in T treatment and V-AA soils (615.16 ± 64.88 mg kg^{-1}, on average), with marginally significant differences, and 53% less Zn than in V treatment (741.78 ± 115.74 mg kg^{-1}), with significant differences. In terms of As uptake (Figure 4.8c), there were no marginal or significant differences among simple treatments, where plants accumulated (on average) 53% more than in V-AA soils (23.08 ± 2.49 mg kg^{-1}). Finally, the plants from V accumulated 28% less Pb than the plants from G, M or T (105.78 ± 7.59 mg kg^{-1}, on average), with marginal differences, but they all had significantly higher values than plants from V-AA soils (42.52 ± 4.36 mg kg^{-1}) (Figure 4.8d). The addition of vermicompost (v) to simple treatments increased the concentration of Zn in plants for any treatment (Figure 4.8b), with marginally significant differences, and the concentration of Pb for Vv (33%) and Tv (56%) treatments (Figure 4.8d), whereas in the rest of cases, it did not promote significant changes.

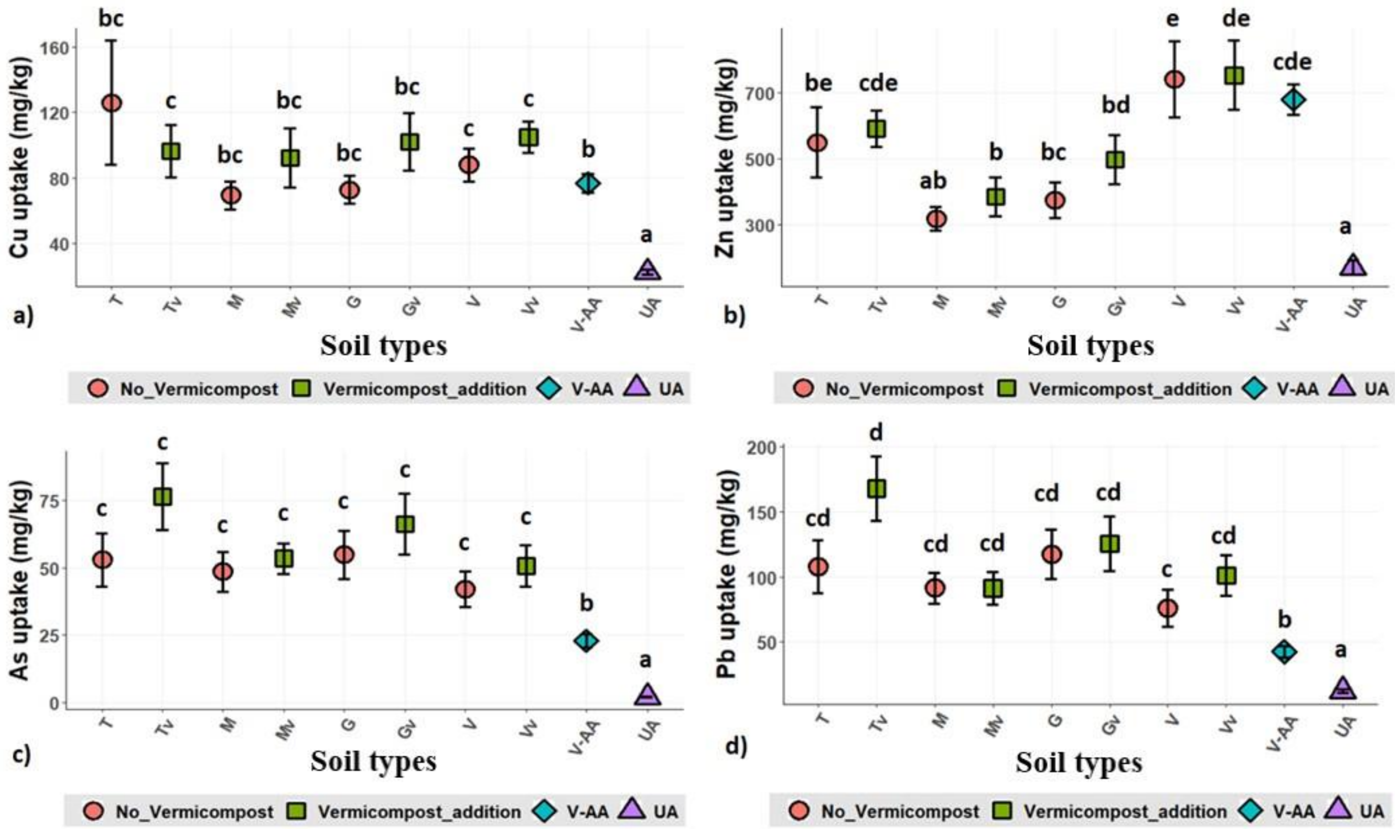


Figure 4.8. PHE uptake (mean ± SE) by key species in soils. PHEs: **a)** Cu**; b)** Zn; **c)** As; **d)** Pb. Soil types: i) **UA**: unaffected soils; ii) **V-AA**: vegetated affected soils; and iii) soil treatments: 1. **G**: Addition of gypsum mining spoil; 2. **M**: Addition of marble sludge; 3. **T**: Tillage; 4. **V**: mixture of UV-AA* and V-AA; 5. **Gv**: G plus vermicompost (v); 6. **Mv**: M + v; 7. **Tv**: T + v; 8. **Vv**: V + v. Different letters above symbols represent statistically significant differences ($p < 0.05$) for the *post-hoc* Tukey tests performed after the LMMs. It should be noted that the vertical scale is different for each graph. *UV-AA: unvegetated affected soil.

3.4. Effect of soil treatments on PHE translocation and bioaccumulation in plants

In the unaffected (UA) and vegetated affected (V-AA) areas, plants accumulated significantly more PHEs in roots than in shoots (Appendix 4.8). Nevertheless, the plants from any treated soil presented PHE concentrations in shoots similar to those in roots, except for Mv, which preferentially accumulated Cu (64 %) in their roots (Appendix 4.8a).

Regarding PHE translocation from roots to shoots (Translocation Factor, TF, Figure 4.9), we observed that it was similar in plants from both UA and V-AA (Cu: 0.71 ± 0.01, Zn: 0.90 ± 0.00, As: 0.85 ± 0.10 and Pb: 0.61 ± 0.25, on average), with just marginally significant differences between the two areas, and with values below 1. Comparing UA area with simple treatments, the TF for Cu (Figure 4.9a) was only higher in the plants from V treatment, with significant differences and values around 1; TF for Zn (Figure 4.9b) was similar in all cases but in T, M and V treatments, it presented values higher than 1; and TF for As (Figure 4.9c) and Pb (Figure 4.9d) in any treatment presented marginal and significant differences, respectively, and values higher than 1. The addition of vermicompost (v) increased TF in Tv and Vv treatments for Cu (Figure 4.9a) and As (Figure 4.9c), in Vv treatment for Zn (Figure 4.9b), and in Tv and Mv treatment for Pb (Figure 4.9d).

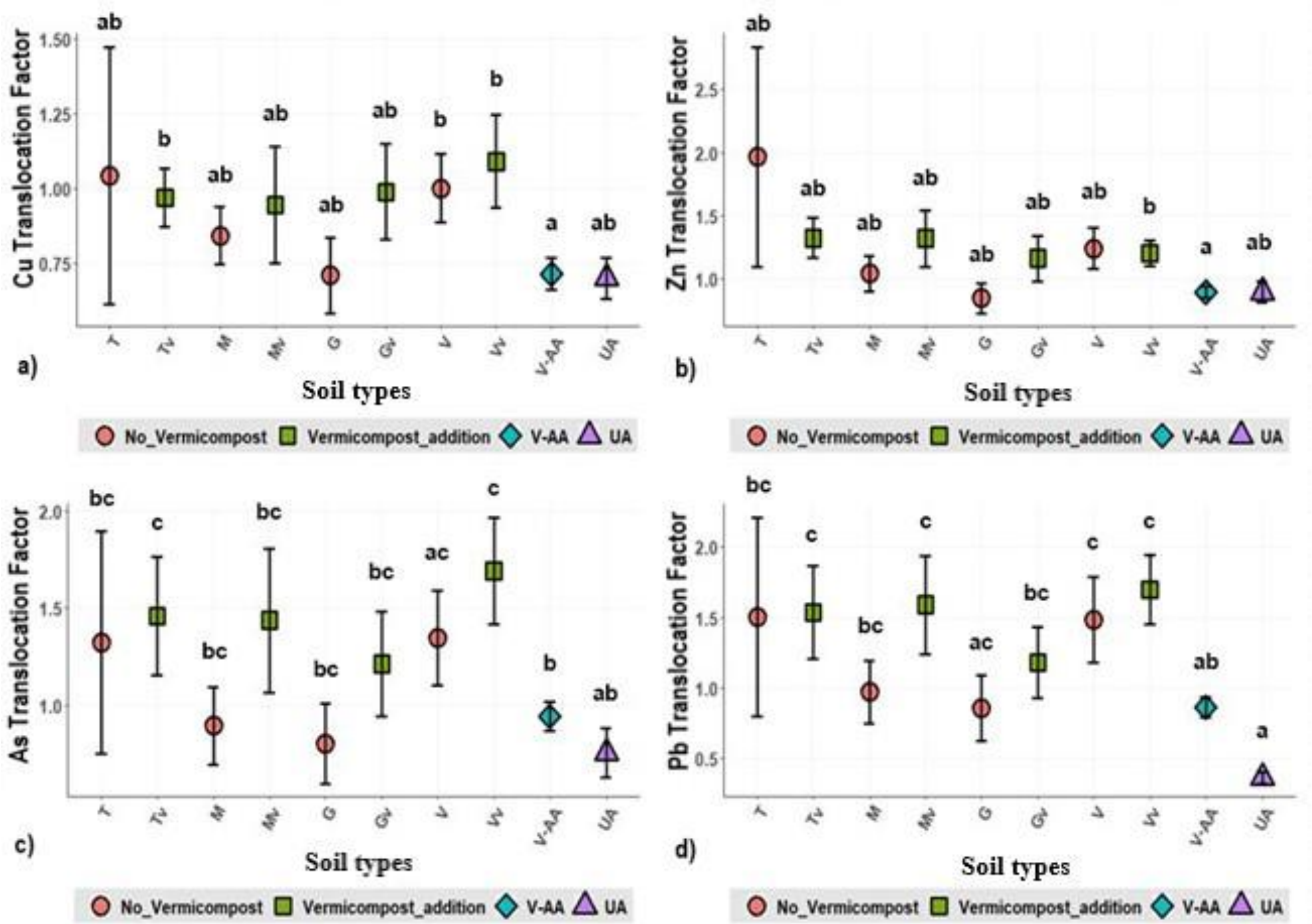


Figure 4.9. Translocation factor (mean ± SE) of PHEs from roots to shoots in plants from each soil type: i) **UA**: unaffected soils; ii) **V-AA**: vegetated affected soils; and iii) soil treatments: 1. **G**: Addition of gypsum mining spoil; 2. **M**: Addition of marble sludge; 3. **T**: Tillage; 4. **V**: mixture of UV-AA* and V-AA; 5. **Gv**: G plus vermicompost (v); 6. **Mv**: M + v; 7. **Tv**: T + v; 8. **Vv**: V + v. PHEs: **a)** Cu; **b)** Zn; **c)** As; **d)** Pb. Different letters above symbols represent statistically significant differences ($p < 0.05$) for the *post-hoc* Tukey tests performed after the LMMs. It should be noted that the vertical scale is different for each graph. *UV-AA: Unvegetated Affected Soil.

Regarding the relation between PHE availability in soils and accumulation in plant shoots (Figure 4.10), we observed that UA soils had the lowest Bioaccumulation Factor (BAF) for Cu (0.54 ± 0.05) and As (0.12 ± 0.02), although they did not present significant differences with those in V-AA area or any treated soils. The BAF for Pb (Figure 4.10d) did not have significant differences among treatments either, nevertheless, soil transfer of Pb into plants tended to be higher in UA area. In the case of Zn (Figure 4.10b), and compared with UA (2.01 ± 0.25), BAF was similar in V (2.30 ± 0.45) and G (1.50 ± 0.19) treatments, lower in M treatment (1.19 ± 0.14), with marginally significant differences, and higher in T treatment (2.86 ± 0.62), with marginally significant differences; in any case, BAF values were higher than 1. The addition of vermicompost (v) did not significantly change the BAF for any PHE in any treatment.

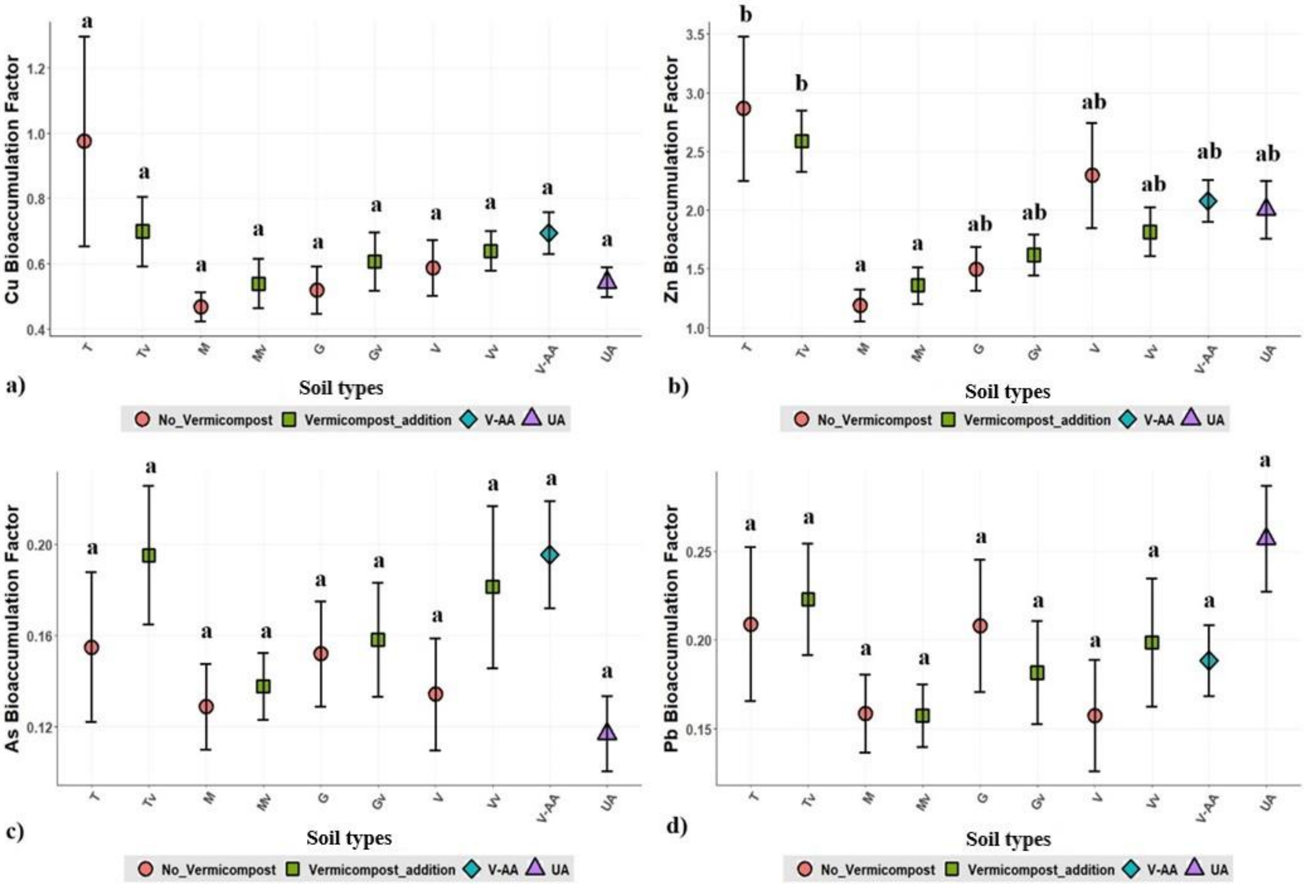


Figure 4.10. Bioaccumulation Factor (mean ±SE) of PHEs from soils to shoots in each soil type: i) **UA**: unaffected soils; ii) **V-AA**: vegetated affected soils; and iii) soil treatments: 1. **G**: Addition of gypsum mining spoil; 2. **M**: Addition of marble sludge; 3. **T**: Tillage; 4. **V**: mixture of UV-AA* and V-AA; 5. **Gv**: G plus vermicompost (v); 6. **Mv**: M + v; 7. **Tv**: T + v; 8. **Vv**: V + v. PHEs: **a)** Cu; **b)** Zn; **c)** As; **d)** Pb. Different letters above symbols represent statistically significant differences ($p < 0.05$) for the *post-hoc* Tukey tests performed after the LMMs. It should be noted that the vertical scale is different for each graph. *UV-AA: Unvegetated Affected Soil.

Finally, the quotient (Q) between PHE concentrations in plants from the affected (V-AA and treatments) and the unaffected areas (UA) were less than 10 times higher in the case of Cu (Figure 4.11a) and Zn (Figure 4.11b), and around 10 times for Pb, (except in Tv that was 18 times, Figure 4.11d); in the case of Zn, there were significant differences between treatments, presenting G and M treatments the lowest values, and V treatment the highest. The accumulation of As in the plants from any treatment was more than 20 times higher than in UA, with no significant differences among treatments (Figure 4.11c). The addition of vermicompost (v) to simple treatments did not produce any significant change, except in Tv and Vv treatments for Pb (Figure 4.11d), where the quotient marginally increased.

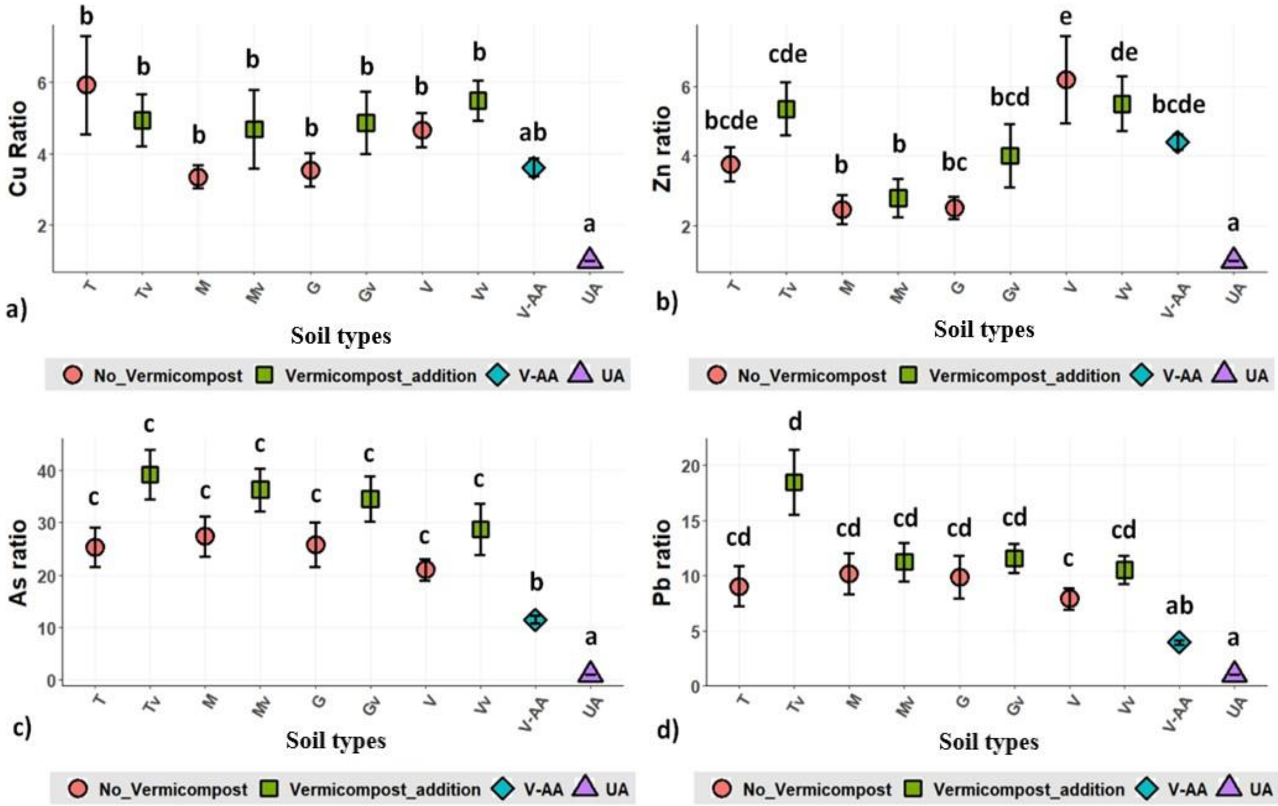


Figure 4.11. Quotient (mean ±SE) between PHE concentrations in plants from each soil type: i) **V-AA**: vegetated affected soils; ii) **UA**: unaffected soils; and iii) soil treatments: 1. **G**: Addition of gypsum mining spoil; 2. **M**: Addition of marble sludge; 3. **T**: Tillage; 4. **V**: mixture of UV-AA* and V-AA; 5. **Gv**: G plus vermicompost (v); 6. **Mv**: M + v; 7. **Tv**: T + v; 8. **Vv**: V + v. PHEs: **a)** Cu; **b)** Zn; **c)** As; **d)** Pb. Different letters above symbols represent statistically significant differences ($p < 0.05$) for the *post-hoc* Tukey tests performed after the LMMs. It should be noted that the vertical scale is different for each graph. *UV-AA: Unvegetated Affected Soil.

