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Functional and ecotoxicological soil regeneration in abandoned metal(loid) mine tailings from semiarid Mediterranean environments spontaneously colonized by vegetation

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

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“¿Por qué nos caemos, Bruce? Para aprender a levantarnos”.

Thomas Wayne (Batman Begins)

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Algo que tienen en común todas las historias, películas, novelas, comics o incluso videojuegos es que antes o después siempre acaban llegando a su final, sin importar lo difícil que haya podido resultar el camino para los protagonistas. Si hace escasamente tres meses alguien me hubiera dicho que hoy me encontraría escribiendo estas palabras, difícilmente me lo habría creído o habría sido capaz de no soltar una carcajada. Sin embargo, aquí estoy, sin saber exactamente muy bien como hemos llegado hasta este punto, y creo que aún sin ser consciente del todo de qué punto es ese. Pero aquí estoy, y algo de lo que sí que soy consciente es que no podría haber llegado hasta este "final" sin ayuda.

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Héctor ha sido también una parte muy importante de este trabajo, tanto en la planificación de algunas cosas, como en la puesta en práctica de otras. Sin tu ayuda a lo mejor todavía estaría subiendo bolsas de enmienda a aquel depósito minero, gracias por compartir conmigo las agujetas de aquella semana. Gracias también por tener siempre la puerta de tu despacho abierta para lo que hiciera falta. Quiero dar las gracias también a María Ángeles Ferrer y a Antonio Calderón por todas las veces que me abrieron su laboratorio. A Mª José Delgado por toda su ayuda con el experimento de las cámaras climáticas. Y a Juanjo Martínez y Pablo Bielza porque siempre tuvieron palabras de ánimo.

No me quiero olvidar de todas las personas que en mayor o menor medida me han ayudado con todo el trabajo del laboratorio. Empezando por Isa que, aunque no coincidimos mucho tiempo, siempre estuviste dispuesta a resolver cualquier duda en la que me pudieras ayudar, incluso cuando ya no estabas en la universidad. Y continuando por todos aquellos a los que sumergí en montones de muestras para procesar y analizar, Carmen, Paloma, Irene, Lázaro, Ibo, Pedro, Cristian, Ana Vanesa, Ana Belén y Vicente. La mayoría de vosotros no solamente me ayudó con las muestras acumuladas, también hizo que el tiempo empleado para ello fuera mucho más agradable. Especialmente David Sánchez Bisquert, alguien que siempre estuvo dispuesto para ayudar en lo que hiciera falta, aunque sé que venirte a muestrear al monte rodeados de garrapatas nunca te hizo mucha gracia. Aun así, siempre lo hiciste todo con una sonrisa y con un sentido del humor único. Pero sobre todo a David lo que quiero agradecerle es su amistad, y también su interés durante todo este tiempo por saber cuándo iba a terminar por fin la tesis. Ya está David, ya la he dejado entregada.

Quiero agradecer también a todas las personas que tuve la suerte de conocer y que me acogieron tan bien durante las estancias que realicé. Al Dr. Kees van Gestel por aceptarme y darme la bienvenida en su grupo en Ámsterdam. A Rudo Verweij, el mejor técnico de laboratorio que nunca había conocido, algún día Pepe te hará una oferta que no podrás rechazar y acabarás trabajando en la UPCT. Al Dr. Matty Berg y a Jurgen van Hal, por toda su ayuda con la identificación de invertebrados. A Francesco Boatta por todas las cervezas que nos tomamos juntos y a Michella Ligtelijn por todos los lugares que me enseñaste, aún tienes pendiente una visita a España. De mi experiencia en Aveiro quiero agradecer a la Dra. Susana Loureiro por acogerme en su grupo y hacerme sentir uno más. Gracias también a Sara Peixoto, Isabel Silva y Abel Ferreira por toda su ayuda. Abel, es muy posible que tú también acabes trabajando en la UPCT junto a Rudo. Gracias a Ana Sousa, Marta Monteiro y Carla Quintaneiro por dejarme acompañaros de ruta al monte y a comer cada día durante un mes. Y gracias también, Carla, por prestarme la bici que casi no te devuelvo. Por último, un agradecimiento especial para mis dos portuguesas favoritas, Catarina Malheiro y Rita Silva, espero que sigáis practicando español, aunque con vuestra técnica secreta ya casi lo teníais dominado. Tengo que reconocer que al final Oporto no estaba tan mal, pero ahí sitios mejores Catarina.

Pero esta historia no solamente se ha desarrollado rodeado de matraces y pipetas, durante estos años también ha habido momentos para comidas, cervezas y charlas de terapia con gente con problemas similares. Lo que viene siendo el típico grupo de apoyo, que tardó un poco en formarse pero que finalmente estuvo compuesto por Antonio, Yolanda y Carolina. Ahora mismo el grupo está un poco disperso por el mundo, pero espero que no pase mucho tiempo hasta la próxima reunión. Y si tarda demasiado, la culpa será de Bryan que se ha llevado a Carolina secuestrada para que se convierta en la próxima superheroína. Lo importante Carolina, es que estemos donde estemos cada uno "juntos somos más fuertes".