3.5. Treatment effectiveness

As a summary, Table 4.2 shows the most effective treatments according to the variables studied.

Table 4.2. List of soil treatments with the best results according to the studied variables. Soil treatments: **G**: Addition of gypsum mining spoil; **M**: Addition of marble sludge; **T**: Tillage; **V**: mixture of UV-AA and V-AA; **Gv**: G plus vermicompost (v); **Mv**: M + v; **Tv**: T + v; **Vv**: V + v. [1]Natural plants refers to the plants growing in UA. [2]Polluted plants refers to the plants growing in V-AA and treatments. UA: Unaffected soils; V-AA: Vegetated Affected soils.

Variables		Treatment(s) with the best results
Plant cover		Vv
Plant species richness		Vv
Plant diversity (H')		Vv
Effective Number of Species (ENS)		Vv
PHE uptake	Cu	M and G
	Zn	M
	As	No significant differences among treatments
	Pb	V
Translocation Factor (TF)	Cu	G, Gv, M, MV and T
	Zn	No significant differences among treatments
	As	No significant differences among treatments
	Pb	T, M, G and Gv
Bioaccumulation Factor (BAF)	Cu	No significant differences among treatments
	Zn	M and Mv
	As	No significant differences among treatments
	Pb	No significant differences among treatments
Quotient natural plants[1]/polluted plants[2] (Q)	Cu	M and G
	Zn	M and Mv
	As	M and V
	Pb	V

4. DISCUSSION

4.1. Effect of soil treatments on plant cover, richness, and diversity

The soils from the vegetated affected areas (V-AA) were completely covered by herbaceous vegetation, similarly to the soils where the spill never reached (UA), suggesting the effectiveness of the intense rehabilitation program carried out after the mining accident, which included, not only cleaning and remediation, but also afforestation (CMA, 2003). Moreover, these areas registered the highest diversity of herbaceous species; in the case of V-AA, richness was even higher than in the surrounding vegetated affected areas within the Guadiamar Green Corridor (GGC) described in previous studies (Belt 1 in García-Carmona et al, 2019). These results indicate that soil conditions in V-AA are rather remediated, favouring the recovery of a developed plant cover. These vegetated affected areas (V-AA) are acting as propagule donors (Pérez-de-Mora et al., 2011) through vegetation with high seed production, effective dispersal mechanisms (Burgos et al., 2013) and PHE tolerance (Del Río et al., 2002; Poniedzialek et al., 2005; Burgos et al., 2008; Hernández and Pastor, 2008; Sierra et al. 2008; Gawronski et al., 2011; Pérez-de-Mora et al., 2011; Montiel-Rozas et al., 2016; García-Carmona et al., 2019). Nevertheless, the still extreme soil conditions in the unvegetated affected areas (UV-AA), mainly acidic pH and high PHE concentrations, have completely inhibited seed emergence and plant re-establishment (Becker and Brändel, 2007) despite the proximity to propagule donors (García-Carmona et al., 2019).

After treatment application in UV-AA soils, plant emergence was reactivated, similarly to what Madejón et al. (2006) reported in the area, and plant cover, richness and diversity were recovered at different levels depending on the treatment. Although marble sludge (M), gypsum mining spoil (G) and tillage (T) promoted plant emergence, they still resulted quite ineffective in comparison with the unaffected (UA) and vegetated affected (V-AA) areas, presenting rather low plant cover, richness and diversity, and suggesting that soil conditions are not completely recovered there yet; for instance, the diversity index (H’) for these treatments was under the normal values usually obtained for empirical data (1.5-3.5, Magurran, 2004). The unvegetated affected soils contained their own seeds from the surrounding vegetated areas (seed sink) prior to treatment application, but it has been inactive for more than 20 years, blocking organic matter input, what entailed a constraining factor for the evolution of a healthy soil ecosystem (Bot and Benites, 2005). As neither marble sludge, gypsum mining spoil or tillage contained organic matter, they could not improve soil organic carbon levels (Sánchez et al., 2010), limiting the availability of essential nutrients, and then resulting in low plant covers. Moreover, these treatments presented other limitations. In the case of marble sludge, despite the elevated $CaCO_3$ content, carbonate particles may have been isolated to some extent by iron hydroxysulphate coatings, reducing the capacity of marble sludge to neutralize the acidic pH and PHE fixation (Martín et al., 2003; Simón et al., 2005); in the case of gypsum mining spoil, the solubility of sulphate ions can promote the relative mobility of PHEs and produce toxicity to plants (Grattan and Grieve, 1999); and in the case of tillage, although crust breaking may enhance aeration and soil water infiltration, no other properties are improved to reduce PHE toxicity in comparison to the unvegetated affected soils (UV-AA), which explains the poor plant performance in this treatment (García-Carmona et al., 2017). On the contrary, the mixture with vegetated topsoil presented the highest plant cover, richness and diversity, facilitated by both the seed bank and organic matter content brought from V-AA; in this vein, in a short period of time, plant species richness in this treatment was similar to that in the surrounding vegetated affected areas (V-AA) described by García-Carmona et al. (2019).

The addition of vermicompost enhanced the properties of simple treatments by increasing the organic matter content, rising their capacity to improve soil physicochemical properties such as pH and structure, and thus, the re-establishment of plant cover, richness and diversity (Clemente et al., 2015). The most effective treatment was, the mixture with vegetated topsoil and vermicompost addition, presenting a similar richness to that in vegetated affected soils in the area (Belt 1 in García-Carmona et al., 2019); this topsoil contained, not only the highest organic matter content, but also the seed bank from V-AA. On the contrary, plant species richness in the soils treated with marble sludge and vermicompost or with gypsum mining spoil and vermicompost was half the richness in vegetated affected soils in the area (Belt 1 in García-Carmona et al., 2019). Nevertheless, the rate at which plant cover, richness and diversity increased after vermicompost addition to tillage (Tv), and especially for marble sludge and gypsum mining spoil with vermicompost (Mv and Gv, respectively), was much higher than in the mixture with vegetated topsoil and vermicompost (Vv). This much higher relative increase was probably related to the fact that the lack of organic matter was a serious limiting factor in soils treated with tillage, marble sludge and gypsum mining spoil. Moreover, the combination of organo-mineral amendments has proved very effective in similar scenarios (Alvarenga et al., 2008; Jiménez-Moraza et al., 2006). On one hand, organic matter provides nutrients, give structure to the soil and has the capacity to bind and make less available cationic PHEs (Bot and Benites, 2005; Gall et al., 2015); on the other hand, marble sludge and gypsum mining spoil also help restructuring the soil and are both rich in calcium (and in sulphur, in the case of G), essential macronutrient(s) for plants (Maathuis 2009). Anyway, the effect of organic matter on the mobility of PHEs should be monitored over time, because an increase in the availability of some elements related to the rise in soil organic matter was also described in the area (Nakamaru et al., 2017; Sierra-Aragón et al., 2019).

4.2. Effect of soil treatments on species composition.

Our key species, *Anthemis arvensis*, *Chrysanthemum coronarium, Lamarckia aurea*, *Plantago coronopus*, *Plantago lagopus*, and *Spergularia rubra*, appeared in all the vegetated soil types (UA, V-AA and treatments). Nevertheless, the relative cover of each of them varied with soil properties after treatment application. *L. aurea* and *S.rubra* cover was very low in the unaffected (UA) and vegetated affected (V-AA) areas, whereas it increased in treated soils, especially in treatments with vermicompost and in the mixture with vegetated topsoil, which had similar OC content to that in UA and V-AA, and higher PHE load. Both species seem to be opportunistic generalist species with a high ability to get adapted to high loads of pollutants, what may affect their capacity to compete in non-polluted environments (Büchi and Vuilleumier, 2014). Despite *P. coronopus*, *P. lagopus*, *A. arvensis* and *C. coronarium* have been previously described as PHE-tolerant species (Del-Río et al., 2002; Montiel-Rozas et al., 2016; Serrano et al., 2017), their presence and cover in the most degraded soils seem to have been more affected by limiting soil properties (mainly the low OC content) than by PHE load.

Observing the overall species composition, the adverse soil properties seem to have acted as a filter for plant species emergence and development. For instance, as soil acidification and pollution are a threat for plant diversity, in the soils with the least effective treatments (T, G and M) we mainly found herbaceous species that could get adapted to the new acidic conditions and high PHE loads, and which belong to families frequently used in phytoremediation for their tolerance to PHEs, such as Asteraceae, Brassicaceae, Caryophyllaceae, Fabaceae, Plantaginaceae or Poaceae (Broadley et al., 2001; Gawronski et al. 2011; Gutiérrez-Ginés et al., 2015). A higher effectiveness of the treatment is linked to higher plant species diversity; hence, we could think that the addition of vermicompost has made simple treatments more effective, especially in combination with gypsum mining spoil and marble sludge. In general terms, most of the species found in our treatments did not show a relation with the treatment itself but with the improvement of the soil properties. Nevertheless, there are a few cases that seem to be affected by a certain treatment; for instance, *Raphanus raphanistrum* could be calcium-dependent, mainly appearing in those treatments with gypsum mining spoil and marble sludge, and *Lolium sp*. only appeared in those treatments with marble sludge (M and Mv), suggesting that the application of amendments with significant concentrations of Ca could increase the uptake of this element at the expense of the absorption of PHEs (Madejón et al., 2006). On the contrary, *Vulpia spp.* appeared in all the treated soils, except in M and Mv; although *Vulpia spp.* grows extremely well in infertile and acidic soils (Tozer, 2004), it has a low calcium requirement (Loneragan et al., 1967), which may limit the presence of this species in soils with high Ca content. Anyway, although an excess in calcium may promote nutrient imbalances (Tuna et al., 2007), in a scenario of soil pollution such as Aznalcóllar case study, the protective action of Ca against the toxicity of PHEs (Mengel and Kirkby, 1987) seem to be more important. Thus, the addition of organo-mineral amendments seems to enhance the spontaneous emergence of herbaceous species coming from the surrounding areas by the reduction of PHE mobility and toxicity.

4.3. Effect of soil treatments on the uptake of Potentially Harmful Elements by plants

Most of the species (or families) growing in our treatments (Appendix 4.6) seem to have the ability to deal with high amounts of PHEs (Broadley et al., 2001; Del-Río et al., 2002; Madejón et al., 2006; Burgos et al., 2008, 2013; Hernández and Pastor, 2008; Sierra et al. 2008; Pérez-de-Mora et al., 2011; Gutiérrez-Ginés et al., 2015; Montiel-Rozas et al., 2016; Serrano et al., 2017; García-Carmona et al., 2019).

Our key species accumulated PHEs differently, probably due to morpho-physiological differences (Gupta et al., 2019), being *L. aurea*, *S. rubra* and *P. coronopus* the species with the highest PHE concentrations in tissues, indicating they are good PHE accumulators (Del-Río et al., 2002; Madejón et al., 2006; Hernández and Pastor, 2008; Gutiérrez-Ginés et al., 2015; Serrano et al., 2017; Midhat et al., 2017). Moreover, key species grew in most of the soil types in this study, except in the unvegetated affected areas, where no plant emergence was registered, suggesting they are pseudometallophyte species with a high capacity to cope with soil pollution and, thus, to get adapted to adverse soil properties, such as salinity or acidity, what has also been supported by other authors' research, both within the Guadiamar Green Corridor (Del-Río et al., 2002; Burgos et al., 2008, 2013; Pérez-de-Mora et al., 2011; Montiel-Rozas et al., 2016; García-Carmona et al., 2019) and in other areas with similar scenarios of pollution (Hernández and Pastor, 2008; Fernández-Caliani and Barba-Briso, 2010; Gutiérrez-Ginés et al., 2013; Midhat et al., 2017).

Apart from the capacity of plant species to deal with pollution and further adverse soil properties, PHE accumulation in plants also depends on abiotic factors such as soil pH, soil exchange capacity, soil organic matter content and concentration of available PHEs in soils (Gupta et al., 2019). As the soil from the unvegetated affected areas still presents extreme conditions, as acidic pH, and total As and Pb surpassing the regional thresholds (NGR) (BOE 2015), no plant development was registered there, which is further evidence that residual pollution and soil degradation are still too high in certain areas in the Guadiamar Green Corridor, and that additional rehabilitation measures are required.

Reference values for plants are highly variable (Kabata-Pendias, 2011), but the range for normal levels are: Cu: 5-30, Zn: 25-150, As: 1-1.5, and Pb: 5-10, in mg kg^{-1}; and for toxic levels are: Cu: 20-100, Zn: 100-400, As: 5-20, and Pb: 30-300, in mg kg^{-1}). According to them, the key species that grew in the unaffected area accumulated Cu, Zn, As and Pb at normal levels, or very close to them, and at phytotoxic levels in the vegetated affected areas, highlighting the presence of residual pollution in these soils twenty years after the accident. Regarding experimental plots, key species accumulated Cu, Zn, As and Pb at phytotoxic levels in any treatment, with no significant differences among treatments. Even though, the treatments with gypsum mining spoil and marble sludge have been the most effective reducing Cu and Zn uptake. This correlates with the effect of these amendments on the reduction of Cu and Zn availability in soils. Kabata-Pendias (2011) detected Cu and Zn immobilization in soils rich in calcium and sulphur, and Garrido et al. (2005) observed how the Al-hydroxy polymers formed in gypsum amended soils immobilized Zn. Antoniadis et al. (2017) reported that the addition of calcium-rich amendments could be a successful strategy to reduce the availability of cationic PHEs such as Cu and Zn, and that even low carbonate content gives enormous buffering capacity to soils through pH rise and the formation of insoluble compounds. In terms of As uptake reduction, none of the treatments showed a positive effect, presenting similar uptake by plants in all treatments, and higher to that in the vegetated affected areas, probably due to the remobilization of this element through pH increase after treatment application (Martín Peinado et al., 2012). Finally, Pb concentration in plant's tissues was also similar in all treatments, just presenting slightly lower values in the mixture with vegetated topsoil as a result of a dilution effect. Nevertheless Pb uptake in treatments was higher than in the vegetated affected areas, despite Pb bioavailability was lower; on one hand, the acidic pH in most treated soils compared to the vegetated affected soils could have mobilize Pb (Romero-Freire et al., 2015); on the other hand, although Pb bioavailability could rise in direct relation with the increase of organic matter content (García-Carmona et al., 2019), the higher availability of macronutrients in the vegetated affected area may have interfered with Pb, favouring nutrient uptake and reducing Pb accessibility for plants (Kabata-Pendias, 2011; Antoniadis et al., 2017). Finally, the addition of vermicompost to simple treatments did not significantly affect PHE uptake, although an increasing trend was observed. Organic matter has controversial effects on PHE mobility; although organic matter tends to promote PHE immobilization as a result of the formation of organo-metallic ligands of high molecular weight (Antoniadis et al., 2017), some authors have observed right the opposite behavior depending on the type of organic matter added (Kumpiene et al., 2008; De-la-Fuente et al., 2011; Sierra-Aragón et al., 2019).

As the plants that grew in the experimental plots still accumulated significantly more Cu, Zn, As and Pb than the plants from the unaffected soils, further management of our experimental plots (i.e. repeated tillage and/or amendment application over time) should be accomplished, similar to what other authors observed in the area (Madejón et al., 2018).

4.4. Effect of soil treatments on PHE translocation and bioaccumulation in plants

A well-established vegetation cover with low capacity to translocate and accumulate PHEs in shoots is of major interest, since it would promote PHE stabilization in soil and would hinder its transfer to the food web (Clemente et al., 2015).

The edible parts (shoots) of the plants from the affected areas, both vegetated and experimental, surpassed the toxic levels proposed by Chaney (1989) for all PHEs, except for As (any treatment and V-AA), for Zn in marble sludge and gypsum mining spoil (with and without vermicompost in both cases), and for Pb in V-AA. All these evidence that the prohibition of introducing herbivores in the Guadiamar Green Corridor is still required, and that further rehabilitation techniques based on PHE immobilization in soils (i.e. amendment application and phytostabilization) must be implemented.

As the toxic potential of each PHE is not the same, the use of contamination indexes is especially recommended when it refers to multi-element pollution, in order to compare among elements in a more realistic way (Antoniadis et al., 2017). In our case, we are especially interested in low translocation (TF) and bioaccumulation (BAF) factors (values below 1) to guarantee PHE stabilization in soils (Mehes-Smith et al., 2013). These low values were found for the vegetated affected areas, suggesting that the rehabilitation activities implemented after the spill have been rather effective stabilizing pollutants (Madejón et al., 2018). In the case of our experimental plots, considering that the concentrations of Zn, As and Pb in plant shoots were similar to those in roots and that TF values were generally above 1, there was a certain translocation of these pollutants in most treatments. Moreover, the plants in the affected soils accumulated more PHEs than the plants in the unaffected soils, and it was especially remarkable for As, which was more than 20 times higher in the former, indicating As was rather available for plants regardless of the treatment. Nevertheless, BAF values for Cu, As and Pb were below 1 in nearly all the cases, with no significant differences among the studied soils and treatments, suggesting that plants did not hyperaccumulate these elements. On the contrary, BAF for Zn was above 1 in all the studied soils and treatments, what could be related to the essential metabolic role that Zn plays in plants; moreover, tolerant species may accumulate Zn up to 1% in storage tissues, limiting the presence of this metal in cellular locations and reducing phytotoxicity (Kabata-Pendias, 2011). Focusing on treatments, tillage (with and without vermicompost) was the only one with higher BAF values for Zn than the unaffected area, what could be due to the strong acidity of these soils promoting Zn availability (García-Carmona et al., 2019).

5. CONCLUSIONS

Mixing the uppermost 10 cm of vegetated affected soils (V-AA) with the unvegetated soils (UV-AA) and vermicompost resulted the most effective rehabilitation technique in order to promote the emergence of herbaceous species, and the recovery of developed plant cover and diversity in the unvegetated affected soils in the Guadiamar Green Corridor; this treatment strongly improved soil properties, and supported vegetation recovery by the presence of an active seed bank from V-AA. The addition of marble sludge with vermicompost also showed a positive effect, achieving the highest plant cover and diversity with the lowest PHE uptake and translocation, except in the case of Pb, for which the best option was the mixture with vegetated topsoil. All this suggests that, along with acidity, salinity, and high load of PHEs, the lack of available nutrients and active seed bank are also key limiting factors for plant emergence and development. Tillage was the least effective treatment and should be used in combination with other soil amendments or treatments. The mixture of marble sludge and vermicompost is also a cost-effective and environmentally friendly rehabilitation option for large polluted areas since it is based on the reuse of wastes coming from other human activities. Notwithstanding, further studies should be conducted in order to improve the positive effects of this organo-mineral amendment, such as sowing native species after amendment application or considering retreatment over time, actions that could also enhance the amending properties of gypsum mining spoil (with or without vermicompost). The assessment of the transfer and bioavailability of PHEs to plants also needs to be monitored over time to avoid the potential risk of pollution into the food chain.