Pero si hablamos de grupos de apoyo para sobrellevar los momentos más difíciles de esta tesis tengo que hablar de mis amigos. Que muchas veces ni apoyo ni nada, pero sí que ayudaban a que la cabeza se despejase fácilmente. Por un lado, Paco, Peche, Félix y Salmi, con vosotros me sacaba tres carreras más si hiciera falta. Puedo decir que sois lo mejor que saqué de estudiar en la universidad. Por otro lado, Noelia, Jesús y Roca, lo sorprendente es que siendo amigo vuestro haya sido capaz de sacarme una carrera y de acabar una tesis. Sois los mejores para conseguir que una tarde normal se convierta en un disparate impredecible en cinco segundos, así que ¡por Christopher Robin! Y, por último, esos que han estado ahí desde que me alcanza la memoria Zamora, Julián, Paco Pepe y Francisco. Con vosotros la cosa más simple, como rodar por una ladera y coger una pelota, se convierte en una aventura que merece la pena vivir, aunque a veces pueda suponer un riesgo para la integridad física. Todas las decisiones importantes tienen que ser tomadas o bien por una camarera que elija una tarjeta al azar o bien mediante "piedra, papel o tijera". Y evidentemente, quien no tenga claro que para calentar hay que sacar piedra queda descalificado. A Estefanía gracias por su interés y su apoyo. A las Truchas os diría, si os hubiera conocido antes que a ellos, que huyerais lo más rápido y lejos posible. Sin embargo, como ellos tienen prioridad, les diré: agarradlas fuerte y que no se os escapen mientras no sepan el error que cometen. A todos vosotros gracias por Ser y Estar.

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Como podéis ver, esta historia ha sido larga y en muchos momentos difícil. Para ir terminando quiero utilizar las palabras que Samsagaz Gamyi le dedicó a Frodo Bolsón en el desarrollo de su propia tesis para puntualizar algo:

«No deberíamos ni haber llegado hasta aquí, pero henos aquí, igual que en las grandes historias, señor Frodo, las que realmente importan, llenas de oscuridad y de constantes peligros. Ésas de las que no quieres saber el final, porque ¿cómo van a acabar bien? ¿Cómo volverá el mundo a ser lo que era después de tanta maldad como ha sufrido? Pero al final, todo es pasajero. Como esta sombra, incluso la oscuridad se acaba, para dar paso a un nuevo día. Y cuando el sol brilla, brilla más radiante aún. Esas son las historias que llenan el corazón, porque tienen mucho sentido, aun cuando eres demasiado pequeño para entenderlas. Pero creo, señor Frodo, que ya lo entiendo. Ahora lo entiendo. Los protagonistas de esas historias se rendirían si quisieran. Pero no lo hacen: siguen adelante, porque todos luchan por algo.»

Sin Sam el anillo no habría sido destruido, sin todos vosotros esta tesis no habría sido posible.

Gracias.

Resumen

La minería metálica es una de las actividades más perjudiciales para el medio ambiente a nivel mundial, entre otros motivos debido a la alta cantidad de residuos potencialmente peligrosos con elevada concentración de metales y metaloides (elementos potencialmente tóxicos, PTEs) que produce. Además, las excavaciones y movimientos de tierra pueden llevar a la destrucción completa de las áreas afectadas y sus alrededores. Los depósitos de almacenamiento de residuos (grandes pilas al aire libre que almacenan residuos fangosos procedentes de lavaderos de mineral) son especialmente preocupantes, ya que presentan las mayores concentraciones de PTEs y muchas veces se abandonan *in situ* tras el cese de la actividad minera sin ninguna medida de restauración.

Los suelos de los depósitos mineros se forman a partir de los residuos que éstos almacenan. Aparte de presentar niveles extremadamente altos de PTEs, se caracterizan generalmente por una alta salinidad, valores extremos de pH (de ácido a alcalino), baja disponibilidad de materia orgánica y nutrientes, y falta de estructura edáfica que conduce a una escasa capacidad de retención de agua y de aireación. Entre otras cosas, esto dificulta el crecimiento de la vegetación provocando que muchas veces la superficie de los depósitos permanezca desnuda, favoreciendo la propagación de PTEs a las áreas adyacentes. Las condiciones hostiles impuestas por los residuos mineros no sólo obstaculizan la colonización de las plantas sino también la biodiversidad del suelo, lo que a menudo da lugar a escasa actividad biológica en los suelos de los depósitos. Son suelos con baja capacidad para albergar organismos vivos y, por consiguiente, para desarrollar procesos biológicos. Esto provoca que la funcionalidad de los suelos de los depósitos mineros sea muy limitada, así como su capacidad para proporcionar servicios ecosistémicos.

Las técnicas convencionales de remediación de depósitos mineros se basan en soluciones de ingeniería tales como operaciones de remoción y/o aislamiento *in situ* mediante sellado y plantación. Sin embargo, estas opciones son generalmente costosas y/o técnicamente difíciles de implementar y eso dificulta su implantación. Otras opciones, que pueden ser complementarias a las técnicas de ingeniería, son las técnicas de fitomanejo. El fitomanejo comprende un conjunto de alternativas más baratas y respetuosas con el medio ambiente que buscan manejar el sistema suelo-planta mediante el uso de enmiendas de suelo y/o la introducción de especies de plantas tolerantes a las

condiciones extremas de estos ambientes, para controlar la dispersión de PTEs. Dentro del fitomanejo, la fitoestabilización tiene como objetivo reducir la movilidad de los PTEs y/o su (bio)disponibilidad a través de la absorción y/o fijación en las raíces, y/o la inmovilización en el suelo por fenómenos como la precipitación, adsorción, oxidación, reducción, para evitar su entrada en la cadena trófica, así como su migración a las aguas subterráneas. Un proceso que puede ayudar a implementar las técnicas de fitoestabilización es la denominada restauración pasiva, que se basa en la capacidad de las plantas nativas para colonizar y crecer espontáneamente en lugares perturbados. Este fenómeno conduce a la formación de parches de vegetación (llamados a veces islas de fertilidad) cuyos suelos suelen mostrar un mayor contenido de C y N orgánicos y una mayor actividad microbiana que las áreas desnudas adyacentes. Esto es particularmente interesante cuando los depósitos mineros están ubicados en mitad de áreas naturales (por ejemplo, zonas boscosas) rodeados de vegetación que puede esparcir propágulos y semillas que alcanzan con facilidad los depósitos abandonados. En este sentido, favorecer el establecimiento de especies nativas en los depósitos ayuda a crear puntos de nucleación en éstos y a desencadenar la expansión de la vegetación en su superficie. Entre otros, este proceso podría verse favorecido por la adición de enmiendas orgánicas al suelo, como biochar y residuos sólidos urbanos (RSU), que inmovilicen los metal(oid)es y mejoren las condiciones de los suelos.

El **objetivo general de la Tesis Doctoral** fue profundizar en el conocimiento de los aspectos físicos, fisicoquímicos, funcionales y ecotoxicológicos en suelos de depósitos abandonados de minería metálica de ambientes semiáridos Mediterráneos y su relación con la colonización vegetal espontánea, y valorar si la adición de enmiendas orgánicas contribuye a mejorar estos ecosistemas al promover la recuperación de la funcionalidad del suelo y desencadenar la colonización espontánea de la vegetación. Para lograr este objetivo general, se plantearon tres **objetivos específicos**:

1. Evaluar en qué grado pueden modificarse las condiciones del suelo tras la colonización espontánea de la vegetación en depósitos abandonados de minería metálica, y aportar evidencias sobre el interés de esta colonización para el fitomanejo de estos depósitos.

2. Evaluar en qué medida la colonización vegetal espontánea de depósitos abandonados de minería metálica da lugar a una mejora funcional del suelo e identificar,

si es posible, un nivel crítico que indique que esta funcionalidad se aproxima a la de los suelos naturales con vegetación de las áreas circundantes.

3. Evaluar la eficacia de una enmienda orgánica compuesta por biochar (procedente de podas de árboles) y compost de RSU para mejorar las condiciones de suelos ácidos sin cubierta vegetal en depósitos de residuos mineros y valorar si los efectos de dicha enmienda persisten estacionalmente durante un año y favorecen la colonización vegetal espontánea.

El área de estudio seleccionada fue el antiguo distrito minero de Cartagena-La Unión (≈50 km^2; Región de Murcia, SE España), cuya actividad finalizó en 1991. La zona presenta un clima semiárido Mediterráneo (precipitación media anual ≈200-300 mm, temperatura media anual ≈17 °C, y tasa media anual de evapotranspiración ≈850 mm). La vegetación natural está constituida principalmente por arbustos xerófilos mediterráneos y bosques de pino carrasco (*Pinus halepensis*). Los principales metales extraídos en la zona fueron Fe, Pb y Zn, obtenidos de minerales como carbonatos, sulfuros y sulfatos. En la actualidad, permanecen en la zona 89 depósitos mineros abandonados, la mayoría de ellos sin ningún tipo de intervención de restauración. El estudio se llevó a cabo en dos de estos depósitos, separados por una distancia de ≈2000 m y construidos a mediados de los años 60 para almacenar los residuos de las minas de galena (PbS), y en las áreas forestales circundantes. Ambos depósitos (ubicados a ≈170-200 m sobre el nivel del mar y situados en pequeños valles de orientación NW a SE) fueron abandonados hace ≈40 años y han sido parcialmente colonizados por la vegetación nativa formando parches irregulares que cubren entre ≈20% y ≈50% de las superficies. A finales de abril-inicios mayo de 2017 se seleccionaron seis ambientes diferentes para el estudio, estableciendo cuatro parcelas de muestreo de 2 m x 2 m en cada uno de ellos:

A) Dentro de los depósitos mineros. 1. Suelos desnudos (B); 2. Parches con pequeños grupos de árboles de *P. halepensis* creciendo dispersos de ≈2,5-5 m de alto (P); 3. Parches formados por árboles aislados de *P. halepensis* creciendo dispersos de >≈4-5 m de alto arbustos y hierbas bajo su copa (P+S); 4. Parches densos que incluyen varios árboles de *P. halepensis* de >≈4-5 m de altura y arbustos y hierbas bajo su copa (DP+S).

B) Fuera de los depósitos mineros. 5. Bosque situado junto a los depósitos mineros con árboles de *P. halepensis* de >≈5 m de altura y arbustos y hierbas bajo su copa (FN);

6. Bosque situado lejos de los depósitos mineros (≈1600-1800 m) con árboles de *P. halepensis* de >≈5 m de altura y arbustos y hierbas bajo su copa (FA).

Además, en los suelos desnudos dentro de los depósitos se establecieron cuatro parcelas adicionales para un experimento de adición de enmiendas al suelo (AB).