6. ACKNOWLEDGEMENTS

The authors would like to thank KNAUF-GmbH and Tatiana-Pérez-de-Guzmán-el-Bueno Foundation for the financial support. We also would like to thank Francisco Javier Martínez Garzón, Yasuo Mitsui Nakamaru, Mariano Simón, Miguel Ballesteros, Olivia Lorente Casalini, Sandra Redondo, Regina Berjano Pérez and Mario Paniagua for their valuable help setting up the experimental plots, sampling, and the lab work.

This work was supported by the Research Project RTI 2018-094327-B-I00 (Ministry of Science, Innovation and Universities).

Helena García Robles was financed by Tatiana-Pérez-de-Guzmán-el-Bueno Foundation PhD grant Programme 2016.

7. REFERENCES

Adriano, D.C., Wenzel, W., Vangronsvel, J., Bolan, N., 2004. Role of assisted natural remediation in environmental cleanup. Geoderma, 122 (2): 121-142. https://doi.org/10.1016/j.geoderma.2004.01.003

Alvarenga, P., Gonçalves, A.P., Fernandes, R.M., de Varennes, A., Vallini, G., Duarte, E., Cunha-Queda, A.C., 2008. Evaluation of composts and liming materials in the phytostabilization of a mine soil using perennial ryegrass. Sci. Total Environ., 406(1-2): 43-56. https://doi.org/10.1016/j.scitotenv.2008.07.061

Ahmed, A., Ugai, K., Kamei, T., 2011. Investigation of recycled gypsum in conjunction with waste plastic trays for ground improvement. Constr. Build. Mater., 25: 208-217. https://doi.org/10.1016/j.conbuildmat.2010.06.036

Alastuey, A., García-Sánchez, A., López, F., Querol, X., 1999. Evolution of pyrite mud weathering and mobility of heavy metals in the Guadiamar valley after the Aznalcóllar spill, south-west Spain. Sci. Total Environ., 642: 41-55. https://doi.org/10.1016/S0048-9697(99)00375-7

Antoniadis, V., Levizou, E., Shaheen, S.M., Ok, Y.S., Sebastian, A., Baum, C., Prasad, M.N.V., Wenzel, W.W., Rinklebe, J., 2017. Trace elements in the soil-plant interface: Phytoavailability, translocation, and phytoremediation- A review. Earth-Sci. Rev., 171: 621-645. https://doi.org/10.1016/j.earscirev.2017.06.005

Arienzo, M., Adamo, P., Cozzolino, V., 2004. The potential of *Lolium perenne* for revegetation of contaminated soil from a metallurgical site. Sci. Total Environ., 319: 13-25. https://doi.org/10.1016/S0048-9697(03)00435-2

Baker, A., Ernst, W., Van der Ent, A., Malaisse, F., Ginocchio, R., 2010. Metallophytes: The unique biological resource, its ecology and conservational status in Europe, central Africa and Latin America, in: Batty, L. Hallberg, K., (Eds.), Ecology of Industrial Pollution. Ecological Reviews. Cambridge: Cambridge University Press, pp. 7-40
https://doi.org/10.1017/CBO9780511805561.00

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

Barton, K., 2016. Multi-model inference. R package version 1.15.6. Available at: https://cran.r-project.org/web/packages/MuMIn/index.html

Bates, D., Maechler, M., Bolker, B., Walker, S., 2015. Fitting Linear Mixed-Effects Models Using lme4. J. Stat. Softw., 67(1): 1-48. https://doi.org/10.18637/jss.v067.i01

Becerra-Castro, C., Monterroso, C., Prieto-Fernández, A., Rodríguez-Lamas, L., Loureiro-Viñas, M., Acea, M.J., Kidd, P.S., 2012. Pseudometallophytes colonising Pb/Zn mine tailings: A description of the plant-microorganism-rhizosphere soil system and isolation of metal-tolerant bacteria. J. Hazard. Mater., 217-218: 350-359. https://doi.org/10.1016/j.jhazmat.2012.03.039

Becker, T., Brändel, M., 2007. Vegetation-environment relationships in a heavy Metal-dry grassland complex. Folia Geobot., 42: 11-28. https://doi.org/10.1007/BF02835100

Bini, C., Bech, J., 2014. PHEs, environment and human health: Potentially harmful elements in the environment and the impact on human health. Springer Dordrecht Heidelberg New York London. https://doi.org/10.1007/978-94-017-8965-3

Bolan, N.S., Park, J.H., Robinson, B., Naidu, R., Huh, K.Y., 2011. Phytostabilization: A green approach to contaminant containment, in: Donald, L.S. (Ed.), Advances in Agronomy. Chapter 4. Academic Press, pp. 145-204. https://doi.org/10.1016/B978-0-12-385538-1.00004-4

Boletín Oficial del Estado (BOE) 38, 2015. Decreto 18/2015 por el que se aprueba el reglamento que regula el régimen aplicable a los suelos contaminados. Consejería de Medioambiente y Ordenación del Territorio, 38: 28-64.

Bot, A., Benites, J., 2005. The importance of soil organic matter key to drought-resistant soil and sustained crop production. FAO (Food and Agriculture Organization of the United Nations), Soils Bulletin 80. Rome.

Broadley, M., Willey, M.J., Wilkins, J.C., Baker, A.J.M., Mead, A., White, P.J., 2001. Phylogenetic variation in heavy metal accumulation in angiosperms. New Phytol., 152: 9-27. https://doi.org/10.1046/j.0028-646x.2001.00238.x

Büchi, L., Vuilleumier, S., 2014. Coexistence of specialist and generalist species is shaped by dispersal and environmental factors. Am. Nat., 183(5): 612-624. https://doi.org/10.1086/675756

Burgos, P., Pérez-de-Mora, A., Madejón, P., Cabrera, F., Madejón, E., 2008. Trace elements in wild grasses: a phytoavailability study on a remediated field. Environ. Geochem. Health., 30: 109-114. https://doi.org/10.1007/s10653-008-9135-3

Burgos, P., Madejón, P., Madejón, E., Girón, I., Cabrera, F., Murillo, J.M., 2013. Natural remediation of an unremediated soil twelve years after a mine accident: Trace element mobility and plant composition. J. Environ. Manage., 114: 36-45. https://doi.org/10.1016/j.jenvman.2012.10.060

Busari, M.A., Kukal, S.S., Kaur, A., Bhatt, R., Dulazi, A.A., 2015. Conservation tillage impacts on soil, crop and the environment. Int. Soil Water Conserv. Res., 3: 119-129. https://doi.org/10.1016/j.iswcr.2015.05.002

Chaney, R.L., 1989. Toxic element accumulation in soils and crops: Protecting soil fertility and agricultural food-chains, in: Bar-Yosef, B., Barrow, N.J., Goldshmid, J. (Eds.), Inorganic Contaminants in the Vadose Zone. Ecological Studies (Analysis and Synthesis). Springer, Berlin, Heidelberg, pp. 140-158. https://doi.org/10.1007/978-3-642-74451-8_10

Clemente, R., Walker, D.J., Roig, A., Bernal, M.P., 2003. Heavy metal bioavailability in a soil affected by mineral sulphides contamination following the mine spillage at Aznalcóllar (Spain). Biodegradation, 14: 199-205. https://doi.org/10.1023/A:1024288505979

Clemente, R., Pardo, T., Madejón, P., Madejón, E., Bernal, M.P., 2015. Food byproducts as amendments in trace elements contaminated soils. Food Res. Int., 73: 176-189. https://doi.org/10.1016/j.foodres.2015.03.040

CMA (Consejería de Medio Ambiente), 2003. Ciencia y restauración del río Guadiamar. PICOVER 1998-2002. Junta de Andalucía, Sevilla, Spain.

Cooke, J.A., Johnson, M.S., 2002. Ecological restoration of land with particular reference to the mining of metals and industrial minerals: A review of theory and practice. Environ. Rev., 10: 41-71. https://doi.org/10.1139/a01-014

Crouzeilles, R., Ferreira, M.S., Chazdon, R.L., Lindenmayer, D.B., Sansevero, J.B.B., Monteiro, L., Iribarrem, A., Latawiec, A.E., Strassburg, B.B.N., 2017. Ecological restoration success is higher for natural regeneration than for active restoration in tropical forests. Sci. Adv., 3: e1701345. https://doi.org/10.1126/sciadv.1701345

De-la-Fuente, C., Clemente, R., Martínez-Alcalá, I., Tortosa, G., Bernal, M.P., 2011. Impact of fresh and composted solid olive husk and their water-soluble fractions on soil heavy metal fractionation; microbial biomass and plant uptake. J. Hazard. Mater., 186: 1283-1289. https://doi.org/10.1016/j.jhazmat.2010.12.004

Del Río, M., Font, R., Almela, C., Vélez, D., Montoro, R., de Haro-Bailón, A., 2002. Heavy metals and arsenic uptake by wild vegetation in the Guadiamar river area after the toxic spill of the Aznalcóllar mine. J. Biotechnol., 98: 125-137. https://doi.org/10.1016/S0168-1656(02)00091-3

Domínguez, M.T., Alegre, J.M., Madejón, P., Madejón, E., Burgos, P., Cabrera, F., Marañón, T., Murillo, J.M., 2016. River banks and channels as hotspots of soil pollution after large-scale remediation of a river basin. Geoderma, 261: 133-140. https://doi.org/10.1016/j.geoderma.2015.07.008

Elzinga, C.L., Salzer, D.W., Willoughby, J.W., 2015. Measuring and monitoring plant populations. Denver: U.S. Dept. of the Interior, Bureau of Land Management Nature Conservancy.

Fernández-Caliani, J.C., Barba-Brioso, C., 2010. Metal immobilization in hazardous contaminated mine soils after marble slurry waste application. A field assessment at the Tharsis mining district (Spain). J. Hazard. Mater., 181: 817-826. https://doi.org/10.1016/j.jhazmat.2010.05.087

Franzen, D., Rehm, G., Gerwing, J., 2006. Effectiveness of gypsum in the North-Central Region of the U.S. NDSU Extension Service, North Dakota.

Gall, J.E., Boyd, R.S., Rajakaruna, N., 2015. Transfer of heavy metals through terrestrial food webs: a review. Environ. Monit. Assess., 187: 201. https://10.1007/s10661-015-4436-3

García-Carmona, M., Romero-Freire, A., Sierra Aragón, M., Martínez Garzón, F.J., Martín Peinado, F.J., 2017. Evaluation of remediation techniques in soils affected by residual contamination with heavy metals and arsenic. Journal Environ. Manage., 191: 228-236. http://doi.org/10.1016/j.jenvman.2016.12.041

García-Carmona, M., García-Robles, H., Turpín Torrano, C., Fernández Ondoño, E., Lorite Moreno, J., Sierra Aragón, M., Martín Peinado, F.J., 2019. Residual pollution and vegetation distribution in amended soils 20 years after a pyrite mine tailings spill (Aznalcóllar, Spain). Sci. Total Environ., 650: 933-940. https://doi.org/10.1016/j.scitotenv.2018.09.092

Garrido, F., Illera, V., García-González, M.T., 2005. Effect of the addition of gypsum- and lime-rich industrial by-products on Cd, Cu and Pb availability and leachability in metal-spiked acid soils. Appl. Geochem., 20: 397-408. https://doi.org/10.1016/j.apgeochem.2004.08.001

Gawronski, S.W., Greger, M., Gawronska, H., 2011. Plant taxonomy and metal phytoremediation, in: Sherameti, I., Varma, A. (Eds.), Detoxification of heavy metals. Chapter 5, pp. 91-109. Springer, Berlin-Heidelberg. https://doi.org/10.1007/978-3-642-21408-0_5

Grattan, S.R., Grieve, C.M., 1999. Mineral nutrient acquisition and response by plants grown in saline environments. Handbook of plant and crop stress, 2(1): 203-229. https://doi.org/10.1201/9780824746728.ch9

Greyson, J., 2007. An economic instrument for zero waste, economic growth and sustainability. J. Clean. Prod., 15(13-14): 1382-1390. https://doi.org/10.1016/j.jclepro.2006.07.019

Grimalt, J.O., Ferrer, M., Macpherson, E., 1999. The environmental impact of the mine tailing accident in Aznalcóllar. Sci. Total Environ., 242: 1-337.

Gupta, N., Yadav, K.K., Kumar, S., Chadd, R.P., Kumar, A., 2019. Trace elements in soil-vegetables interface: Translocation, bioaccumulation, toxicity and amelioration - A review. Sci. Total Environ., 651: 2927-2942. https://doi.org/10.1016/j.scitotenv.2018.10.047

Gutiérrez-Ginés, M.J., Pastor, J., Hernández, A.J., 2013. Assessment of field portable X-ray fluorescence spectrometry for the in situ determination of heavy metals in soils and plants. Environ. Sci.: Processes Impacts, 15: 1545. https://doi.org/10.1039/c3em00078h

Gutiérrez-Ginés, M.J., Pastor, J., Hernández, A.J., 2015. heavy metals in native Mediterranean grassland species growing at abandoned mine sites: ecotoxicological assessment and phytoremediation of polluted soils, in: Sherameti, I., Varma, A., (Eds.), Heavy metal contamination of soils. Monitoring and Remediation. Chapter 9. Springer International Publishing Switzerland. https://doi.org/10.1007/978-3-319-14526-6

Hardie, M., Doyle, R., 2012. Measuring soil salinity, in: Shabala, S., Cuin, T.A., (Eds.), Plant salt tolerance: Methods and Protocols. Methods in Molecular Biology, Chapter 28, 913: 424. Springer Science & Business Media. https://doi.org/10.1007/978-1-61779-986-0_28

Hernández, A.J., Pastor, J., 2008. Relationship between plant biodiversity and heavy metal bioavailability in grasslands overlying an abandoned mine. Environ. Geochem. Health, 30: 127-133. https://doi.org/10.1007/s10653-008-9150-4

Hobbs, R.H., Streit, B., 1986. Heavy metal concentrations in plants growing on a copper mine spoil in the Grand Canyon, Arizona. Am. Midl. Nat., 115: 277-281. https://www.jstor.org/stable/2425864

Hooda, P., 2010. Trace elements in soils, first ed. John Wiley and Sons Ltd, Chichester, West Sussex.

Hothorn, T., Bretz, F., Westfall, P., 2008. Simultaneous inference in general parametric models. Biom. J., 50 (3): 346-363. https://doi.org/10.1002/bimj.200810425

Iskandar, I.K., Adriano, D.C., 1997. Remediation of soils contaminated with metals. Science Reviews, Northwood, UK, pp. 1-26.

IUSS Working Group WRB, 2015. World Reference Base for Soil Resources 2014, update 2015. International soil classification system for naming soils and creating legends for soil maps. World Soil Resources Reports No. 106. FAO, Rome.

Jiménez-Moraza, C., Iglesias, N., Palencia, I., 2006. Application of sugar foam to a pyrite-contaminated soil. Miner. Eng., 19(5): 399-406. https://doi.org/10.1016/j.mineng.2005.10.011

Jost, L., 2006. Entropy and diversity. Oikos, 113: 363-375.

Kabata-Pendias, A., 2011. Trace elements in soils and plants, fourth ed. CRC Press, Boca Raton.

Kumpiene, J., Lagerkvist, A., Maurice, C., 2008. Stabilization of As, Cr, Cu, Pb and Zn in soil using amendments - A review. Waste Manage., 28: 215-225. https://doi.org/10.1016/j.wasman.2006.12.012

Liu, L., Li, W., Song, W., Guo, M., 2018. Remediation techniques for heavy metal-contaminated soils: Principles and applicability. Sci. Total Environ., 633: 206-219. https://doi.org/10.1016/j.scitotenv.2018.03.161

Loneragan, J.F., Gladstone, J.S., Simmons, W.J., 1967. Mineral elements in temperate and pasture plants. II. Calcium. Aust. J. Agr. Res., 19: 353-364. https://doi.org/10.1071/AR9680353

Maathuis, J.M.F., 2009. Physiological functions of mineral macronutrients. Curr. Opin. Plant Biol., 12: 250-258. https://doi.org/10.1016/j.pbi.2009.04.003

MacArthur, R.H., 1965. Patterns of Species Diversity. Biol. Rev., 40 (4): 510-533.

Macnair, M.R., 2003. The hyperaccumulation of metals by plants. Adv. Bot. Res., 40: 63-105. https://doi.org/10.1016/S0065-2296(05)40002-6

Madejón, P., Murillo, J.M., Marañón, T., Cabrera, F., Soriano, M.A., 2003. Trace element and nutrient accumulation in sunflower plants two years after the Aznalcóllar mine spill. Sci. Total Environ., 307: 239-257. https://doi.org/10.1016/S0048-9697(02)00609-5

Madejón, E., Pérez-de-Mora, A., Felipe, E., Burgos, P., Cabrera, F., 2006. Soil amendments reduce trace element solubility in a contaminated soil and allow regrowth of natural vegetation. Environ. Pollut., 139: 40-52. https://doi.org/10.1016/j.envpol.2005.04.034

Madejón, P., Domínguez, M.T., Madejón, E., Cabrera, F., Marañón, T., Murillo-Carpio, J.M., 2018. Soil-plant relationships and contamination by trace elements: A review of twenty years of experimentation and monitoring after the Aznalcóllar (SW Spain) mine accident. Sci. Total Environ., 625: 50-63. https://doi.org/10.1016/j.scitotenv.2017.12.277

Magurran, A.E. 2004. Measuring Biological Diversity. Blackwell.

Mallmann, F.J.K., dos Santos, D.R., Ceretta, C.A., Cella, C., Minella, J.P.G., Guma, R.L., Filipovié, V., van Oort, F., Šimůnek, J., 2014. Soil tillage to reduce surface metal contamination - model development and simulations of zinc and copper concentration profiles in a pig slurry-amended soil. Agric. Ecosyst. Environ., 196: 59-68. https://doi.org/10.1016/j.agee.2014.06.024

Manzano, M., Custodio, E., Ayora, C., Navarrete, P., 2000. Contamination of the Guadiamar river aquifer after the Aznalcóllar mine accident, SW Spain. Boletín Geológico y Minero, 111 (6): 93-106.

Martín, F., Simón, M., García, I., Bouza, P., Dorronsoro, C., Aguilar, J., 2003. Efecto del encalado en suelos contaminados por lodos piríticos. Edafología, 10(2): 177-183.

Martín Peinado, F.J., Romero Freire, A., Arco Lázaro, E., Sierra Aragón, M., Ortiz-Bernad, I., Abbaslou, H., 2012. Assessment of arsenic toxicity in spiked soils and water solutions by the use of bioassays. Span. J. Soil Sci., 2(3): 45-56. https://doi.org/10.3232/SJSS.2012.V2.N3.05

Martín-Peinado, F.J., Romero-Freire, A., García-Fernández I., Sierra-Aragón, M., Ortiz-Bernad, I., Simón-Torres, M., 2015. Long-term contamination in a recovered area affected by a mining spill. Sci. Total Environ., 514: 219-223. https://doi.org/10.1016/j.scitotenv.2015.01.102

Mehes-Smith, M., Nkongolo, K., Cholewa, E., 2013. Coping mechanisms of plants to metal contaminated soils, in: Silvern, S., Young, S., (Eds.), Environmental change and sustainability. Chapter 3. InTech, pp. 53. https://doi.org/10.5772/55124

Mengel, K., Kirkby, E.A., 1987. Principles of plant nutrition, fourth ed. International Potash Institute, Bern.