Los **resultados de la Tesis Doctoral** se presentan en tres capítulos:

Capítulo 5. Incluye el trabajo planificado para responder al primer objetivo específico, basado en una campaña de trabajo de campo realizada en el verano de 2017. Se evaluaron un conjunto de indicadores del suelo (físicos, fisicoquímicos y biológicos) en condiciones de campo y laboratorio en los ambientes B, P, P+S, DP+S, FN y FA. Los resultados evidencian el interés de la colonización espontánea por parte de la vegetación nativa para el fitomanejo de depósitos de minería metálica, en términos de proporcionar funcionalidad al ecosistema. En los parches de vegetación dentro de los depósitos se encontraron especies de plantas pioneras y nodrizas (favorecedoras de la sucesión vegetal) y los índices ecológicos de vegetación en P+S y DP+S fueron similares a FN y FA. En los suelos de los parches se encontraron evidencias de pedogénesis, como el desarrollo de estructura edáfica y el aumento de la capacidad de intercambio catiónico y el C y N orgánicos, siguiendo un patrón de aumento/mejora B-P-P+S-DP+S. Sin embargo, los contenidos de metal(oid)es del suelo no siguieron el mismo patrón de variación. Por ejemplo (en mg kg^{-1}): P mostró el valor máximo de Cu (≈277) y Zn (≈17860) totales, mientras que P+S de As (≈1250) y Pb (≈14570) totales. B tuvo el máximo de Pb (≈4) y Zn (≈207) solubles en agua, mientras que FA de As (≈0,192) y Cu (≈0,149). El C de la biomasa microbiana del suelo, la actividad enzimática, la respiración de suelo (emisión de CO_2), la descomposición de la materia orgánica y la actividad alimentaria de los invertebrados edáficos indicaron una actividad biológica similar o incluso superior en P+S y DP+S que en FN y FA. De hecho, FA mostró alto riesgo de ecotoxicidad del suelo (reproducción reducida del invertebrado *Enchytraeus crypticus*), atribuible a las altas concentraciones de As soluble en agua. Por lo tanto, de estos resultados se deduce que la vegetación que coloniza espontáneamente los depósitos de residuos mineros puede modificar eficazmente sus suelos, que adquieren la capacidad de proporcionar ciertas funciones del ecosistema. Los resultados de este trabajo fueron publicados en el artículo científico: Álvarez-Rogel, J., Peñalver-Alcalá, A., Jiménez-Cárceles, F.J., Tercero, M.C., González-Alcaraz, M.N. 2021. Evidence supporting the value of spontaneous vegetation for phytomanagement of soil ecosystem functions in abandoned metal(loid) mine tailings. Catena 201, 105191; doi: https://doi.org/10.1016/j.catena.2021.105191.

Capítulo 6. Este capítulo incluye el trabajo planificado para responder al segundo objetivo específico, basado en una campaña de trabajo de campo realizada en la primavera de 2018. Se estudiaron los índices ecológicos de la vegetación, las formas de vida de las plantas y los roles funcionales de éstas, junto con parámetros fisicoquímicos y funcionales de los suelos, en los ambientes B, P, P+S, DP+S, FN y FA. Los parches de vegetación solo mostraron pequeñas diferencias en los parámetros fisicoquímicos relacionados con las condiciones de estrés abiótico del suelo (pH, salinidad y metales), independientemente de la vegetación. Sin embargo, parches de vegetación con mayor diversidad y riqueza de especies y presencia de plantas con mayor contraste de formas de vida y rasgos funcionales más diversos que facilitan el crecimiento de especies menos tolerantes al estrés, mostraron un aumento de la funcionalidad microbiana del suelo (mayor C de la biomasa microbiana, actividad β-glucosidasa, actividad metabólica bacteriana y diversidad funcional). Además, estos parches de vegetación mostraron un estado funcional del suelo comparable al de los bosques fuera de los depósitos mineros. Los resultados de este trabajo fueron publicados en el artículo científico: Peñalver-Alcalá, A., Álvarez-Rogel, J., Peixoto, S., Silva, I., Silva, A.R.R., González-Alcaraz, M.N. 2021. The relationships between functional and physicochemical soil parameters in metal(loid) mine tailings from Mediterranean semiarid areas support the value of spontaneous vegetation colonization for phytomanagement. Ecological Engineering 168: 106293; doi: https://doi.org/10.1016/j.ecoleng.2021.106293.

Capítulo 7. Este capítulo incluye el trabajo planificado para responder al tercer objetivo específico. En abril de 2017 se aplicó una enmienda orgánica consistente en una mezcla de 3:1 de biochar y RSU compostado (dosis de 3% peso seco) a suelos ácidos (pH ≈5,5) sin vegetación de uno de los depósitos mineros. Las concentraciones de metal(oid)es totales en los suelos enmendados fueron (en mg kg^{-1}): As ≈220, Cd ≈40, Mn ≈1800, Pb ≈5300 y Zn ≈8600. Dos meses después de la adición de la enmienda ya se observaron mejoras en las propiedades químicas y fisicoquímicas de los suelos (reducción de la acidez, salinidad y metales solubles en agua y aumento del contenido de C orgánico y nutrientes), que resultó en una reducción de la ecotoxicidad para el invertebrado edáfico *Enchytraeus crypticus*. El C orgánico recalcitrante proporcionado por el biochar permaneció en el suelo, mientras que los compuestos orgánicos lábiles proporcionados por el RSU se consumieron con el tiempo. Estas mejoras fueron consistentes durante al menos un año y condujeron a una menor densidad aparente, mayor capacidad de retención de agua y mayores niveles para los parámetros microbianos y relacionados con funcionalidad (C de la biomasa microbiana, actividad microbiana catabólica y emisión de CO_2) en el suelo enmendado del depósito. El crecimiento espontáneo de la vegetación nativa se favoreció con la adición de enmiendas, pero fue necesario un periodo de tres años para que las plantas colonizadoras persistieran y alcanzaran tamaño adulto. Los resultados de este trabajo fueron publicados en el artículo científico: Peñalver-Alcalá, A., Álvarez-Rogel, J., Conesa, H.M., González-Alcaraz, M.N. 2021. Biochar and urban solid refuse ameliorate the inhospitality of acidic mine tailings and foster effective spontaneous plant colonization under semiarid climate. Journal of Environmental Management 292: 112824; https://doi.org/10.1016/j.jenvman.2021.112824.

Las **conclusiones generales de la Tesis Doctoral** son tres:

1) La colonización vegetal espontánea de depósitos abandonados de minera metálica de zonas semiáridas Mediterráneas induce mejora de las condiciones físicas, fisicoquímicas, funcionales y ecotoxicológicas del suelo, independientemente de los niveles de meta(oid)es totales.

2) La riqueza y diversidad de especies vegetales con formas de vida diferentes y rasgos funcionales distintos parecen ser factores clave para lograr una mejora funcional efectiva del suelo en los parches de vegetación de depósitos mineros espontáneamente colonizados.

3) La combinación de biochar de podas de árboles y RSU compostado es, en general, una enmienda adecuada para mejorar los suelos ácidos de depósitos de residuos mineros, promoviendo su recuperación funcional y desencadenando la colonización espontánea de la vegetación.

Summary

Metal mining is one of the most environmentally detrimental activities worldwide, among others, due to the high load of (hazardous) wastes disposed. Mining can cause extreme impacts on the affected ecosystem(s), which can lead to multi-elemental pollution problems and, in some cases, to the complete destruction of the affected areas and their surroundings. Of particular concern are the so-called mine tailings (open-air piles that store muddy residues), which are, on many occasions, abandoned *in situ* after the mining activity ceases without any restoration.

Mine tailings soils are formed from the mine wastes they store. Apart from extremely high metal(loid) levels, they are generally characterized by high salinity, extreme pH values (from acid to alkaline), low availability of organic matter and nutrients, and lack of physical structure that leads to reduced water retention capacity and aeration. Among others, this tends to cause lack of vegetation and that tailing surfaces remain bare, favoring the spread of potentially toxic elements (PTEs) to adjacent areas. The hostile conditions imposed by tailing wastes not only hinder plant colonization but also soil biodiversity, often leading to low-biologically active tailing soils (soils with low capacity to shelter living organisms and, consequently, to support biological processes). Thereby, the functionality of mine tailings soils is often restricted, as well as their capacity to provide ecosystem services.

Conventional mine tailings remediation techniques are based on engineering solutions such as removal operations and/or on-site isolation by sealing and afforestation. However, these options are generally expensive and/or technically difficult to implement. Other options, which can be complementary to engineering ones, are phytomanagement techniques. Phytomanagement comprises a set of cheaper and environmentally friendly alternatives that seek to manipulate the soil-plant system (by using soil amendments and/or tolerant plants) to control the fluxes of pollutants in the environment. Within phytomanagement, phytostabilization aims to reduce pollutants mobility and (bio)availability via root uptake, precipitation or reduction to prevent their entry into the food chain as well as their migration to groundwater. Phytostabilization can take advantage of the so-called passive restoration that relies on the capability of native plants to colonize and grow spontaneously in disturbed places. This phenomenon leads to the formation of vegetated patches (fertility islands) whose soils usually show higher contents

of soil organic C and N and higher microbial activity than the adjacent surrounding barren areas. This is particularly interesting when mine tailings are embedded in vegetated areas that can spread propagules and seeds to tailings. In this sense, favoring the natural recruitment of native species might help to create nucleation spots and to trigger vegetation expansion within mine tailings. Among others, this process could be favored by the addition of soil organic amendments, as biochar and urban solid refuse (USR), which immobilize metal(loid)s and improve the conditions of tailing soils.

The **general objective of the PhD Thesis** was to deepen the knowledge of physical, physicochemical, functional and ecotoxicological aspects in soils of abandoned metal(loid) mine tailings from Mediterranean semiarid environments and their relationship with spontaneous plant colonization, and whether the addition of organic amendments contributes to improving these ecosystems by promoting the recovery of soil functionality and triggering the spontaneous colonization of vegetation. To achieve this general objective, three **specific objectives** were raised:

1. To evaluate in which degree soil conditions can be modified following spontaneous vegetation colonization in abandoned metal(loid) mine tailings, and to provide evidence about the interest of this colonization for the phytomanagement of these structures.

2. To assess to what degree spontaneous plant colonization of abandoned metal(loid) mine tailings led to functional soil improvement and to identify, if possible, a critical level indicating that this functionality was moving towards that of the natural vegetated soils from the surrounding areas.

3. To assess the effectiveness of an organic amendment composed of biochar from pruning trees and compost from USR to ameliorate the conditions of barren metal(loid) acidic mine tailings soils and if these effects persist seasonally over a year, and whether the organic amendment favors spontaneous plant colonization.