Midhat, L., Ouazzani, N., Esshaimi, M., Ouhammou, A., Mandi, L., 2017. Assessment of heavy metals accumulation by spontaneous vegetation: Screening for new accumulator plant species grown in Kettara mine-Marrakech, Southern Morocco. Int. J. Phytoremediat., 19 (2): 191-198. http://doi.org/10.1080/15226514.2016.1207604

Montiel-Rozas, M.M., López-García, A., Kjøller, R., Madejón, E., Rosendahl, S., 2016. Organic amendments increase phylogenetic diversity of arbuscular mycorrhizal fungi in acid soil contaminated by trace elements. Mycorrhiza, 26: 575-585. https://doi.org/10.1007/s00572-016-0694-3

Morales, M., 2017. Sciplot: Scientific graphing functions for factorial designs. Available at: https://cran.r-project.org/web/packages/sciplot/index.html

Nakamaru, Y.M., Martín-Peinado, F.J., 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

Nirola, R., Megharaj, M., Beecham, S., Aryal, R., Thavamani, P., Vankateswarlu, K., Saint, C., 2016. Remediation of metalliferous mines, revegetation challenges and emerging prospects in semi-arid and arid conditions. Environ. Sci. Pollut. Res., 23: 20131-20150. https://doi.org/10.1007/s11356-016-7372-z

Nocete, F., Sáez, R., Bayona, M.R., Nieto, J.M., Peramo, A., López, P., Gil-Ibarguchi, J.I., Inácio, N., García, S., Rodríguez, J., 2014. Gold in the southwest of the Iberian Peninsula during the 3rd millennium BC. J. Archaeol. Sci., 41: 691-704. https://doi.org/10.1016/j.jas.2013.10.006

Nunes, A., Oliveira, G., Mexia, T., Valdecantos, A., Zucca, C., Costantini, E.A.C., Abraham, E.M., Kyriazopoulos, A.P., Salah, A., Prasse, R., Correia, O., Milliken, S., Kotzen, B., Branquinho, C., 2016. Ecological restoration across the Mediterranean Basin as viewed by practitioners. Sci. Total Environ., 566-567: 722-732. https://doi.org/10.1016/j.scitotenv.2016.05.136

Parra, A., Zornoza, R., Conesa, E., Gómez-López, M.D., Faz, A., 2014. Seedling emergence, growth and trace elements tolerance and accumulation by Lamiaceae species in a mine soil. Chemosphere, 113: 132-140. https://doi.org/10.1016/j.chemosphere.2014.04.090

Pérez-de-Mora, A., Madejón, P., Burgos, P., Cabrera, F., Lepp, N.W., Madejón, E., 2011. Phytostabilization of semiarid soils residually contaminated with trace elements using by-products: Sustainability and risks. Environ. Pollut., 159: 3018-3027. https://doi.org/10.1016/j.envpol.2011.04.015

Poniedziałek, M., Sękara, A., Ciura, J., Jędrszczyk, E., 2005. Nickel and manganese accumulation and distribution in organs of nine crops. Folia Horticulturae, 17(1): 11-22.

Poschenrieder, C., Bech, J., Llugany, M., Pace, A., Fenés, E., Barceló, J., 2001. Copper in plant species in a copper gradient in Catalonia (North East Spain) and their potential for phytoremediation. Plant Soil, 230: 247-256. https://10.1023/A:1010374732486

Prach, K., Hobbs, R.J., 2008. Spontaneous succession versus technical reclamation in the restoration of disturbed sites. Restor. Ecol., 16(3): 363-366. https://doi.org/10.1111/j.1526-100X.2008.00412.x

R Core Team, 2017. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available at: https://www.r-project.org

Rayed, A., Omrane, B., Chokri, S., Mohamed-Amine, K., Abdeliazim-Mustafa M., 2019. Effects of incorporation of marble powder obtained by recycling waste sludge and limestone powder on rheology, compressive strength, and durability of self-compacting concrete. Adv. Mater. Sci. Eng., Volume 2019. https://doi.org/10.1155/2019/4609353

Reeves, R.D., Baker, A.J.M., Jaffré, T., Erskine, P.D., Echevarria, G., Van der Ent, A., 2018. A global database for plants that hyperaccumulate metal and metalloid trace elements. New Phytol, 218: 407-411. https://doi.org/10.1111/nph.14907

Rodríguez-Jordá, M.P., Garrido, F., García-González, M.T., 2012. Effect of the addition of industrial by-products on Cu, Zn, Pb and As leachability in a mine sediment. J. Hazard. Mater., 213-214: 46-54. https://doi.org/10.1016/j.jhazmat.2012.01.049

Romero-Freire, A., Martín-Peinado, F.J., Van Gestel, C.A.M., 2015. Effect of soil properties on the toxicity of Pb: Assessment of the appropriateness of guideline values. J. Hazard. Mater., 289: 46-53. https://doi.org/10.1016/j.jhazmat.2015.02.034

Sah, R.N., Miller, R.O., 1992. Spontaneous reaction for acid dissolution of biological tissues in closed vessels. Anal. Chem., 64(2): 230-233.

Salvany, J.M., Mediavilla, C., Mantecón, R., Manzano, M., 2001. Geología del Valle del Guadiamar y áreas colindantes. Boletín Geológico y Minero. Vol. Especial, 57-68.

Sánchez, J.A., García, I., del Moral, F., de Haro, S., González, V., Simón, M., Martín, F., Iriarte, A., 2010. Utilización de lodos de corte y pulido del mármol en la recuperación de escombreras de mármol. Revista de Ciencias Agrarias.

Saxena, G., Purchase, D., Mulla, S.I., Saratale, G.D., Bharagava, R.N., 2020. Phytoremediation of heavy metal-contaminated sites: eco-environmental concerns, field studies, sustainability issues, and future prospects. Rev. Environ. Contam. T., 249: 71-131. https://doi.org/10.1007/398_2019_24

Serrano, H.C., Cotrim, H., Pinto, M.J., Martins-Loução, M.A., Branquinho, C., 2017. Metal hyperaccumulation patterns within *Plantago* phylogeny (Plantaginaceae). Plant Soil, 411: 227-241. https://doi.org/10.1007/s11104-016-3024-9

Shannon, C., 1948. A mathematical theory of communication. Bell System Tech. J., 27: 379-423, 623-656. https://doi.org/10.1002/j.1538-7305.1948.tb01338.x

Sherene, T., 2010. Mobility and transport of heavy metals in polluted soil environment. Biol. Forum, 2(2): 112-121.

Sierra, M.J., Milan, R., Esteban, E., Cardona, A.I., Schmid, T., 2008. Evaluation of mercury uptake and distribution in *Vicia sativa* L. applying two different study scales: greenhouse condition and lysimeter experiments. J. Geochem. Explor., 96: 203-209. https://doi.org/10.1016/j.gexplo.2007.04.013

Sierra-Aragón, M., Mitsui-Nakamaru, Y., García-Carmona, M., Martínez-Garzón, F.J., Martín-Peinado, F.J., 2019. The role of organic amendment in soils affected by residual pollution of potentially harmful elements. Chemosphere, 237: 124549. https://doi.org/10.1016/j.chemosphere.2019.124549

Simon, L., 2014. Potentially harmful elements in agricultural soils, in: Bini, C., Bech, J., (Eds). PHEs, Environment and Human Health. Potentially harmful elements in the environment and the impact on human health, Chapter: 3. Springer Science and Business Media, Dordrecht. https://doi.org/10.1007/978-94-017-8965-3_3

Simón, M., Ortiz, I., García, I., Fernández, E., Fernández, J., Dorronsoro, C., Aguilar, J., 1999. Pollution of soils by the toxic spill of a pyrite mine (Aznalcóllar, Spain). Sci. Total Environ., 242: 105-115. https://doi.org/10.1016/S0048-9697(99)00378-2

Simón, M., Martín, F., García, I., Bouza, P., Dorronsoro, C., Aguilar, J., 2005. Interaction of limestone grains and acidic solutions from the oxidation of pyrite tailings. Environ. Pollut., 135: 65-72. https://doi.org/10.1016/j.envpol.2004.10.013

Simón, M., García, I., Martín, F., Díez, M., del Moral, F., Sánchez, J.A., 2008. Remediation measures and displacement of pollutants in soils affected by the spill of a pyrite mine. Sci. Total Environ., 407: 23-39. https://doi.org/10.1016/j.scitotenv.2008.07.040

Tozer, K., 2004. Ecology and management of *Vulpia* spp. G.C. Gmelin in perennial pastures of southern Australia. Melbourne: Doctoral Thesis, University of Melbourne, 2004.

Tuna, A.L., Kaya, C., Ashraf, M., Altunlu, H., Yokas, I., Yagmur, B., 2007. The effects of calcium sulphate on growth, membrane stability and nutrient uptake of tomato plants grown under salt stress. Environmental and Experimental Botany, 59: 173-178. https://doi.org/10.1016/j.envexpbot.2005.12.007

Van der Ent, A., Baker, A.J.M., Reeves, R., Pollard A.J., Schat, H., 2012. Hyperaccumulators of metal and metalloid trace elements: Facts and fiction. Plant Soil, 362(1-2): 319-334. https://doi.org/10.1007/s11104-012-1287-3

Viehweger, K., 2014. How plants cope with heavy metals. Bot. Stud., 55: 35. http://www.as-botanicalstudies.com/content/55/1/35

Wickham, H., 2011. The split-apply-combine strategy for data analysis. J. Stat. Softw., 40(1). https://doi.org/10.18637/jss.v040.i01

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

Whittaker, R. 1972. Evolution and measurement of species diversity. Taxon, 21: 213-251.

Zuur, A.F., Ieno, E.N., Elphick, C.S., 2010. A protocol for data exploration to avoid common statistical problems. Methods Ecol. Evol., 1: 3-14. https://doi.org/10.1111/j.2041-210X.2009.00001.x

APPENDICES

Appendix 4.1. Main soil properties and constituents (mean ± SE). Soil types: i) **UA**: Unaffected soils; ii) **UV-AA**: Unvegetated Affected soils; iii) **V-AA**: Vegetated Affected soils; and iv) soil treatments: **G**: Addition of gypsum mining spoil; **M**: Addition of marble sludge; **T**: Tillage; **V**: mixture of UV-AA and V-AA; **Gv**: G plus vermicompost (v); **Mv**: M + v; **Tv**: T + v; **Vv**: V + v. Different letters (columns) represent statistically significant differences ($p < 0.05$) for the *post-hoc* Tukey tests performed after the LMMs. N= 24.

Soil types	Soil properties						
	pH	**EC** (dS m^{-1})	**CaCO$_3$** (%)	**OC** (%)	**Fe** (mg kg^{-1})	**Ca** (mg kg^{-1})	**S** (mg kg^{-1})
T	3,98 ± 0,16 a	3,24 ± 0,30 c	0,71 ± 0,08 b	0,53 ± 0,02 a	71194,79 ± 2828,49 cd	15524,13 ± 922,74 ab	22025,51 ± 2202,00 bc
Tv	4,03 ± 0,14 a	3,15 ± 0,21 c	0,74 ± 0,09 b	0,88 ± 0,03 b	75304,53 ± 3175,60 d	15422,30 ± 1253,97 ab	21677,50 ± 1812,70 bc
M	6,13 ± 0,19 c	2,74 ± 0,11 bc	4,00 ± 0,54 e	0,59 ± 0,04 a	70548,84 ± 3285,04 cd	38543,85 ± 3406,76 b	21952,72 ± 2312,39 bc
Mv	6,50 ± 0,20 c	2,70 ± 0,14 bc	3,47 ± 0,37 e	0,92 ± 0,06 b	71766,01 ± 3136,12 cd	39191,07 ± 2324,71 b	23266,20 ± 2514,16 bc
G	5,04 ± 0,22 b	3,34 ±0,27 c	1,34 ± 0,09 cd	0,52 ± 0,03 a	71598,56 ± 2555,42 cd	21697,06 ± 1448,78 ab	25023,29 ± 2617,76 c
Gv	5,21 ± 0,21 b	3,12 ± 0,19 c	1,22 ± 0,10 cd	0,88 ± 0,05 b	72466,9 ± 2759,33 bd	21428,03 ± 1532,28 ab	24340,28 ± 2263,47 c
V	4,89 ± 0,26 b	2,83 ± 0,18 bc	1,40 ± 0,22 cd	0,87 ± 0,07 b	63746,07 ± 2091,63 bc	20412,68 ± 1753,30 ab	18388,77 ± 1866,35 bc
Vv	5,02 ± 0,22 b	2,74 ± 0,15 bc	1,39 ± 0,27 cd	1,21 ± 0,07 c	62372,77 ± 2469,87 c	17608,50 ± 1406,68 ab	16336,83 ± 1545,51 b
UV-AA	3,43 ± 0,05 a	3,29 ± 0,21 c	0,92 ± 0,08 bc	0,51 ± 0,031 a	73620,15 ± 3308,34 d	16831,01 ± 1608,87 ab	22445,85 ± 1874,68 bc
V-AA	6,34 ± 0,33 c	0,74 ± 0,13 ab	2,30 ± 0,61 d	1,84 ± 0,16 d	49370,46 ± 3088,78 a	21027,97 ± 4204,15 ab	3203,78 ± 504,02 a
UA	6,34 ± 0,09 bc	0,09 ± 0,01 a	0.00 ± 0.00 a	3,37 ± 0,03 e	37117,42 ± 1726,10 a	9931,94 ± 1526,97 a	499,38 ± 24,20 a

Appendix 4.2. Total concentration (mean ± SE) of main pollutants (Cu, Zn, As and Pb) in soils. Soil types: i) **UA**: Unaffected soils; ii) **UV-AA**: Unvegetated Affected soils; iii) **V-AA**: Vegetated Affected soils; and iv) soil treatments: **G**: Addition of gypsum mining spoil; **M**: Addition of marble sludge; **T**: Tillage; **V**: mixture of UV-AA and V-AA; **Gv**: G plus vermicompost (v); **Mv**: M + v; **Tv**: T + v; **Vv**: V + v. Different letters (columns) represent statistically significant differences ($p < 0.05$) for the *post-hoc* Tukey tests performed after the LMMs. N= 24.

	Total PHEs			
Soil types	Cu	Zn	As	Pb
T	199,71 ± 16,99 bc	311,46 ± 26,15 bc	458,89 ± 26,28 de	778,36 ± 55,80 cd
Tv	195,34 ± 16,31 bc	273,12 ± 25,48 b	529,42 ± 47,66 e	784,17 ± 47,47 cd
M	195,30 ± 14,72 bc	283,39 ± 17,67 bc	454,55 ± 27,09 de	701,11 ± 48,83 bd
Mv	185,22 ± 16,25 ab	298,82 ± 26,43 bc	462,02 ± 25,67 e	740,26 ± 62,89 bd
G	212,08 ± 18,96 c	378,37 ± 40,71 cd	479,49 ± 26,93 e	723,16 ± 32,70 bd
Gv	193,56 ± 15,61 bc	315,50 ± 31,83 bc	490,51 ± 39,78 e	728,89 ± 25,65 bd
V	187,23 ± 13,70 ab	392,51 ± 26,03 d	369,73 ± 16,38 cd	638,54 ± 34,49 bc
Vv	172,87 ± 11,69 ac	386,17 ± 21,18 d	337,61 ± 16,61 ac	612,58 ± 33,52 b
UV-AA	175,84 ± 14,87 ab	293,49 ± 32,95 b	478,52 ± 28,03 e	818,46 ± 57,60 d
V-AA	150,49 ± 21,88 a	429,76 ± 43,74 d	169,91 ± 25,88 b	293,26 ± 36,28 a
UA	37,37 ± 5,05 ab	87,04 ± 3,12 a	19,23 ± 2,14 a	61,27 ± 10,62 a

Appendix 4.3. Ratio (mean ± SE) between soluble and total concentrations of main pollutants (Cu, Zn, As and Pb) in soils. Soil types: i) **UA**: Unaffected soils; ii) **UV-AA**: Unvegetated Affected soils; iii) **V-AA**: Vegetated Affected soils; and iv) Soil treatments: **G**: Addition of gypsum mining spoil; **M**: Addition of marble sludge; **T**: Tillage; **V**: mixture of UV-AA and V-AA; **Gv**: G plus vermicompost (v); **Mv**: M + v; **Tv**: T + v; **Vv**: V + v. Different letters (columns) represent statistically significant differences ($p < 0.05$) for the *post-hoc* Tukey tests performed after the LMMs. N=24.

Ratio Soluble/ Total PHEs				
Soil types	Cu	Zn	As	Pb
T	0,11 ± 2,81E^{-02} ef	0,31 ±0,05 de	2,38E^{-04} ± 4,671E^{-05} a	0,22 ± 0,03 c
Tv	0,06 ± 1,56E^{-02} df	0,26 ± 0,04 de	1,89E^{-04} ± 3,43E^{-05} a	0,19 ± 0,03 bc
M	0,01 ± 5,35E^{-03} ab	0,06 ± 0,02 ab	1,46E^{-04} ± 1,83E^{-05} a	0,15 ± 0,02 b
Mv	0,01 ± 7,02E^{-03} a	0,04 ± 0,03 ab	1,76E^{-04} ± 1,61E^{-05} a	0,15 ± 0,02 b
G	0,07 ± 2,31E^{-02} bcd	0,21 ± 0,05 b	3,12E^{-04} ± 9,72E^{-05} a	0,21 ± 0,03 c
Gv	0,02 ± 9,31E^{-03} ac	0,14 ± 0,04 bc	1,72E^{-04} ± 2,54E^{-05} a	0,18 ± 0,02 bc
V	0,04 ± 1,41E^{-02} cd	0,20 ± 0,04 bc	2,78E^{-04} ± 4,84E^{-05} a	0,19 ± 0,03 bc
Vv	0,02 ± 7,37E^{-03} ad	0,16 ± 0,03 bd	2,14E^{-04} ± 4,18E^{-05} a	0,17 ± 0,02 bc
UV-AA	0,09 ± 1,33E^{-02} e	0,37 ± 0,03 e	2,09E^{-04} ± 2,55E^{-05} a	0,18 ± 0,02 bc
V-AA	4,35E^{-03} ± 4,94E^{-04} a	0,02 ± 4,95E^{-03} ab	1,52E^{-03} ± 2,34E^{-04} b	0,18 ± 0,02 bc
UA	5,25E^{-03} ± 4,26E^{-04} a	3,22E^{-03} ± 2,47E^{-04} a	1,90E^{-03} ± 2,44E^{-04} b	0,01 ± 9,00E^{-04} a

Appendix 4.4. Ratio (mean ± SE) between bioavailable and total concentrations of main pollutants (Cu, Zn, As and Pb) in soils. Soil types: i) **UA**: Unaffected soils; ii) **UV-AA**: Unvegetated Affected soils; iii) **V-AA**: Vegetated Affected soils; and iv) Soil treatments: **G**: Addition of gypsum mining spoil; **M**: Addition of marble sludge; **T**: Tillage; **V**: mixture of UV-AA and V-AA; **Gv**: G plus vermicompost (v); **Mv**: M + v; **Tv**: T + v; **Vv**: V + v. Different letters (columns) represent statistically significant differences ($p < 0.05$) for the *post-hoc* Tukey tests performed after the LMMs. N=24.

Ratio Bioavailable/ Total PHEs				
Soil types	Cu	Zn	As	Pb
T	0,39 ± 0,04 c	0,05 ± 0,02 ac	$5{,}20E^{-04} \pm 1{,}69E^{-04}$ a	$3{,}66E^{-04} \pm 5{,}96E^{-05}$ a
Tv	0,35 ± 0,03 cd	0,08 ± 0,03 bce	$6{,}31E^{-04} \pm 2{,}30E^{-04}$ a	$4{,}97E^{-04} \pm 8{,}53E^{-05}$ ab
M	0,25 ± 0,03 ab	0,04 ± 0,02 a	$5{,}98E^{-04} \pm 2{,}49E^{-04}$ a	$5{,}55E^{-04} \pm 1{,}34E^{-04}$ a
Mv	0,25 ± 0,03 bd	0,05 ± 0,02 ab	$2{,}09E^{-03} \pm 8{,}32E^{-04}$ a	$7{,}41E^{-04} \pm 1{,}14E^{-04}$ ab
G	0,36 ± 0,05 bc	0,04 ± 0,01 bcd	$6{,}26E^{-04} \pm 2{,}07E^{-04}$ a	$8{,}61E^{-04} \pm 2{,}39E^{-04}$ ab
Gv	0,34 ± 0,04 bc	0,07 ± 0,02 bce	$1{,}53E^{-03} \pm 5{,}96E^{-04}$ a	$2{,}86E^{-03} \pm 1{,}70E^{-03}$ b
V	0,33 ± 0,04 bc	0,07 ± 0,03 ce	$1{,}25E^{-03} \pm 4{,}85E^{-04}$ a	$7{,}34E^{-03} \pm 1{,}82E^{-03}$ c
Vv	0,30 ± 0,03 bc	0,09 ± 0,04 ce	$1{,}87E^{-03} \pm 1{,}22E^{-03}$ a	$7{,}16E^{-03} \pm 2{,}30E^{-03}$ c
UV-AA	0,38 ± 0,03 c	0,13 ± 0,05 de	$2{,}92E^{-03} \pm 2{,}35E^{-03}$ a	$5{,}03E^{-04} \pm 1{,}04E^{-04}$ a
V-AA	0,17 ± 0,02 a	0,06 ± 0,02 e	$2{,}15E^{-03} \pm 4{,}82E^{-04}$ b	$3{,}39E^{-02} \pm 6{,}59E^{-03}$ d
UA	0,18 ± 0,01 abc	0,09 ± 0,01 ace	$6{,}82E^{-03} \pm 8{,}10E^{-04}$ ab	$2{,}03E^{-01} \pm 1{,}85E^{-02}$ d

Appendix 4.5. Physicochemical properties and PHE content (mean±SD) of soil amendments. Cu, Zn, As and Pb: Potentially Harmful Elements (PHEs). T: total concentrations (in mg kg^{-1}) of PHEs, Fe, Ca, S and Al. NA= Not Available.

Properties		Soil amendments: Vermicompost [1](mean±SD)	Marble sludge[2]	Gypsum mining spoil [3](mean±SD)
pH		7.88 ± 0.12	9.1	7.5 ± 0.2
EC (dS m^{-1})		4.18 ± 0.25	2.03	2.90 ± 0.04
Organic carbon (%)		23.16 ± 1.87	0.06	0.04 ± 0.03
CaCO3 (%)		NA	99	27.25 ± 3.52
Total forms (mg kg^{-1})	**TFe**	NA	1100	9501.07 ± 713.12
	TCa	NA	386900	186153.44 ± 4288.77
	TS	NA	NA	126094.49 ± 4552.09
	TAl	NA	1900	2529.57 ± 532.98
	TCu	44.38 ± 3.56	1.67	10.10 ± 0.84
	TZn	17.18 ± 2.58	7.05	18.07 ± 2.06
	TAs	1.81 ± 0.27	3.76	3.50 ± 0.41
	TPb	3.90 ± 0.72	1.18	4.39 ± 0.73

[1]Nakamaru and Martín-Peinado, 2017; [2]Sanchez et al., 2010; [3]Ballesteros, 2018.

Appendix 4.6. List of species found at the study area in each soil type: i) **UA**: Unaffected soils; ii) **UV-AA**: Unvegetated Affected soils; iii) **V-AA**: Vegetated Affected soils; and iv) Soil treatments: **G**: Addition of gypsum mining spoil; **M**: Addition of marble sludge; **T**: Tillage; **V**: mixture of UV-AA and V-AA; **Gv**: G plus vermicompost (v); **Mv**: M + v; **Tv**: T + v; **Vv**: V + v. Presence is marked with a cross.

Family	Species	UA	V-AA	UV-AA	T	Tv	M	Mv	G	Gv	V	Vv
Asteraceae	*Andryala ssp.*					X		X		X	X	X
	Anthemis arvensis L.	X	X		X	X	X	X	X	X	X	X
	Calendula officinalis L.	X	X							X		
	Calendula tripterocarpa Rupr.	X									X	
	Chrysanthemum coronarium L.	X	X		X	X	X	X	X	X	X	X
	Crepis sp.		X									
	Filago sp.										X	
	Galactites sp.	X										
	Leontodon sp.	X	X								X	X
	Senecio sp.	X	X					X		X	X	X
Boraginaceae	*Echium plantagineum* L.	X	X									
Brassicaceae	*Alyssum sp.*	X								X		
	Capsella bursa-pastoris L.							X				X
	Diplotaxis sp.	X	X								X	X
	Hirschfeldia incana (L.) Lagr. -Foss.	X	X			X	X	X		X		
	Raphanus raphanistrum L.	X	X				X	X	X	X		X
Caryophyllaceae	*Dianthus lusitanicus* Brot.											X
	Silene gallica L.										X	X
	Spergularia rubra (L.) C. Presl	X	X		X	X	X	X	X	X	X	X
Chenopodiaceae	*Atriplex halimus* L.							X				
	Chenopodium sp.	X	X			X		X		X	X	
Euphorbiaceae	*Euphorbia sp.*	X	X									
Fabaceae	*Astragalus sp.*	X										
	Lupinus albus L.	X										
	Medicago ssp.	X	X			X	X	X		X	X	X
	Trifolium ssp.	X	X								X	X
	Vicia sp.	X	X									
Geraniaceae	*Erodium ssp.*	X	X			X	X	X		X		X
Juncaceae	*Juncus sp.*							X			X	
Lamiaceae	*Lamium amplexicaule* L.	X	X									
Malvaceae	*Lavatera sp.*											X
	Malva sp.							X				
Oxalidaceae	*Oxalis pes caprae* L.	X	X									
Plantaginaceae	*Plantago coronopus* L.	X	X		X	X	X	X	X	X	X	X
	Plantago lagopus L.	X	X		X	X	X	X	X	X	X	X
	Plantago lanceolate L.										X	

Appendix 4.6. (Cont.) List of species found at the study area in each soil type: i) **UA**: Unaffected soils; ii) **UV-AA**: Unvegetated Affected soils; iii) **V-AA**: Vegetated Affected soils; and iv) Soil treatments: **G**: Addition of gypsum mining spoil; **M**: Addition of marble sludge; **T**: Tillage; **V**: mixture of UV-AA and V-AA; **Gv**: G plus vermicompost (v); **Mv**: M + v; **Tv**: T + v; **Vv**: V + v. Presence is marked with a cross.

Family	Species	UA	V-AA	UV-AA	T	Tv	M	Mv	G	Gv	V	Vv
Poaceae	*Agrostis sp.*	X	X					X				X
	Avena sterilis L.	X	X								X	
	Bromus ssp.	X	X			X		X	X	X	X	X
	Chaetopogon fasciculatus (Link) Hayek				X	X					X	
	Gastridium ssp.							X				X
	Holcus ssp.	X	X							X	X	X
	Hordeum ssp.		X			X		X		X	X	
	Lamarckia aurea (L.) Moench	X	X		X	X	X	X	X	X	X	X
	Lolium sp.					X	X	X				
	Phalaris minor Retz.											X
	Poa sp.											X
	Polypogon maritimus Willd.							X		X		X
	Trisetaria panicea (Lam.) Paunero	X	X					X		X	X	X
	Vulpia ssp.	X	X		X	X			X	X	X	X
Polygonaceae	*Rumex bucephalophorus* L.	X										
Primulaceae	*Lysimachia arvensis* (L.) U.Manns & Anderb.	X										X
Rosaceae	*Sanguisorba sp.*	X										
Rubiaceae	*Rubia sp.*	X	X									
TOTAL		36	30	0	8	16	11	24	9	21	25	29

Appendix 4.7. Species common to all the vegetated soils types (UA, V-AA, and treatments) (key species).

Lamarckia aurea (L.) Moench

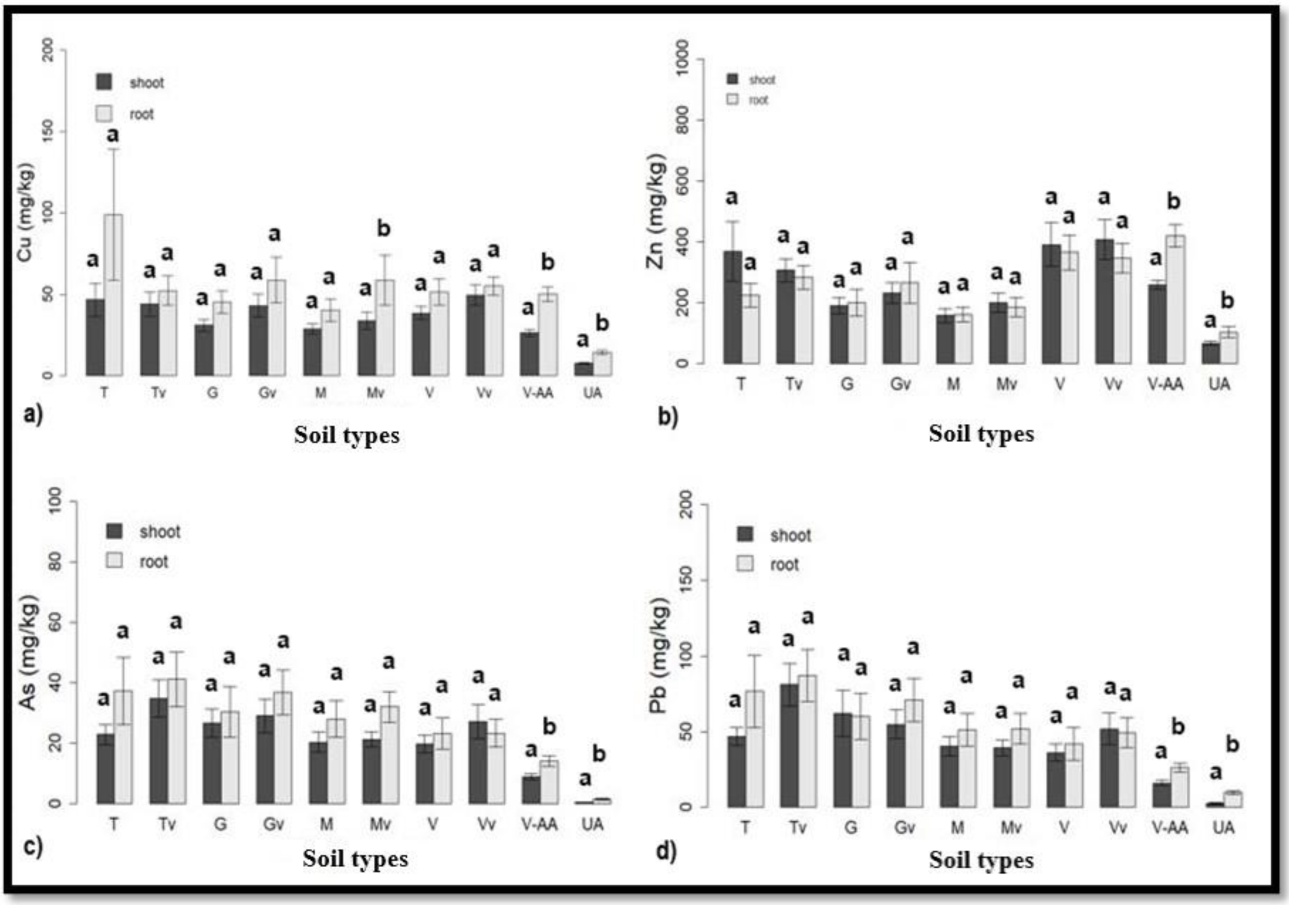
Spergularia rubra (L.) J. Presl & C. Presl

Plantago coronopus L.

Plantago lagopus L.

Anthemis arvensis L.

Chrysanthemum coronarium L.



Appendix 4.8. PHE content (mean ±CI in mg kg^{-1}) accumulated in shoots and roots in plants from each soil type: i) **UA**: unaffected soils; ii) **V-AA**: vegetated affected soils; and iii) soil treatments: 1. **G**: Addition of gypsum mining spoil; 2. **M**: Addition of marble sludge; 3. **T**: Tillage; 4. **V**: mixture of UV-AA* and V-AA; 5. **Gv**: G plus vermicompost (v); 6. **Mv**: M + v; 7. **Tv**: T + v; 8. **Vv**: V + v. PHEs: **a)** Cu; **b)** Zn; **c)** As; **d)** Pb. Different letters above symbols represent statistically significant differences ($p < 0.05$) for the *post-hoc* Tukey tests performed after the LMMs. *UV-AA: Unvegetated affected soil.

GENERAL DISCUSSION

GENERAL DISCUSSION

1. Soil pollution and habitat rehabilitation

Soils are essential for life and play a critical role in supporting ecosystem services (Robinson et al., 2013). An intense and unsustainable use of soil by human beings is leading to the intense degradation of many terrestrial ecosystems. Many anthropogenic activities are causing soil pollution, which poses a serious threat for the environment and humans' health (Rodríguez-Eugenio et al., 2018) and, thus, the restoration of the soil ecosystems is one of the biggest challenges to face ever (Liu, et al., 2018). Restoration activities frequently take a long time to pay off, especially in highly degraded ecosystems and/or where the climatic conditions act as limiting factors, hindering natural restoration. Numerous are the cases in which, even after the implementation of intense restoration measures, residual pollution and ecosystem degradation remain a concern. This is certainly the case of the Guadiamar Green Corridor (GGC)(SW Spain).

Throughout this thesis, we have assessed the current toxicity and recovery status of the Guadiamar Green Corridor in the sector closest to the Aznalcóllar pyrite mine, by means of a detailed study of soils and vegetation. According to previous studies (Martín-Peinado et al., 2015), the area is still affected by residual pollution heterogeneously distributed, and certain spots remain barren and unvegetated.

These unvegetated soils are characterized by high concentrations of Potentially Harmful Elements (PHEs), acidic pH, high presence of soluble salts and surface crust formation during the dry season. Total concentrations of PHEs (Cu, Zn, As and Pb) exceeded the background values reported by Simón et al. (1999) for the unaffected soils in the area (from 5- to 25- fold, depending on the element), especially in the case of As and Pb, which in many residual areas, even surpassed by far (13- and 3- fold, respectively) the levels for declaring a soil as potentially polluted (NGR) by the Regional Government in Andalusia (Spain) (BOE, 2015). Remediation measures considerably improved soil properties, especially pH and organic carbon (OC) content, facilitating vegetation growth in most parts of the affected area. Even though, PHE total concentrations also surpassed background values in the vegetated affected areas and, although to a lesser extent, intervention thresholds for As and Pb.

In order to assess the current mobility and bioavailability of PHEs in the affected soils, selective extractions of pollutant elements were analyzed (Chapters 1, 2 and 3), from short- (soluble in water and exchangeable forms) to long-term mobile forms (bioavailable), and unavailable fractions (bounded to amorphous Fe/Mn oxides forms and residual fraction). Differences among affected areas were found (Chapter 1), with the highest values generally concentrated in the unvegetated ones, especially for soluble and exchangeable forms of Cu, Zn and Cd, and for bounded to Fe/Mn oxides forms of Cu and As. On the contrary, the most densely vegetated areas showed the lowest values of PHE mobile forms, except for bioavailable and bounded to Fe/Mn oxides Pb forms, what could have been related to the increase of pH caused by the raise in $CaCO_3$ content (Simón et al., 2005), and to the co-precipitation with Fe as long-term and unavailable extracted forms. Arsenic presented a very low mobility in all cases, mainly due to the retention of As by the amorphous iron oxides (Acosta et al., 2015; Manaka, 2006) present in these soils (Aguilar et al., 2007). According to our results (Chapter 1, 2 and 3), the risk of pollutant dispersion of some PHEs is still present in the area, and further remediation and monitoring actions are required (García-Carmona et al., 2019; Pastor-Jáuregui et al., 2020).

Chemical analyses and selective extractions give us information about the potential mobility and availability of PHEs in these polluted soils; however, the assessment of potential toxicity of PHEs is essential to evaluate the potential ecological risk in the area (Alvarenga et al., 2008). In this vein, toxicity bioassays are a useful tool for the assessment of soil quality (Conder et al., 2001; Van Gestel et al., 2001) and to obtain indicators for the restoration of habitat functions (Brown et al., 2005; ISO/DIS 17402, 2006). Toxicity bioassays can be made using different organisms as bioindicators, being *Lactuca sativa, Eisenia andrei* and *Vibrio fischeri* among the most commonly used (García-Carmona et al., 2017) because they are representative of three major groups of soil organisms (primary producers, detritivores, and microbes).

In our case, we conducted a short-term toxicity bioassay with *Lactuca sativa* L. controlling seed germination and root elongation (Chapter 3). According to our results, the most important factors related to the toxicity over *L. sativa* were low pH, low OC and $CaCO_3$ content, and high electrical conductivity (EC) and PHE solubility in soils from the unvegetated areas. Otherwise, despite the presence of relatively high concentration of PHEs in the vegetated affected areas, *L. sativa* germination and root elongation were similar to control (unpolluted) samples, indicating no phytotoxicity in the vegetated affected areas and suggesting that the evolution of soil properties after remediation actions are positive for plant performance. Conversely, toxicity bioassays suggested the existence of PHE toxicity in the unvegetated affected soils, as previously observed by other authors in the area (Romero-Freire et al., 2015; García-Carmona et al., 2017; Pastor-Jáuregui et al. 2020). The application of additional remediation actions and monitoring of the potential toxicity in these areas are needed and should be a priority in future studies.

Finally, information about vegetation distribution, species richness or PHE uptake and translocation was required to assess the recovery of the Guadiamar Green Corridor (Chapter 1 and 4). As far as we could observed, both the soils from the area where the spill never reached and from the vegetated affected area were completely covered by vegetation. Moreover, they presented similar species richness and diversity, which suggests that soil conditions in the vegetated affected areas are rather remediated, favouring the recovery of vegetation. According to these results, the intense rehabilitation program carried out after the mining accident was rather effective, favoured by soil amendment application and phytostabilization (Pastor-Jáuregui et al., 2020) through native tree and shrub species plantation (CMA, 2003). The natural evolution of the rehabilitation process promoted the recovery of herbaceous species through the arrival of propagules from the surrounding unaffected areas (Pérez-de-Mora et al., 2011; Burgos et al., 2013). Nevertheless, spontaneous revegetation depends on abiotic and biotic factors such as soil properties, the abundance and quality of seeds, and the capacity of local species to tolerate harsh conditions (Córdova et al., 2011; Gupta et al., 2019), and although many of the species found in the study area present physiological mechanisms to cope with PHE pollution, the still extreme soil conditions in the unvegetated affected areas have completely inhibited seed emergence and plant establishment (Tiller and Merry, 1981; Becker and Brändel, 2007; Undersander et al., 2011; Delgado-Caballero et al., 2017; Chapters 1, 2 and 4). According to our results, the key species that grew in the unaffected area accumulated Cu, Zn, As and Pb in the range of normal levels established by Kabata-Pendias (2011), or very close to them. Due to the presence of residual pollution in the vegetated affected soils, some plants accumulated PHEs at phytotoxic levels (Kabata-Pendias, 2011), highlighting the capacity of these species to cope with these pollutants. Moreover, although translocation (TF) and bioaccumulation (BAF) factors were low, the edible parts (shoots) of some plants surpassed toxic levels for livestock in all PHEs (Chaney, 1989), except for Pb. In this sense, although Pb tended to be more available for plants in the vegetated affected areas than in the unvegetated affected ones due to the higher OM content, the also higher availability of macronutrients in this vegetated area may have interfered with Pb, favouring nutrient uptake and reducing Pb accessibility for plants (Kabata-Pendias, 2011; Antoniadis et al., 2017). A well-established vegetation cover with low capacity to translocate and accumulate PHEs in shoots is of major interest, since it would promote PHE stabilization in soil and would hinder its transference to the food web (Clemente et al., 2015). Anyway, according to our results, the Guadiamar Green Corridor is not yet prepared to host herbivores and requires further rehabilitation and monitoring actions (Chapter 4).

2. Gypsum mining spoil as soil amendment; benefits and limitations

Many studies have been conducted in the area (Madejón et al., 2018) aiming at getting a better understanding of how soil pollution behaves in those Mediterranean ecosystems affected by mining activities. The development of innovative remediation techniques that would facilitate the complete rehabilitation of the area and that could help in similar scenarios has also been a focus of interest. In this vein, *in-situ* techniques based on immobilization of Potentially Harmful Elements (PHEs) in soils (i.e. amendment application and phytostabilization) would be essential to diminish the potential toxicity risk for the food chain (Mendez and Maier, 2008).

In order to offer a sustainable solution for the rehabilitation of polluted areas based on ecological aspects and zero waste strategy, the use of innocuous waste materials as amendment of polluted soils is highly recommended, as well as a cost-effective and eco-efficient option (Rodríguez-Jordá et al., 2012; Liu et al., 2018). In this sense, gypsum is among the largest mined natural minerals (Escavy et al., 2012), producing large quantities of gypsum mining spoil that require sustainable management. The benefits of gypsum for certain types of degraded soils have already been confirmed by other authors. For instance, red gypsum and phosphogypsum (gypsum-rich waste materials) have been used as raw material to fertilize poor soils and to amend polluted ones (Sumner et al., 1990; Garrido et al., 2003; Illera et al., 2004; Rodríguez-Jordá et al., 2012; Dinake and Kelebemang, 2019), but there are no available references for gypsum mining spoil so far. Considering that gypsum has the capacity to decrease soil crusting, to adjust pH, to reclaim sodic and saline-sodic soils, to rise plant essential nutrient availability and to reduce PHE availability (Sharma et al., 1974; Kabata-Pendias, 2011; Amezketa et al., 2005; Franzen et al., 2006; Gadepalle et al., 2007), we have evaluated the potential role of gypsum mining spoil as amendment of soils affected by acidification, salinization and PHEs, such as those found in the Guadiamar Green Corridor (GGC), aiming at promoting and enhancing the potential ecological value of this mining waste material along with the natural remediation of polluted soils and their associated plant communities.