The selected study area was the former metal mining district of Cartagena-La Unión (≈50 km^2; Murcia Region, SE Spain), which ended its activity in 1991. The area presents a Mediterranean semiarid climate (mean annual precipitation ≈200-300 mm, mean annual temperature ≈17 °C, and mean annual evapotranspiration rate ≈850 mm) and the natural vegetation is mainly constituted by xerophytic shrubs and small formations of pine trees (*Pinus halepensis*). The principal metals extracted were Fe, Pb and Zn, obtained from minerals such as carbonates, sulfides, and sulfates. At present, 89 mine tailings remain in the area, most of them abandoned without any type of intervention. Specifically, the study was conducted in two mine tailings ≈2000 m apart built by mid-60's to store wastes from mines exploiting galena ore, and in the surrounding forest areas. Both tailings (similar altitude, ≈170-200 m a.s.l., and embedded in small valleys NW to SE facing) were abandoned ≈40 years ago and have been partially colonized by native vegetation in a patchy structure with covers between ≈20% and ≈50%. One environment devoid of vegetation and five different types of vegetated environments inside and outside of the mine tailings were selected in April-May 2017. The environments studied were (four plots of 2 m x 2 m per environment):

A) Four inside the mine tailings. 1. Bare soils (B); 2. Patches with small groups of *P. halepensis* trees ≈2.5-5 m high growing scattered (P); 3. Patches formed by isolated *P. halepensis* trees >≈4-5 m high growing scattered with shrubs and herbs under the canopy (P+S); 4. Dense patches including several *P. halepensis* trees >≈4-5 m high and shrubs and herbs under the canopy (DP+S).

B) Two outside the mine tailings. 5. Forest located next to the mine tailings with *P. halepensis* trees >≈5 m high and shrubs and herbs under the canopy (FN); 6. Forest located away from the mine tailings (≈1600-1800 m) with *P. halepensis* trees >≈5 m high and shrubs and herbs under the canopy (FA).

In addition, in bare soils, four additional plots were established for a soil amendment addition experiment (AB).

The following three chapters include the **results of the PhD Thesis**:

<u>Chapter 5.</u> This chapter includes the work planned to respond to the first specific objective, based on the field work campaign carried out in summer 2017. A set of soil indicators (physical, physicochemical and biological) were evaluated under field and laboratory conditions in B, P, P+S, DP+S, FN and FA environments. The results provide evidence about the interest of spontaneous colonization by native vegetation for the phytomanagement of abandoned metal(loid) mine tailings in terms of providing ecosystem functions. Pioneer and nurse plant species (which are facilitation plants that favor succession phenomena) were mainly found inside the tailings, although vegetation ecological indexes in P+S and DP+S were similar to FN and FA. Pedogenesis evidence such as structure development and increase in cation exchange capacity, organic C and N were found in tailing soils from B to DP+S. However, soil metal(loid)s did not follow the

same variation pattern. For example (in mg kg^{-1}): P showed the maximum total Cu (≈277) and Zn (≈17,860), while P+S of As (≈1250) and Pb (≈14,570). B had the maximum water soluble Pb (≈4) and Zn (≈207), while FA of As (≈0.192) and Cu (≈0.149). Soil microbial biomass C, enzyme activity, CO_2 emission, organic matter decomposition and feeding activity of soil dwelling organisms indicated similar, or even higher, biological activity in P+S and DP+S than in FN and FA. In fact, FA showed the highest soil ecotoxicity risk (reduced reproduction of the soil invertebrate *Enchytraeus crypticus*). Therefore, mine tailing soils can be effectively modified following spontaneous vegetation colonization, achieving conditions with capacity to provide certain ecosystem functions. The results of this work were published in the following scientific paper: Álvarez-Rogel, J., Peñalver-Alcalá, A., Jiménez-Cárceles, F.J., Tercero, M.C., González-Alcaraz, M.N. 2021. Evidence supporting the value of spontaneous vegetation for phytomanagement of soil ecosystem functions in abandoned metal(loid) mine tailings. Catena 201, 105191; doi: https://doi.org/10.1016/j.catena.2021.105191.

Chapter 6. This chapter includes the work planned to respond to the second specific objective, based on the field work campaign carried out in spring 2018. Vegetation ecological indexes, plant life forms and species functional roles, together with physicochemical and functional soils parameters, were studied in B, P, P+S, DP+S, FN and FA environments. Vegetated patches showed only small differences in physicochemical parameters related to soil abiotic stress conditions (pH, salinity and metals), regardless of the vegetation. However, vegetated patches with greater species diversity and richness and presence of plants with contrasted life forms and functional traits that facilitate the growth of less stress-tolerant species showed an increase of soil microbial functionality (higher microbial biomass C, β-glucosidase activity, bacterial metabolic activity, and functional diversity). Moreover, these vegetated patches showed a functional soil status comparable to that of the forests outside the mine tailings. The results of this work were published in the following scientific paper: Peñalver-Alcalá, A., Álvarez-Rogel, J., Peixoto, S., Silva, I., Silva, A.R.R., González-Alcaraz, M.N. 2021. The relationships between functional and physicochemical soil parameters in metal(loid) mine tailings from Mediterranean semiarid areas support the value of spontaneous vegetation colonization for phytomanagement. Ecological Engineering 168: 106293; doi: https://doi.org/10.1016/j.ecoleng.2021.106293.

Chapter 7. This chapter includes the work planned to respond to the third specific objective. In April 2017, an organic amendment consisting of a 3:1 mixture of biochar and composted USR was applied (3% d.w. dose) to acidic barren areas of one of the mine tailings (pH ≈5.5). Total metal(loid) concentrations in the amended soils were (in mg kg^{-1}): As ≈220, Cd ≈40, Mn ≈1800, Pb ≈5300, and Zn ≈8600. Two months after amendment addition were enough to observe improvements in chemical and physicochemical tailing soil properties (reduced acidity, salinity and water soluble metals and increased organic C and nutrients content), which resulted in lowered ecotoxicity for the soil invertebrate *E. crypticus*. Recalcitrant organic C provided by biochar remained in soil whereas labile organic compounds provided by USR were consumed over time. These improvements were consistent for at least one year and led to lower bulk density, higher water retention capacity and higher scores for microbial/functional-related parameters (microbial biomass C, microbial catabolic activity, and CO_2 emission) in the amended tailing soil. Spontaneous growth of native vegetation was favored with amendment addition, but adult plants of remarkable size were only found after three years. The results of this work were published in the following scientific paper: Peñalver-Alcalá, A., Álvarez-Rogel, J., Conesa, H.M., González-Alcaraz, M.N. 2021. Biochar and urban solid refuse ameliorate the inhospitality of acidic mine tailings and foster effective spontaneous plant colonization under semiarid climate. Journal of Environmental Management 292: 112824; https://doi.org/10.1016/j.jenvman.2021.112824.

The **general conclusions of the PhD Thesis** are three:

1) Spontaneous vegetation colonization of abandoned metal(loid) mine tailings from Mediterranean semiarid areas induces improvement of physical, physicochemical, functional and ecotoxicological soil conditions regardless of total metal(loid) levels.

2) Plant species richness and diversity with contrasting life forms and functional traits seem to be key factors for achieving effective functional soil improvement in spontaneously vegetated mine tailings patches.

3) The combination of biochar from pruning trees and composted URS is, in general, a suitable amendment to improve barren acidic mine tailing soils, by promoting their functional recovery and triggering spontaneous vegetation colonization.

ÍNDEX

PART I. GENERAL ASPECTS AND METHODOLOGY

PART II. RESULTS AND DISCUSSION

PART III. CONCLUSIONS

PART I

GENERAL ASPECTS AND METHODOLOGY

CHAPTER 1

Introduction

1.1. Impacts of metal mining

Metal mining is one of the most environmentally detrimental activities worldwide, among others, due to the high load of (hazardous) wastes disposed (Dudka and Adriano, 1997; Lottermoser, 2010). Mining activities can cause extreme impacts on the affected ecosystem(s), which can lead to multi-elemental pollution problems and, in some cases, to the complete destruction of the affected areas and their surroundings (Dudka and Adriano, 1997; Conesa and Schulin, 2010). Metal mining is particularly detrimental not only for the release of potentially toxic elements (PTEs) to the environment such as metal(loid)s, but also for: i) land degradation (e.g., vegetation removal, topographic alterations, topsoil loss, soil compaction); ii) sheer amount of wastes produced (the valuable portion of the ore represents a small fraction of the total volume of excavated materials); iii) unfavorable conditions mine wastes offer to soil biota. In fact, the restoration of areas affected by metal mining implies the reconstruction of the whole ecosystem, similar to the earliest stages of primary succession after major disturbances (Huang et al., 2012). This is a global issue if we consider that ≈1% of worldwide land surface is impacted by mining activities and that ≈700 million tons of metalliferous wastes are laid down annually (Walker, 1999; Warhurst, 2000; FAO and ITPS, 2015).

Metal mine wastes can be classified into overburden (ore-free rocks), wastes from ore processing (residual fraction after ore extraction) and mine water (water used to extract and process ore and/or acid mine drainage) (Salomons and Forstner, 1988; Dudka and Adriano, 1997; Lottermoser, 2010) (Fig. 1.1). All these wastes are, on many occasions, abandoned *in situ* after the mining activity ceases. Of particular concern are the so-called metal(loid) mine tailings (hereafter mine tailings), which are open-air piles that store muddy residues (Fig. 1.2). They are a major source of pollution for surrounding areas due to their high content in metal(loid)s (Mendez and Maier, 2008; Conesa and Schulin, 2010; Kossoff et al., 2014; Wang et al., 2019).