We tested the effect of different doses of gypsum mining spoil on soil and vegetation recovery under controlled conditions (Chapter 2). In a greenhouse experiment, we observed that the addition of gypsum mining spoil significantly increased soil pH, especially the dose of 50%, due to the high amounts of calcium carbonate it contains (27%), but no change was registered for EC values, probably because gypsum solubility maintains slight saline condition and increases available salts (Casas-Castro and Casas-Barba, 1999). Total concentrations of PHEs were reduced after the application of 50% gypsum mining spoil by a dilution effect, whereas smaller doses were not effective in general terms. This dilution effect was also assessed after the spill, during the implementation of the rehabilitation plan, and was used as a treatment itself, either through the application of liming amendments or deep tillage (Aguilar et al., 2004a). We observed that gypsum mining spoil significantly reduced Cu, Zn, Cd, As and Pb soluble fractions in direct relation with its dose, mainly due to the rise in pH, but increased Sb solubility, probably due to the relatively high concentration of soluble Sb in the amendment. Regarding PHE availability for plants, Sb and Pb bioavailable fractions were similar to those in the unvegetated affected areas after the addition of gypsum mining spoil, whereas the bioavailability of Zn and As was reduced at any dose; on one hand, calcium may have reduced the availability of cationic PHEs such as Zn by competing effects (Antoniadis et al., 2017). On the other hand, sulphur and iron may have retained As by the formation of iron hydroxysulfates (García et al., 2009). Cu and Cd seemed to be dose-dependent since their bioavailability was only reduced by the highest dose of gypsum mining spoil (50%), probably due to the presence of significant quantities of $CaCO_3$ which would have significantly raised soil pH, promoting the precipitation of these elements (García-Carmona et al., 2017; Madejón et al., 2018). In terms of vegetation, the addition of gypsum mining spoil promoted high emergence of seeds and also increased seedling growth and survival, all in direct relation with the dose and as a result of the improvement of the overall soil properties. Nevertheless, plants from any treatment accumulated Zn, As and Pb at phytotoxic levels (Kabata-Pendias, 2011), and Cu concentrations were close in the treatments with the lowest doses of gypsum mining spoil. Even though, PHEs were mainly retained in roots (except for Zn), what would limit the toxicity risk for the food web.

Considering the positive results obtained in the greenhouse experiment for gypsum mining spoil, we conducted a field experiment to test the suitability of gypsum mining spoil in the assisted natural remediation of the soils affected by residual pollution in the GGC and their associated vegetation (Chapters 3 and 4). We were especially interested in testing the capacity of gypsum mining spoil to increase acidic pH; to reduce salinity and PHE toxicity; to enhance native plant species propagation, vegetation growth and species richness; and to reduce PHE uptake by plants. In this case, the PHEs selected for this study were Cu, Zn, As and Pb, as it has been reported that, after intense remediation, soils are still affected by relatively high concentrations of these elements (Martín-Peinado et al., 2015). Galán et al. (2002) suggested Cd and Sb were not a great concern, considering that their total concentrations were under the regional thresholds, whereas As exceeded intervention values (Aguilar et al., 1999), requiring reclamation, and the total concentrations of Cu, Zn and Pb indicated that further investigation in some areas would be advisable. Moreover, according to the results in Chapter 2, our plants accumulated Cu, Zn, As and Pb under phytotoxic levels (Kabata-Pendias, 2011), while Cd and Sb were below. Consequently, Cd and Sb were not considered for this study.

Concerning acidity remediation (Chapter 3), the addition of gypsum mining spoil (rich in calcium carbonate) to unvegetated polluted soils in the GGC proved to significantly increase soil acidic pH; moreover, gypsum can minimize the phytotoxic conditions related to the excess of soluble aluminum in acid soils by reacting with Al^{3+} and reducing its toxic effects (Shainberg et al., 1989).

In terms of amending soil salinity (Chapter 3), the effect of gypsum mining spoil on EC was negligible in this case. It has been described that gypsum solubility releases available ions that are exchangeable with PHEs, what contributes to maintain a buffered EC (Wallace and Wallace, 1995; Casas-Castro and Casas-Barba, 1999). Nevertheless, gypsum is considered the most common amendment for sodic soil reclamation (Lwin et al., 2018), and its beneficial effects may improve soil physical and chemical properties to greater depth compared to calcitic lime (more limited to the soil surface), due to the relatively much higher solubility of gypsum (Toma et al., 1999); hence, the improvement of rooting depth would allow plants to have more available water and nutrients (Chen et al., 2005).

Regarding PHE pollution (Chapter 3), gypsum mining spoil significantly reduced Cu solubility and Zn soluble and bioavailable fractions mainly due to pH rise and co-precipitation on carbonates (Hooda, 2010; Simón et al., 2010; Kabata-Pendias, 2011; García-Carmona et al., 2019). Moreover, gypsum mining spoil could have promoted the formation of Al-hydroxy polymers, immobilizing Cu (Garrido et al., 2005). Nonetheless, gypsum mining spoil could not reduce Cu bioavailability, probably due to the low OC content in soil. It has been described that

Cu complexation by soil organic matter can effectively control Cu bioavailability (Matijevic et al., 2014), but in this case, as gypsum mining spoil was poor in organic matter, the OC content in the polluted soil remained low after amendment application. The dose of gypsum mining spoil applied in field was not effective in reducing total concentrations of PHEs. Consequently, total concentrations of PHEs far exceeded background values and, in the case of Pb and As, also the current regulatory thresholds (12- and 25- fold, respectively) set by the Regional Government of Andalusia (BOE, 2015). In this sense, this dose of gypsum mining spoil could not reduce the soluble and bioavailable fractions of As and Pb. In our study, Pb soluble fraction was under toxic levels (100 µg Pb kg^{-1}) (Ewers, 1991), but in the case of As, the mean values of the soluble fraction reached the toxic levels estimated by Bohn et al. (1985) (40 µg As kg^{-1}), probably due to the competence between gypsum sulphates and As for sorption sites in the iron oxides (Hering et al., 1997; Meng et al., 2000).

The different results obtained in the greenhouse and field experiments for gypsum mining spoil, especially in terms of PHE reduction, could mainly be due to the fact that the doses applied in the greenhouse experiment, which were higher than in field, generally produced a dilution effect in direct relation with increasing doses. Moreover, controlled conditions could have also influenced the results; for instance, pots were daily watered, possibly generating higher leaching in our substrates, reducing total, soluble and bioavailable concentrations of PHEs. As well, plants would have had a higher effect on pots than in field, where the sampled soil was not necessarily covered by vegetation.

According to toxicity bioassays with *L. sativa* (Chapter 3), gypsum mining spoil decreased PHE toxicity, but it was not as effective as expected (mean germination and elongation rates under 60% compared to the unpolluted control). We observed that the phytotoxicity in soils treated with gypsum mining spoil was influenced by soluble Cu, Zn and Pb, probably because these elements are strongly related to soluble salts, mainly sulphates (Romero-Freire et al., 2016); the EC in this treatment remained high, negatively affecting *L. sativa*'s performance; in this respect, Van Lierop and Mackenzie (1975) observed that lettuce yield in gypsum-amended soils were low, probably due to sulphate ion toxicity.

With respect to plant performance (Chapter 4), plant emergence was reactivated after gypsum mining spoil application to the polluted soils, similarly to what Madejón et al. (2006) reported in the area, as a result of the improvement of soil properties. Nevertheless, plant cover, richness and diversity were still rather low compared to those in the vegetated affected and unaffected areas, suggesting that soil conditions are not completely recovered yet; for instance, the diversity index (H') was under the normal values usually obtained for empirical data (Magurran, 2004). The unvegetated affected soils, acting as seed sink, contained seeds from the surrounding vegetated areas prior to treatment application, but they had been inactive for more than 20 years, blocking organic matter input, what entailed a constraining factor for the evolution of a healthy soil ecosystem (Bot and Benites, 2005). Being a mineral amendment, gypsum mining spoil does not contain organic matter in substantial quantities and, thus, soil organic carbon levels could not have been improved, limiting the availability of essential nutrients and, then, resulting in low plant cover. Moreover, $CaCO_3$ content in gypsum mining spoil may have been isolated to some extent by iron hydroxysulphates, also reducing its capacity to neutralize the acidic pH and PHE fixation (Martín et al., 2003). The still high PHE load in the soil may have negatively interfered with essential nutrient uptake (Madejón et al., 2002), limiting growth or reducing plant survival (Marschner, 1995; Chibuike and Obiora, 2014; Kabata-Pendias, 2011). The sampled plant species accumulated Cu, Zn, As and Pb at phytotoxic levels (Kabata-Pendias, 2011); moreover, PHE translocations was not significantly reduced, and the edible parts (shoots) of the plants surpassed toxic levels for livestock in all PHEs (Chaney, 1989), except for As and Zn. Even though, this treatment showed a positive effect on the reduction of Cu and Zn uptake, what correlates with the effect of gypsum mining spoil on the reduction of Cu and Zn availability in soils (Chapters 2 and 3). The reduction of Cu and Zn uptake was probably due to the protective action of calcium (Carbonell et al., 1998). It has been detected Cu and Zn immobilization in soils rich in calcium, sulphur, and carbonates (Kabata-Pendias, 2011; Antoniadis et al., 2017). Moreover, Garrido et al. (2005) observed how the Al-hydroxy polymers formed in gypsum amended soils immobilized Zn. Nevertheless, gypsum mining spoil was not effective in terms of As uptake reduction, presenting higher plant uptake in this treatment than in the vegetated affected areas, probably due to the remobilization of this element through pH increase after treatment application (Martín-Peinado et al., 2012). Lead uptake was also higher in the soils treated with gypsum mining spoil than in vegetated affected soils, even if Pb bioavailable soil fraction was lower; the still acidic pH in gypsum mining spoil treated soils could have mobilize Pb (Aguilar et al., 2004b). As gypsum solubility is relatively low (Shainberg et al., 1989), the effects on PHE immobilization in the soils treated with gypsum mining spoil may be negligible so far, whereas the increase in salt concentrations in soils under semi-arid conditions, may have influenced phytotoxicity (Grattan and Grieve, 1999). Therefore, *in-situ* experiments more extended in time are needed to assess the effect of gypsum mining spoil on the recovery of soil properties and plant growth.

3. Combination of gypsum mining spoil with organic amendments and comparison with other treatments.

The combination of organo-mineral amendments has proved very effective in similar scenarios (Alvarenga et al., 2008; Jiménez-Moraza et al., 2006). Thus, in order to test the potential improvement of gypsum mining spoil (G) in combination with organic materials for soil remediation purposes, we mixed G with vermicompost (v), obtaining a new amendment (Gv). This combination produced significant and positive changes in OC content, what would have enhanced soil biological activity (Gupta et al., 2016), and provided calcium and sulphur, essential macronutrients for plants (Maathuis 2009). As a result, Gv registered higher plant cover, species richness and species diversity compared to G. Nevertheless, the addition of vermicompost did not improved the other soil chemical properties analyzed (pH, EC and $CaCO_3$) compared to G. Regarding PHEs, Cu soluble forms were reduced, due to the binding capacity of organic matter for Cu (McLaren et al., 1981; Sierra-Aragón et al., 2019), but it did not produce any significant reduction in terms of Zn, As or Pb solubility. The addition of vermicompost neither reduced Cu, Zn, As or Pb bioavailability, although a decreasing trend was observed for Zn bioavailable fraction in direct relation with higher OC values. An increasing trend was observed for Pb bioavailability, probably because $CaCO_3$ and organic matter may be forming co-precipitates with Fe (García-Carmona et al., 2019), releasing Pb from sorption sites. Consequently, this could explain the slight increase in the translocation of Pb from roots to shoots. Moreover, plants absorbed more Zn in Gv; in this sense, Sinha and Prasad (1977) described a rise in Zn uptake after the addition of organic chelating agents to soils rich in $CaCO_3$. PHE uptake by plants, and translocation and bioaccumulation factors for the other elements were similar in G and Gv.

In order to compare and evaluate the effects of gypsum mining spoil in field, we also applied other waste materials and remediation treatments previously used in the area or in similar scenarios (Fernández-Caliani and Barba-Brioso, 2010; García-Carmona et al., 2017; Madejón et al., 2018). The first two treatments were mainly based on physical actions: tillage (T) and mixture of vegetated topsoil from the surrounding V-AA area with unvegetated affected soil (V). Tillage is an inexpensive technique that would break soil crust and dilute pollutants by loosening the soil (Mallmann et al., 2014; Busari et al., 2015), but does not provide any organic or inorganic amendment. In this sense, V treatment would break soil crust, reduce PHE availability and provide unvegetated soils with the essential microbiota and seed bank (García-Carmona et al., 2017). The rest of treatments were based on the *in-situ* application of soil amendments: marble sludge (M) and a mixture of vermicompost (v) with single treatments. Marble sludge has the capacity to rise acidic pH and to immobilize PHEs in soil, whereas vermicompost is a great source of organic carbon.

According to our results (Chapters 3 and 4), G treatment significantly improved pH and calcium carbonate content in relation to T treatment and raised these variables to similar values to those observed in V treatment. Nevertheless, M treatment, which presented the highest concentration of carbonates, was the one that most increased soil pH (Martín et al., 2003; Aguilar et al., 2007), towards similar values to those in the unaffected (UA) and vegetated affected (V-AA) soils. M and V treatments marginally decreased EC, but G or T treatments did not significantly modify soil salinity. V treatment was the only treatment that significantly rise OC through the addition of organic matter from vegetated affected soils. A significant reduction of the total concentration of PHEs was only observed in V treatment (mainly for Pb and As), due to the higher dilution effect of this treatment compared to the others (Galán et al., 2002; Simón et al., 2008; Yang et al., 2012). On the contrary, total Zn concentration was higher in V since the topsoil from V-AA contained high amounts of Zn. Focusing on PHE mobility, Cu and Zn solubility was most effectively reduced by M treatment, due to the great adsorption capacity of carbonates for these elements (Simón et al., 2010; García-Carmona et al., 2019). G and V treatments were less effective than M treatment but also reduced Cu and Zn solubility, while T treatment presented similar values to those in UV-AA soils. Only marble sludge (M treatment) tended to reduce Pb solubility; in this sense, Simón et al. (2005) observed that $CaCO_3$ can promote the precipitation of Pb soluble forms in soils with pH>3.8. Finally, observing PHE bioavailable fractions, M was the treatment that most reduced Cu bioavailability, approaching the values in V-AA and UA, probably due to the presence of $CaCO_3$ and to pH raise (García-Carmona et al., 2017; Madejón et al., 2018). The M, G and T treatments significantly reduced Zn bioavailability, towards similar values to those in V-AA. As and Pb bioavailability were lower in any treated soil than in UA and V-AA soils, especially where G, M and T treatments were applied; nonetheless, V treatment significantly increased Pb bioavailability.

The application of vermicompost (v), which contained 23% of OC, to single treatments (T, M, G and V) produced significant changes in the OC content, especially in Tv, Mv and Gv, but not in the other soil properties analyzed. Moreover, although the dose of vermicompost was not high enough as to produce a significant reduction of PHE total concentrations by dilution effect, it decreased Cu soluble forms, especially in Tv, Gv and Vv, as a result of the capacity of organic matter to bind Cu (McLaren et al., 1981). It slightly reduced Pb solubility in Tv and Gv; in this sense, it has been described the formation of relatively stable complexes between Pb and soil organic matter (Romero-Freire et al., 2015), and sulphate ions could promote the precipitation of Pb as anglesite ($PbSO_4$) (Rehman et al., 2017). On the contrary, it did not produce any significant change in terms of Zn or As solubility, nor in Cu, Zn or As bioavailability. It slightly increased Pb bioavailability in Gv, Mv and Tv, probably due to the interference of $CaCO_3$ and organic matter with Pb for sorption sites in Fe oxides (García-Carmona et al., 2019); even though, Pb solubility was still lower than in the unaffected soil (UA) in the area. Although all the treatments maintained As solubility and bioavailability low, the mobility of this element should be monitored over time, especially in those treatments that contains $CaCO_3$ and organic matter (García-Carmona et al., 2017) such as Mv and Gv, to prevent rise in As mobility over time.

Focusing on the effects of treatments on plant performance, although marble sludge (M), gypsum mining spoil (G) and tillage (T) promoted plant emergence, they still resulted not completely effective when compared to vegetated affected soils in the area, presenting rather low plant cover, richness and diversity, and suggesting that soil conditions have not been completely recovered in the short-term period of this experiment. Hence, in these treatments, we mainly found herbaceous species that could get adapted to acidic soils and to high PHE loads. On the contrary, the soils with V treatment, presented the highest plant cover, richness and diversity, facilitated by both the seed bank and organic matter content brought from vegetated affected soils. Regarding PHE uptake by plants, none of the treatments could significantly reduce it under the phytotoxic levels set by Kabata-Pendias (2011), but a decreasing trend was observed for Cu and Zn in G and M treatments, due to the effect of carbonates, calcium, and sulphur (Garrido et al., 2005; Kabata-Pendias, 2011; Antoniadis et al., 2017). On the contrary, none of the treatments showed a positive effect on As uptake reduction, probably due to the remobilization of this element through pH increase after treatment application (Martín Peinado et al., 2012). Finally, Pb concentration in plant tissues was also similar in all treatments, just presenting slightly lower values in V, probably due to the higher availability of macronutrients which may have interfered with Pb, reducing Pb accessibility for plants (Kabata-Pendias, 2011; Antoniadis et al., 2017). Nevertheless, the plants in treated soils accumulated more PHEs than the plants in the unaffected soils, and it was especially remarkable for As, which was more than 20 times higher, indicating As was rather available for plants regardless of the treatment. The concentrations of Zn, As and Pb in plant shoots were similar to those in roots, suggesting that there was a certain translocation of these pollutants in most of the treatments. Moreover, the edible parts (shoots) of the plants that grew in treatments surpassed toxic levels for livestock (Chaney, 1989) for all PHEs, except for As (any treatment) and for Zn (M, Mv, G and Gv treatments). Finally, bioaccumulation factor values were under 1 in nearly all cases (except in T and Tv treatments for Zn), suggesting that plants did not hyperaccumulate PHEs. The addition of vermicompost increased plant performance, especially in Tv, Mv and Gv treatments when compared to simple treatments, through the enhancement of soil properties and increased soil organic matter content (Clemente et al., 2015). Nevertheless, it did not significantly affect PHE uptake, although an increasing trend was observed. Organic matter has controversial effects on PHE mobility; although organic matter tends to promote PHE immobilization as a result of its metal-chelating ability (Antoniadis et al., 2017), some authors have observed right the opposite behavior depending on the type of organic matter added (Kumpiene et al., 2008; De-la-Fuente et al., 2011; Sierra-Aragón et al., 2019). The addition of vermicompost increased the translocation of Cu and As from root to shoot in Tv and Vv treatments, in Vv treatment for Zn, and in Tv and Mv treatments for Pb. Nevertheless, the addition of vermicompost did not significantly change the bioaccumulation for any PHE in any treatment.

Finally, according to toxicity bioassays, G and Gv decreased toxicity in line with the response in V treatment, whereas M and Mv were the most effective treatments, presenting germination and elongation rates close to those in V-AA and UA; moreover, the slight reduction of EC by M and Mv could have promoted the reduction of Cu, Zn and Pb toxicity compared to G (Romero-Freire et al., 2016).

4. Lessons learnt and future research direction

Soil amendments have been extensively used for the recovery of degraded soils. As their application is focused on the immobilization of PHEs, the assessment of their positive and negative effects, their long-term effects on the environment and their cost effectiveness should be considered before application (Palansooriya et al., 2020). Moreover, the suitable dose should be also carefully studied (Clemente et al., 2012; Moreno-Jiménez et al., 2013; Simón et al., 2010). In this sense, excessive doses of soil amendments could cause negative effects on plants; for instance, special attention should be paid to the nutritional imbalances and, in the case of As-polluted soils, the rise in its availability (Simón et al., 2010). On the contrary, insufficient doses could not have the required effects (Cabrera et al., 2005, 2008). From the greenhouse experiment (Chapter 2) we learnt that the application of low amounts of gypsum mining spoil to PHE-polluted soils resulted rather ineffective, whereas higher doses promoted better plant performance. Consequently, the dose selected for our field experiment was based on effective doses previously applied in the area for calcium-rich amendments (Madejón et al., 2018). Nevertheless, the doses applied *in-situ* seemed to have been insufficient considering that plant cover and diversity in the soils treated with gypsum mining spoil were still low, and that the accumulation of As and Pb in plant tissue was rather high, when compared with the vegetated affected soils from the surrounding areas (Chapter 4). Moreover, the application of gypsum mining spoil to the affected soils on surface and without mixing it in depth could have slow down its effects. On the contrary, the same dose of marble sludge obtained better results, probably because it presents much higher $CaCO_3$ content, promoting higher pH rise. Several studies have highlighted the need to retreat affected areas to maintain the effect of amendments and to prevent new acidification processes, which could remobilize PHEs in soils over time. In this sense, we could maintain the dose of gypsum mining spoil and repeat amendment application over time (Madejón et al., 2018), or mix it with the soil to reach deeper layers. If still insufficient, we could also increase the single dose of the amendment. Furthermore, considering that gypsum has a relatively low solubility rate (Shainberg et al., 1989; Toma et al., 1999), and that the duration of the study was too short (2 years), a longer-term study could enhance the positive effects of the amendment providing better results.

We observed that the soils treated with gypsum mining spoil presented high electrical conductivity and strong soil crust formation. As a result of the weathering of tailings, sulphates releases and precipitates in the soil surface during the dry season (Alastuey et al., 1999). The addition of gypsum mining spoil could have increased the concentrations of sulphates in soil, intensifying soil crusting formation. In this regard, the combination of gypsum mining spoil and tillage over time could reduce surface crusting, although the formation of soil aggregates could facilitate the leachability of excessive salts (Norton and Rhoton, 2007).

The effectiveness of gypsum mining spoil could have also been limited due to the low organic matter content in the polluted soils, which was not compensated after amendment application. Consequently, we tested the combination of gypsum mining spoil and vermicompost in our field experiment (Chapter 3 and 4). In this case, we maintained the dose of gypsum but doubled the dose previously applied in the area for organic amendments, in the light that it resulted insufficient to promote vegetation growth (Cabrera et al., 2005, 2008). Nevertheless, although this mixed amendment registered better results in terms of plant performance, the overall soil conditions were not substantially enhanced, and plant cover and richness remained rather low compared to the surrounding vegetated affected areas. Consequently, further studies should be conducted in order to improve the positive effects of this organo-mineral amendment and to calculate the adequate ratio between both amendments. Moreover, an interesting research line for the future would be to test the combined effect of gypsum mining spoil with other organic waste materials as amendment of polluted soils. In this vein, our group is already investigating the effects of a calcium- and organic -rich amendment composed of gypsum mining spoil and olive mill waste compost with promising results. Furthermore, it would also be interesting to test the role of gypsum mining spoil (as simple or mixed amendment) in combination with seed sowing.

The results obtained throughout this thesis will certainly help to develop better rehabilitation plans for mining affected sites such as the Guadiamar Green Corridor, as well as a better understanding of the potential of gypsum mining spoil as amendment of soils polluted with PHEs. Nevertheless, the monitoring of rehabilitated areas after amendment applications is needed, especially when PHEs cope with the presence of sulphates, carbonates, iron oxides and organic matter, as changes in soil properties over time may produce variations in the mobility and potential toxicity of PHEs.

In order to evaluate the economic viability of gypsum mining spoil, in-depth studies should be performed. The costs associated to gypsum mining spoil are usually low and, as no further processing is required because it is considered a mining waste, gypsum mining spoil expenses are mainly related to its transport, what should be reflected in reduced market prices. Moreover, as its storage in the mine facilities entails costs and space limitations, the commercialization of this gypsum-rich mining waste could constitute a new sustainable and profitable business.

The potential of this material to promote a well-established vegetation cover with low capacity to translocate and accumulate PHEs in shoots is of major interest, so it would reduce PHE biomagnification through the food chain. As well, its contribution to soil physical stabilization, and the improvement of soil chemical properties are promising enough as to consider this amendment a friendly and environmentally sustainable material to use in the rehabilitation of polluted soils.

5. References

Acosta, J.A., Arocena, J.M., Faz, A., 2015. Speciation of arsenic in bulk and rhizosphere soils from artisanal cooperative mines in Bolivia. Chemosphere, 138: 1014-1020. https://doi.org/10.1016/j.chemosphere.2014.12.050

Aguilar, J., Dorronsoro, C., Galán, E., Gómez, L., 1999. Criterios y estándares para declarar un suelo como contaminado en Andalucía. Public. Universidad de Sevilla, p. 252.

Aguilar, J., Bouza, P., Dorronsoro, C., Fernández, E., Fernández, J., García, I., Martín, F., Simón, M., 2004a. Application of remediation techniques for immobilization of metals in soils contaminated by a pyrite tailing spill in Spain. Soil Use Manage., 20(4): 451-453.

Aguilar J., Dorronsoro C., Fernández E., Fernández J., García I., Martín F., Simón M., 2004b. Remediation of Pb-contaminated soils in the Guadiamar river basin (SW Spain). Water Air Soil Pollut., 151: 323-333.

Aguilar, J., Dorronsoro, C., Fernández, E., Fernández, J., García, I., Martín, F., Sierra, M., Simón, M., 2007. Remediation of As-contaminated soils in the Guadiamar river basin (SW, Spain). Water Air Soil Pollut., 180: 109-118. https://doi.org/10.1007/s11270-006-9254-3

Alastuey, A., García-Sánchez, A., López, F., Querol, X., 1999. Evolution of pyrite mud weathering and mobility of heavy metals in the Guadiamar valley after the Aznalcóllar spill, south-west Spain. Sci. Total Environ., 242(1-3): 41-55. https://doi.org/10.1016/S0048-9697(99)00375-7

Alvarenga, P., Gonçalves, A.P., Fernandes, R.M., de Varennes, A., Vallini, G., Duarte, E., Cunha-Queda, A.C., 2008. Evaluation of composts and liming materials in the phytostabilization of a mine soil using perennial ryegrass. Sci. Total Environ., 406(1-2): 43-56. https://doi.org/10.1016/j.scitotenv.2008.07.061

Amezketa, E., Aragues, R., Bazol, R., 2005. Efficiency of sulfuric acid, mined gypsum and two gypsum byproducts in soil crusting prevention and sodic soil reclamation. Agron. J., 97: 983-989. https://doi.org/10.2134/agronj2004.0236

Antoniadis, V., Levizou, E., Shaheen, S.M., Ok, Y.S., Sebastian, A., Baum, C., Prasad, M.N.V., Wenzel, W.W., Rinklebe, J., 2017. Trace elements in the soil-plant interface: Phytoavailability, translocation, and phytoremediation - A review. Earth-Sci. Rev., 171: 621-645. https://doi.org/10.1016/j.earscirev.2017.06.005

Becker, T., Brändel, M., 2007. Vegetation-environment relationships in a heavy Metal-dry grassland complex. Folia Geobot., 42: 11-28. https://doi.org/10.1007/BF02835100

Boletín Oficial del Estado (BOE) 38, 2015. Decreto 18/2015 por el que se aprueba el reglamento que regula el régimen aplicable a los suelos contaminados. Consejería de Medioambiente y Ordenación del Territorio, 38: 28-64.

Bohn, H.I., McNeal, B.L., O'Connor, G.A., 1985. Soil Chemistry. Wiley Interscience, New York. pp. 341.

Bot, A., Benites, J., 2005. The importance of soil organic matter key to drought-resistant soil and sustained crop production. FAO (Food and Agriculture Organization of the United Nations), Soils Bulletin 80. Rome.

Brown, S., Sprenger, M., Maxemchuk, A., Compton, H., 2005. Ecosystem function in alluvial tailings after biosolids and lime addition. J. Environ. Qual., 34(1): 139-148.

Burgos, P., Madejón, P., Madejón, E., Girón, I., Cabrera, F., Murillo, J.M., 2013. Natural remediation of an unremediated soil twelve years after a mine accident: Trace element mobility and plant composition. J. Environ. Manage., 114: 36-45. https://doi.org/10.1016/j.jenvman.2012.10.060

Busari, M.A., Kukal, S.S., Kaur, A., Bhatt, R., Dulazi, A.A., 2015. Conservation tillage impacts on soil, crop and the environment. Int. Soil Water Conserv. Res, 3: 119-129. https://doi.org/10.1016/j.iswcr.2015.05.002

Cabrera, F., Clemente, L., Cordón, R., Hurtado, M.D., López, R., Madejón, P., Marañón, T., Moreno, F., Murillo, J.M., Nagel, I., 2005. Effect of remediation on trace metal pollution in soils of Guadiamar river valley, in: Del Valls, T.A., Blasco, J. (Eds.), Integrated Assessment and Management of the Ecosystems Affected by the Aznalcóllar Mining Spill (SW, Spain). Cátedra UNESCO/Unitwin, Cádiz, pp. 33-40.

Cabrera, F., Ariza, J.L., Madejón, P., Madejón, E., Murillo, J.M., 2008. Mercury and other trace elements in soils affected by the mine tailing spill in Aznalcóllar (SW Spain). Sci. Total Environ., 390: 311-322. https://doi.org/10.1016/j.scitotenv.2007.10.002

Carbonell, A.A., Aarabi, M.A., DeLaune, R.D., Gambrell, R.P., Patrick, W.H.J., 1998. Bioavailability and uptake of arsenic by wetland vegetation: Effects on plant growth and nutrition. J. Environ. Sci. Health, Part A, 33(1): 45-66. https://doi.org/10.1080/10934529809376717

Casas-Castro, A., Casas-Barba, E., 1999. El análisis de suelo-agua-planta y su aplicación en la nutrición de los cultivos hortícolas en la zona del sureste peninsular. Caja Rural De Almería.

Chaney, R.L., 1989. Toxic element accumulation in soils and crops: Protecting soil fertility and agricultural food-chains, in: Bar-Yosef, B., Barrow, N.J., Goldshmid, J. (Eds.), Inorganic contaminants in the Vadose zone. Ecological Studies (Analysis and Synthesis). Springer, Berlin, Heidelberg, pp. 140-158. https://doi.org/10.1007/978-3-642-74451-8_10

Chen, L., Lee, Y.B., Ramsier, C. Bigham, J., Slater, B. Dick, W.A., 2005. Increased crop yield and economic return and improved soil quality due to land application of FGD-gypsum, in: Proceedings of the World of Coal Ash, April 11-15, 2005. Lexington, Ky.

Chibuike, G.U., Obiora, S.C., 2014. Heavy metal polluted soils: effect on plants and bioremediation methods. Appl. Environ. Soil Sci., 2014. https://doi.org/10.1155/2014/752708

Clemente, R., Walker, D.J., Pardo, T., Martínez-Fernández, D., Bernal, M.P., 2012. The use of a halophytic plant species and organic amendments for the remediation of a trace elements-contaminated soil under semi-arid conditions. J. Hazard. Mater., 223-224: 63-71. http://doi.org/10.1016/j.jhazmat.2012.04.048

Clemente, R., Pardo, T., Madejón, P., Madejón, E., Bernal, M.P., 2015. Food byproducts as amendments in trace elements contaminated soils. Food Res. Int., 73: 176-189. https://doi.org/10.1016/j.foodres.2015.03.040

CMA (Consejería de Medio Ambiente), 2003. Ciencia y restauración del río Guadiamar. PICOVER 1998-2002. Junta de Andalucía, Sevilla, Spain.

Conder, J.M., Seals, L.D., Lanno, R.P., 2002. Method for determining toxicologically relevant cadmium residues in the earthworm *Eisenia fetida*. Chemosphere, 49(1): 1-7. https://doi.org/10.1016/S0045-6535(02)00192-3

Córdova, S., Neaman, A., González, I., Ginocchio, R., Fine, P., 2011. The effect of lime and compost amendments on the potential for the revegetation of metal-polluted, acidic soils. Geoderma, 166(1): 135-144. https://doi.org/10.1016/j.geoderma.2011.07.022

De-la-Fuente, C., Clemente, R., Martínez-Alcalá, I., Tortosa, G., Bernal, M.P., 2011. Impact of fresh and composted solid olive husk and their water-soluble fractions on soil heavy metal fractionation; microbial biomass and plant uptake. J. Hazard. Mater., 186: 1283-1289. https://doi.org/10.1016/j.jhazmat.2010.12.004

Delgado-Caballero, M.D.R., Alarcon-Herrera, M.T., Valles-Aragon, M.C., Melgoza-Castillo, A., Ojeda-Barrios, D.L., Leyva-Chavez, A., 2017. Germination of *Bouteloua dactyloides* and *Cynodon dactylon* in a multi-polluted soil. Sustainability, 9(1): 81. https://doi.org/10.3390/su9010081

Dinake, P., Kelebemang, R., 2019. Critical assessment of mechanistic pathways for chemical remediation techniques applied to Pb impacted soils at shooting ranges - A review. Environ. Pollutants Bioavailab., 31 (1): 282-305. https://doi.org/10.1080/26395940.2019.1676170

Escavy, J.I., Herrero, M.J., Arribas, M.E., 2012. Gypsum resources of Spain: Temporal and spatial distribution. Ore Geol. Rev., 49: 72-84. https://doi.org/10.1016/j.oregeorev.2012.09.001

Ewers, W., 1991. Standars, guidelines and legislative regulatory concerning metals and their compounds, in: Merian, E. (Ed.), Metals and their compounds in the environment. VCH Publisher, Weinheim. Germany.

Fernández-Caliani, J.C., Barba-Brioso, C., 2010. Metal immobilization in hazardous contaminated mine soils after marble slurry waste application. A field assessment at the Tharsis mining district (Spain). J. Hazard. Mater., 181: 817-826. https://doi.org/10.1016/j.jhazmat.2010.05.087

Franzen, D., Rehm, G., Gerwing, J., 2006. Effectiveness of gypsum in the North-Central Region of the U.S. NDSU Extension Service, North Dakota.

Gadepalle, V.P., Ouki, S.K., Van Herwijnen, R., Hutchings, T., 2007. Immobilization of heavy metals in soil using natural and waste materials for vegetation establishment on contaminated sites. Soil Sediment. Contam., 16: 233-251. https://doi.org/10.1080/15320380601169441

Galán, E., González, I., Fernández-Caliani, J.C., 2002. Residual pollution load of soils impacted by the Aznalcóllar (Spain) mining spill after clean-up operations. Sci. Total Environ., 286: 167-179. https://doi.org/10.1016/S0048-9697(01)00974-3

García, I., Diez, M., Martín, F., Simón, M., Dorronsoro, C., 2009. Mobility of arsenic and heavy metals in a sandy-loam textured and carbonated soil. Pedosphere, 19(2): 166-175. https://doi.org/10.1016/S1002-0160(09)60106-5

García-Carmona, M., Romero-Freire, A., Sierra Aragón, M., Martínez Garzón, F.J., Martín Peinado, F.J., 2017. Evaluation of remediation techniques in soils affected by residual contamination with heavy metals and arsenic. J. Environ. Manage., 191: 228-236. http://doi.org/10.1016/j.jenvman.2016.12.041

García-Carmona, M., García-Robles, H., Turpín Torrano, C., Fernández Ondoño, E., Lorite Moreno, J., Sierra Aragón, M., Martín Peinado, F.J., 2019. Residual pollution and vegetation distribution in amended soils 20 years after a pyrite mine tailings spill (Aznalcóllar, Spain). Sci. Total Environ., 650: 933-940. https://doi.org/10.1016/j.scitotenv.2018.09.092

Garrido, F., Illera, V., Vizcayno, C., García-González, M.T., 2003. Evaluation of industrial by-products as soil acidity amendments: chemical and mineralogical implications. Eur. J. Soil Sci., 54: 411-422. https://doi.org/10.1046/j.1365-2389.2003.00522.x

Garrido, F., Illera, V., García-González, M.T., 2005. Effect of the addition of gypsum- and lime-rich industrial by-products on Cd, Cu and Pb availability and leachability in metal-spiked acid soils. Appl. Geochem., 20: 397-408. https://doi.org/10.1016/j.apgeochem.2004.08.001

Grattan, S.R., Grieve, C.M., 1999. Mineral nutrient acquisition and response by plants grown in saline environments. Handbook of plant and crop stress, 2(1): 203-229. https://doi.org/10.1201/9780824746728.ch9

Gupta, M., Srivastava, P.K., Shikha, Niranjan, A., Tewari, S.K., 2016. Use of a bioaugmented organic soil amendment in combination with gypsum for *Withania somnifera* growth on sodic soil. Pedosphere, 26: 299-309.

Gupta, N., Yadav, K.K., Kumar, S., Chadd, R.P., Kumar, A., 2019. Trace elements in soil-vegetables interface: Translocation, bioaccumulation, toxicity and amelioration - A review. Sci. Total Environ., 651: 2927-2942. https://doi.org/10.1016/j.scitotenv.2018.10.047

Hering, J.G., Chen, P.Y., Wilkie, J.A., Elimelech, M., 1997. Arsenic removal from drinking water during coagulation. J. Environ. Eng., 123: 800-807. https://doi.org/10.1061/(asce)0733-9372(1997)123:8(800)

Hooda, P., 2010. Trace Elements in Soils, first ed. John Wiley and Sons Ltd, Chichester, West Sussex.

Illera, V., Garrido, F., Serrano, S., García-González, M.T., 2004. Immobilization of the heavy metals Cd, Cu and Pb in an acid soil amended with gypsum- and lime-rich industrial by-products. Eur. J. Soil Sci., 55: 135-145. https://doi.org/10.1046/j.1365-2389.2003.00583.x

ISO/DIS. 2006. 17402: Soil quality. Guidance for the selection and application of methods for the assessment of bioavailability of contaminants in soil and soil materials. ISO, Geneva, Switzerland.

Jiménez-Moraza, C., Iglesias, N., Palencia, I., 2006. Application of sugar foam to a pyrite-contaminated soil. Minerals Engineering, 19(5): 399-406. https://doi.org/10.1016/j.mineng.2005.10.011

Kabata-Pendias, A., 2011. Trace elements in soils and plants, fourth Edition. CRC, Boca Raton.

Kumpiene, J., Lagerkvist, A., Maurice, C., 2008. Stabilization of As, Cr, Cu, Pb and Zn in soil using amendments - a review. Waste Manage., 28(1): 215-225. https://doi.org/10.1016/j.wasman.2006.12.012

Liu, L., Li, W., Song, W., Guo, M., 2018. Remediation techniques for heavy metal-contaminated soils: Principles and applicability. Sci. Total Environ., 633: 206-219. https://doi.org/10.1016/j.scitotenv.2018.03.161

Lwin, C.S., Seo, B.H., Kim, H.U., Owens, G., Kim, K.R., 2018. Application of soil amendments to contaminated soils for heavy metal immobilization and improved soil quality-a critical review. Soil Sci. Plant Nutr., 64: 156-167.

Maathuis, J.M.F., 2009. Physiological functions of mineral macronutrients. Curr. Opin. Plant Biol., 12: 250-258. https://doi.org/10.1016/j.pbi.2009.04.003

Madejón, P., Murillo, J.M., Marañón, T., Cabrera, F., López, R., 2002. Bioaccumulation of As, Cd, Cu, Fe and Pb in wild grasses affected by the Aznalcóllar mine spill (SW Spain). Sci. Total Environ., 290: 105-120. https://doi.org/10.1016/s0048-9697(01)01070-1

Madejón, E., Pérez-de-Mora, A., Felipe, E., Burgos, P., Cabrera, F., 2006. Soil amendments reduce trace element solubility in a contaminated soil and allow regrowth of natural vegetation. Environ. Pollut., 139: 40-52. https://doi.org/10.1016/j.envpol.2005.04.034

Madejón, P., Domínguez, M.T., Madejón, E., Cabrera, F., Marañón, T., Murillo-Carpio, J.M., 2018. Soil-plant relationships and contamination by trace elements: A review of twenty years of experimentation and monitoring after the Aznalcóllar (SW Spain) mine accident. Sci. Total Environ., 625: 50-63. https://doi.org/10.1016/j.scitotenv.2017.12.277

Magurran, A.E. 2004. Measuring Biological Diversity. Blackwell.

Mallmann, F.J.K., dos Santos Rheinheimer, D., Ceretta, C.A., Cella, C., Minella, J.P.G., Guma, R.L., Šimůnek, J., 2014. Soil tillage to reduce surface metal contamination–model development and simulations of zinc and copper concentration profiles in a pig slurry-amended soil. Agric. Ecosyst. Environ., 196: 59-68. https://doi.org/10.1016/j.agee.2014.06.024

Manaka, M., 2006. Amount of amorphous materials in relationship to arsenic, antimony, and bismuth concentrations in a brown forest soil. Geoderma, 136(1): 75-86. https://doi.org/10.1016/j.geoderma.2006.02.002

Marschner, H., 1995. Mineral nutrition of higher plants, second ed. Academic Press, New York.

Martín, F.J., Simón, M., García, I., Bouza, P., Dorronsoro, C., Aguilar, J., 2003. Efecto del encalado en suelos contaminados por lodos piríticos. Edafología, 101(2): 177-183.

Martín-Peinado, F.J., Romero Freire, A., Arco Lázaro, E., Sierra Aragón, M., Ortiz-Bernad, I., Abbaslou, H., 2012. Assessment of arsenic toxicity in spiked soils and water solutions by the use of bioassays. Span. J. Soil Sci., 2(3): 45-56. https://doi.org/10.3232/SJSS.2012.V2.N3.05

Martín-Peinado, F.J., Romero-Freire, A., García-Fernández I., Sierra-Aragón, M., Ortiz-Bernad, I., Simón-Torres, M., 2015. Long-term contamination in a recovered area affected by a mining spill. Sci. Total Environ., 514: 219-223. https://doi.org/10.1016/j.scitotenv.2015.01.102

Matijevic, L., Romic, D., Romic, M., 2014. Soil organic matter and salinity affect copper bioavailability in root zone and uptake by *Vicia faba* L. plants. Environ. Geochem. Health, 36: 883-896. https://doi.org/10.1007/s10653-014-9606-7

McLaren, R.G., Swift, R.S., Williams, J.G., 1981. The adsorption of copper by soil materials at low equilibrium solution concentrations. J. Soil Sci., 32: 247-256. https://doi.org/10.1111/j.1365-2389.1981.tb01704.x

Mendez, M.O., Maier, R.M., 2008. Phytostabilization of mine tailings in arid and semi-arid environments-an emerging remediation technology. Environ. Health Perspect., 116(3): 278. https://doi.org/10.1289/ehp.10608

Meng, X., Bang, S., Korfiatis, G.P., 2000. Effects of silicate, sulfate, and carbonate on arsenic removal by ferric chloride. Water Res., 34 (4): 1255-1261. https://doi.org/10.1016/S0043-1354(99)00272-9

Moreno-Jiménez, E., Peñalosa, J.M., Manzano, R., Carpena-Ruiz, R.O., Gamarra, R., Esteban, E., 2009. Heavy metals distribution in soils surrounding an abandoned mine in NW Madrid (Spain) and their transference to wild flora. J. Hazard. Mater., 162(2): 854-859. https://doi.org/10.1016/j.jhazmat.2008.05.109

Norton, L.D., Rhoton, F., 2007. FGD gypsum influences on soil surface sealing, crusting, infiltration and runoff. Presented at the workshop on Agricultural and Industrial Uses of FGD Gypsum. October 2007, Atlanta, Ga. http://library.acaa-usa.org/5-FGD_Gypsum_Influences_on_Soil_Surface_Sealing_Crusting_Infiltration_and_Runoff.pdf

Palansooriya, K.N., Shaheen, S.M., Chen, S.S., Tsang, D.C.W., Hashimoto, Y., Hou, D., Bolan, N.S., Rinklebe, J., Ok, Y.S., 2020. Soil amendments for immobilization of potentially toxic elements in contaminated soils: A critical review. Environmental International, 134: 105046. https://doi.org/10.1016/j.envint.2019.105046

Pastor-Jáuregui, R., Paniagua-López, M., Martínez-Garzón, J., Martín-Peinado, F., Sierra-Aragón, M., 2020. Evolution of the residual pollution in soils after bioremediation treatments. Appl. Sci., 10: 1006. https://doi.org/10.3390/app10031006

Pérez-de-Mora, A., Madejón, P., Burgos, P., Cabrera, F., Lepp, N.W., Madejón, E., 2011. Phytostabilization of semiarid soils residually contaminated with trace elements using by-products: Sustainability and risks. Environ. Pollut., 159: 3018-3027. https://doi.org/10.1016/j.envpol.2011.04.015

Rehman, M.Z., Rizwan, M., Ali, S., Sabir, M., Sohail, M.I., 2017. Contrasting effects of organic and inorganic amendments on reducing lead toxicity in wheat. Bull. Environ. Contam. Toxicol., 99(5): 642-647. https://doi.org/10.1007/s00128-017-2177-4

Robinson, D.A., Hockley, N., Cooper, D.M., Emmett, B.A., Keith, A.M., Lebron, I., Reynolds, B., Tipping, E., Tye, A.M., Watts, C.W., Whalley, W.R., Black, H.I.J., Warren, G.P., Robinson, J.S., 2013. Natural capital and ecosystem services, developing an appropriate soils framework as a basis for valuation. Soil Biol. Biochem., 57: 1023-1033.

Rodríguez-Eugenio, N., McLaughlin, M., Pennock, D., 2018. Soil Pollution: a hidden reality. Rome, FAO, 142.

Rodríguez-Jordá, M.P., Garrido, F., García-González, M.T., 2012. Effect of the addition of industrial by-products on Cu, Zn, Pb and As leachability in a mine sediment. J. Hazard. Mater., 213-214: 46-54. https://doi.org/10.1016/j.jhazmat.2012.01.049

Romero-Freire, A., Martín Peinado, F., Van Gestel, C.A.M., 2015. Effect of soil properties on the toxicity of Pb: assessment of the appropriateness of guideline values. J. Hazard Mater. 289: 46-53. https://doi.org/10.1016/j.jhazmat.2015.02.034

Romero-Freire, A., García Fernández, I., Simón Torres, M., Martínez Garzón, F.J., Martín Peinado, F.J., 2016. Long-term toxicity assessment of soils in a recovered area affected by a mining spill. Environ. Pollut., 208: 553-561. https://doi.org/10.1016/j.envpol.2015.10.029

Shainberg, I., Sumner, M.E., Miller, W.P., Farina, M.P.W., Pavan, M.A., Fey, M.V., 1989. Use of gypsum on soils. A review. Adv. Soil S., 9: 1-111.

Sharma, A.K., Fehrenbacher, J.B., Jones, B.A., 1974. Effect of gypsum, soil disturbance and tile spacing on the amelioration of Huey silt loam, a nitric soil in Illinois. Soil Sci. Soc. Am. Pro., 38: 628-632. https://doi.org/10.2136/sssaj1974.03615995003800040029x

Sinha, M.K., Prasad, B., 1977. Effect of chelating agents on the kinetics of diffusion of zinc to a simulated root system and its uptake by wheat. Plant Soil, 48(3): 599-612. https://doi.org/10.1007/BF00145770

Sierra-Aragón, M., Mitsui-Nakamaru, Y., García-Carmona, M., Martínez-Garzón, F.J., Martín-Peinado, F.J., 2019. The role of organic amendment in soils affected by residual pollution of potentially harmful elements. Chemosphere, 237: 124549. https://doi.org/10.1016/j.chemosphere.2019.124549

Simón, M., Ortiz, I., García, I., Fernández, E., Fernández, J., Dorronsoro, C., Aguilar, J., 1999. Pollution of soils by the toxic spill of a pyrite mine (Aznalcóllar, Spain). Sci. Total Environ., 242: 105-115. https://doi.org/10.1016/S0048-9697(99)00378-2

Simón, M., Martín, F., García, I., Bouza, P., Dorronsoro, C., Aguilar, J., 2005. Interaction of limestone grains and acidic solutions from the oxidation of pyrite tailings. Environ. Pollut., 135(1): 65-72. https://doi.org/10.1016/j.envpol.2004.10.013

Simón, M., García, I., Martín, F., Díez, M., del Moral, F., Sánchez, J.A., 2008. Remediation measures and displacement of pollutants in soils affected by the spill of a pyrite mine. Sci. Total Environ., 407: 23-39. https://doi.org/10.1016/j.scitotenv.2008.07.040

Simón, M., Diez, M., González, V., García, I., Martín, F., de Haro, S., 2010. Use of liming in the remediation of soils polluted by sulphide oxidation: A leaching-column study. J. Hazard. Mater., 180: 241-246. https://doi.org/10.1016/j.jhazmat.2010.04.020

Sumner, M.E., Radcliffe, D.E., McCray, M., Carter, E., Clark, R.L., 1990. Gypsum as an ameliorant for subsoil hardpans. Soil Technol., 3: 253-258. https://doi.org/10.1016/0933-3630(90)90005-N

Tiller, K.G., Merry, R.H., 1981. Copper pollution of agricultural soils, in: Loneragan, J. F., Robson, A. D., Graham, R. D., (Eds.), Copper in Soils and Plants. Academic Press, Australia, pp. 119.

Toma, M., Sumner, M.E., Weeks, G., Saigusa, M., 1999. Long-term effects of gypsum on crop yield and subsoil chemical properties. Soil Sci. Soc. Am. J., 63: 891-895.

Undersander, U., Hall, H.M., Vassalotti, P., Cosgrove, D., 2011. Alfalfa germination and growth. National alfalfa and forage alliance (NAFA), University of Wisconsin-Extension, Cooperative Extension, Wisconsin.

Van Gestel, C.A., Van der Waarde, J.J., Derksen, J.G.M., Van der Hoek, E.E., Veul, M.F., Bouwens, S., Stokman, G.N., 2001. The use of acute and chronic bioassays to determine the ecological risk and bioremediation efficiency of oil-polluted soils. Environ. Toxicol. Chem., 20(7): 1438-1449. https://doi.org/10.1002/etc.5620200705

Van Lierop, W.M., Mackenzie, A.F., 1975. Effects of calcium carbonate and sulphate on the growth of lettuce and radish in some organic soils of Southwestern Quebec. Can. J. Soil Sci., 55: 205-212.

Wallace, A., Wallace, G.A., 1995. Gypsum is almost a universal soil amendment, Soil amendments and amendment technologies, Wallace Laboratories, El Segundo.

Yang, X., Hou, Q., Yang, Z., Zhang, X., Hou, Y., 2012. Solid-solution partitioning of arsenic (As) in the paddy soil profiles in Chengdu Plain, Southwest China. Geosci. Front., 3 (6): 901-909. https://doi.org/10.1016/j.gsf.2012.03.006

CONCLUSIONS

Ad astra per aspera

Lucius Annaeus Seneca

(4 a.C. - 65 d.C.)

CONCLUSIONS

1. More than twenty years after the Aznalcóllar mine accident, Assisted Natural Remediation is still required in the sector of the Guadiamar Green Corridor closest to the mine, which is affected by residual pollution. This remediation should be based on the immobilization of potentially harmful elements through the addition of soil amendments, followed by phytostabilization with native vegetation.

2. Soils in the unvegetated affected areas are highly acidic, extremely saline, and have low organic carbon content and a high load of potentially harmful elements, especially As and Pb, which strongly inhibit seed emergence, survival and plant growth. Although soil properties improved in the vegetated affected areas and promoted vegetation cover and plant species diversity, the accumulation of potentially harmful elements in plant tissues poses a potential toxicity risk for the food chain that should be monitored.

3. Under controlled conditions (greenhouse experiment), the addition of gypsum mining spoil to soils affected by potentially harmful elements enhances seed emergence, survival and plant growth, and it is especially effective at higher doses. This is directly related to the improvement of soil properties such as the reduction of soil crust, the delivery of essential nutrients for plants (Ca and S) and the rise of pH, which would promote the reduction of the solubility and/or bioavailability of some potentially harmful elements such as Cu, Zn, Cd, As and/or Pb.

4. Amendment application of gypsum mining spoil in field would improve some soil properties and promote the spontaneous recovery of vegetation. It is rather effective amending acidic soils highly polluted by Cu and Zn, but does not seem to alleviate soils affected by Pb or As pollution, nor to produce significant changes in the physical properties of the soil to modify the leaching of soluble salts. Nonetheless, the relatively low solubility of this amendment may improve its positive effects in the long term, requiring further studies to this respect.

5. The application of other amendments and treatments were also studied in comparison with gypsum mining spoil: i) Marble sludge proved to be more effective in terms of soil properties improvement, reduction of the mobility of potentially harmful elements and vegetation recovery. ii) The addition of neighbouring vegetated affected soil presented similar results to

gypsum mining spoil, although the provision of seeds from vegetated soils is a key factor to consider in this case study. iii) The physical treatment of tillage broke soil crusting but did not improve soil conditions or vegetation cover and diversity; as such, using this technique in isolation should be discouraged.

6. The addition of vermicompost appear to produce positive effects in terms of vegetation cover and species diversity when applied to gypsum mining spoil, marble sludge, mixture of vegetated and unvegetated affected soils, and tillage. The effectiveness of treatments would be as followed: mixture of vegetated and unvegetated affected soils (due to the presence of an active seed bank), marble sludge, gypsum mining spoil and, finally, tillage. Further monitoring would be advisable to prevent new acidification processes and the remobilization of potentially harmful elements, and to assess the positive evolution of these treatments.

7. Toxicity bioassay with *Lactuca sativa* L. indicated no potential phytotoxicity in the vegetated affected areas in relation to the unaffected ones, while strong toxicity is still detected in unvegetated affected soils. All the treatments used seem to reduce phytotoxicity in relation to unvegetated areas, with the exception of tillage, being marble sludge (with or without vermicompost) the most effective treatment; gypsum mining spoil and the mixture of vegetated and unvegetated affected soils would present intermediate results. In any case, due to the potential environmental risk derived from treatment application, it would be required to conduct detailed studies of the medium- to long- term effects of carbonates, sulphates and organic matter on soil toxicity.

8. This thesis improves the understanding of the use of gypsum mining spoil and other treatments in the remediation of degraded polluted soils, and provides valuable information about new remediation strategies that could be transposed to similar scenarios all over the world. The improvement of soil properties is the key factor that promotes the spontaneous development of vegetation, laying the basis for the evolution of a healthier ecosystem.

CONCLUSIONES

Ad astra per aspera

Lucius Annaeus Seneca

(4 a.C. - 65 d.C.)

CONCLUSIONES

1. Más de veinte años después del accidente de la mina de Aznalcóllar, todavía es necesaria la remediación natural asistida en el sector del Corredor Verde del Guadiamar más cercano a la mina, el cual está afectado por contaminación residual. Esta remediación debe basarse en la inmovilización de los elementos potencialmente tóxicos mediante la adición de enmiendas al suelo, seguida de su fitoestabilización con vegetación autóctona.

2. Los suelos de las zonas afectadas aún sin vegetación son altamente ácidos, extremadamente salinos, y tienen bajo contenido en carbono orgánico y una alta carga de elementos potencialmente tóxicos, especialmente As y Pb, que inhiben sustancialmente el crecimiento y la supervivencia de las plantas. Aunque las propiedades del suelo mejoraron en las zonas afectadas ya vegetadas promoviendo la cobertura y la diversidad de especies, la acumulación de elementos potencialmente tóxicos en los tejidos de las plantas supone un riesgo potencial de toxicidad para la cadena trófica que debe ser monitorizado.

3. En condiciones controladas (experimento en invernadero), la adición de yeso de rechazo, especialmente a altas dosis, a suelos afectados por elementos potencialmente tóxicos mejora la emergencia de semillas, así como la supervivencia y crecimiento de las plantas. Este hecho está directamente relacionado con la reducción de la costra superficial del suelo, el aporte de nutrientes esenciales para las plantas (Ca y S) y el aumento de pH, lo que promovería la reducción de la solubilidad y/o la biodisponibilidad de algunos elementos potencialmente tóxicos como el Cu, Zn, Cd, As y/o Pb.

4. La aplicación de la enmienda de yeso de rechazo en campo mejoría algunas propiedades del suelo y promovería la recuperación espontánea de la vegetación. Resulta bastante eficaz a la hora de enmendar suelos ácidos con alto contenido en Cu y Zn, pero no parece poder remediar suelos contaminados con Pb o As, ni producir cambios significativos en las propiedades físicas del suelo que limiten el lixiviado de las sales solubles. No obstante, la solubilidad relativamente baja de esta enmienda podría mejorar sus efectos positivos a largo plazo, por lo que sería recomendable la realización de estudios ulteriores al respecto.

5. También se estudió la aplicación de otras enmiendas y tratamientos en comparación con el yeso de rechazo: i) El lodo de mármol demostró ser más eficaz en cuanto a la mejora de las propiedades del suelo, la reducción de la movilidad de los elementos potencialmente tóxicos y la recuperación de la vegetación. ii) La mezcla de suelos contaminados con y sin vegetación también presentó resultados similares a los del yeso de rechazo, aunque el aporte de un banco de semillas a través del suelo vegetado es un factor clave a considerar en estos casos. iii) El arado rompió la costra del suelo, pero no mejoró sus condiciones ni la cobertura vegetal o la diversidad de especies herbáceas; por lo tanto, el uso aislado de esta técnica debe ser desaconsejado.

6. La adición de vermicompost parece producir efectos positivos en términos de cobertura vegetal y diversidad de especies cuando es aplicado en combinación con yeso de rechazo, lodo de mármol, mezcla de suelos contaminados con y sin vegetación, y con el arado del suelo. La efectividad de los tratamientos sería: mezcla de suelos contaminados con y sin vegetación (debido a la presencia de un banco de semillas activo), lodo de mármol, yeso de rechazo y, por último, el arado. Sería aconsejable monitorear los suelos tratados para prevenir nuevos procesos de acidificación y de removilización de los elementos potencialmente tóxicos, así como para evaluar la evolución positiva de estos tratamientos.

7. El bioensayo de toxicidad con *Lactuca sativa* L. no indicó la existencia de fitotoxicidad potencial en las zonas con vegetación afectadas en relación con las no afectadas, mientras que sí se detectó una fuerte toxicidad en los suelos afectados aún sin vegetación. Todos los tratamientos empleados, a excepción del arado, parecen reducir la fitotoxicidad en comparación con las zonas contaminadas no vegetadas, siendo el lodo de mármol (con o sin vermicompost) el tratamiento más eficaz; el yeso de rechazo y la mezcla de suelos contaminados con y sin vegetación presentarían resultados intermedios. En cualquier caso, debido al posible riesgo ambiental derivado de la aplicación de los tratamientos, sería necesario realizar estudios detallados de los efectos a medio y largo plazo de los carbonatos, sulfatos y materia orgánica sobre la toxicidad del suelo.

8. Esta tesis mejora la comprensión del uso del yeso de rechazo y otros tratamientos para la remediación de suelos contaminados y degradados, y proporciona información valiosa sobre nuevas estrategias de remediación que podrían transponerse a escenarios similares en todo el mundo. La mejora de las propiedades del suelo es el factor clave que promueve el desarrollo espontáneo de la vegetación, sentando las bases para un ecosistema más saludable.

LIST OF APPENDICES

6. Mv: M + v; 7. Tv: T + v; 8. Vv: V + v. PHEs: a) Cu; b) Zn; c) As; d) Pb. Different letters above symbols represent statistically significant differences ($p < 0.05$) for the *post-hoc* Tukey tests performed after the LMMs. *UV-AA: Unvegetated affected soil.

LIST OF FIGURES

LIST OF TABLES

"Todavía quedan muchas cosas en el mundo por las que merece la pena luchar. Muchas cosas bellas, mucha gente maravillosa luchando por revertir el daño causado, por ayudar a aliviar el sufrimiento. Y muchísima gente joven dedicada a hacer de este un mundo mejor"

Dra. Jane Goodall

UNIVERSIDAD
DE GRANADA