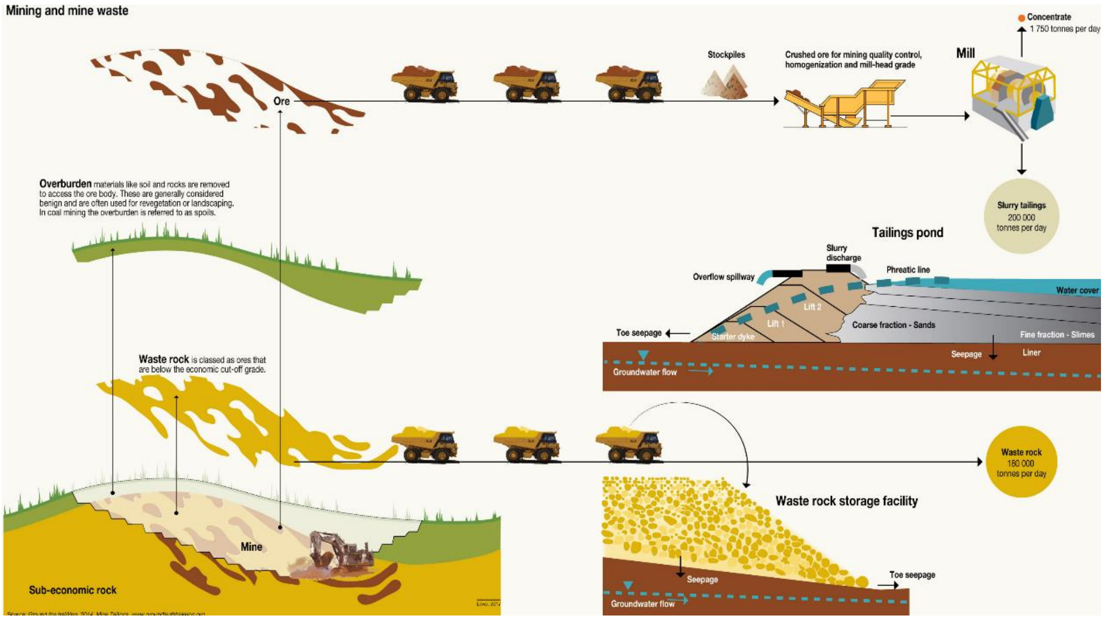

Figure 1.1. Types of wastes generated in mining activity (retrieved from Thygesen, 2017a).

Figure 1.2. Mine tailing abandoned after the cease of mining activity.

1.2. The particular case of mine tailings

1.2.1. Structural problems of mine tailings

One of the major risks of mine tailings is their structural collapse, which consists of the failure of the tailings structure, causing severe environmental damage to the surroundings and, often, loss of lives (Armstrong et al., 2019). Numerous accidents have been registered around the world during the last few decades. However, the majority of mine tailing collapses remain unreported, especially in countries where environmental legislation is too lax (Rico et al., 2008). Among the reported accidents, Rico et al. (2008) detailed how USA and Europe are the places where the greatest number of tailings failure are detected, with storm rain events as the most common cause of failures (Fig. 1.3).

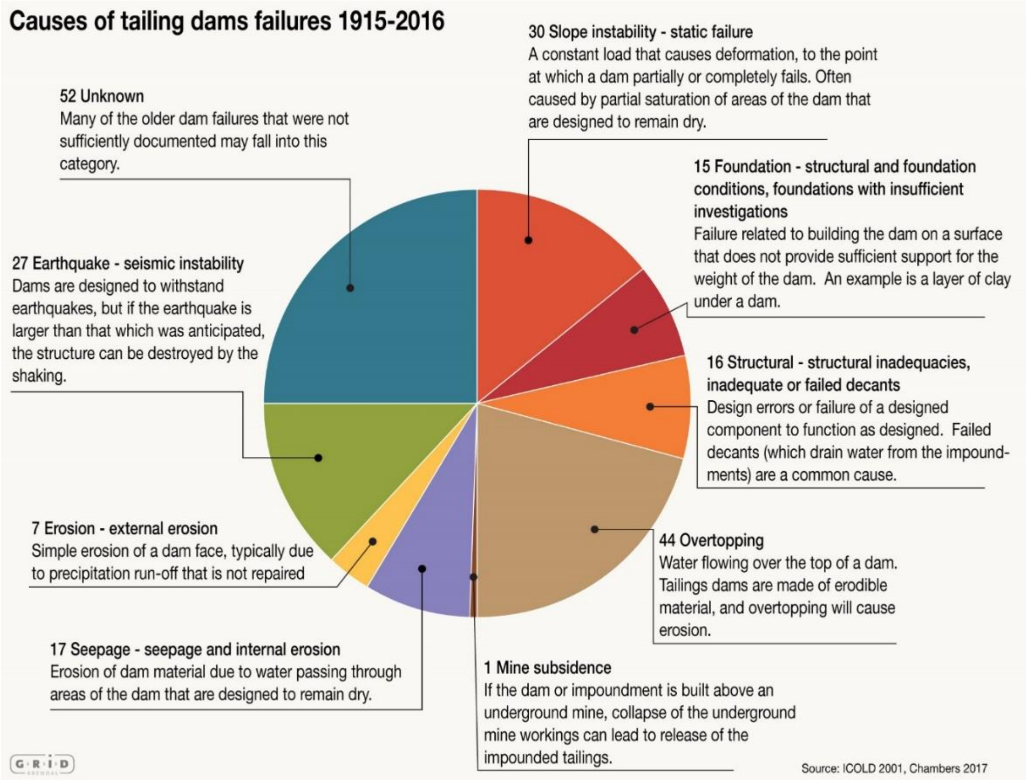

Figure 1.3. Main causes of tailings dams failures between 1915 and 2016 (retrieved from Thygesen, 2017b).

In Spain, the most important and environmentally harmful mine spill occurred in 1998 in Aznalcollar (Andalusia Region) (Fig. 1.4), in which the Agrio, Guadiamar and Guadalquivir Rivers as well as the National Park of Doñana were affected (Olías et al., 2020; Santos et al., 2020). In 2014, in British Columbia (Canada), an accident at Mount Polley Mining Corporation spilled around 25 million m^3 of mine waste that affected the Quesnel Lake (Byrne et al., 2018; Garris et al., 2018; Hatam et al., 2019). In Brazil, the Samarco tailing collapse accident in 2015 killed 19 people and the Brumadinho tailing disaster in 2019 killed around 300 people, spreading huge amounts of mine waste to the surrounding areas (Armstrong et al., 2019; Scotti et al., 2020; Silva Rotta et al., 2020). These accidents cause punctual environmental, social, and economic damage with highly visible repercussions (Fig. 1.5).

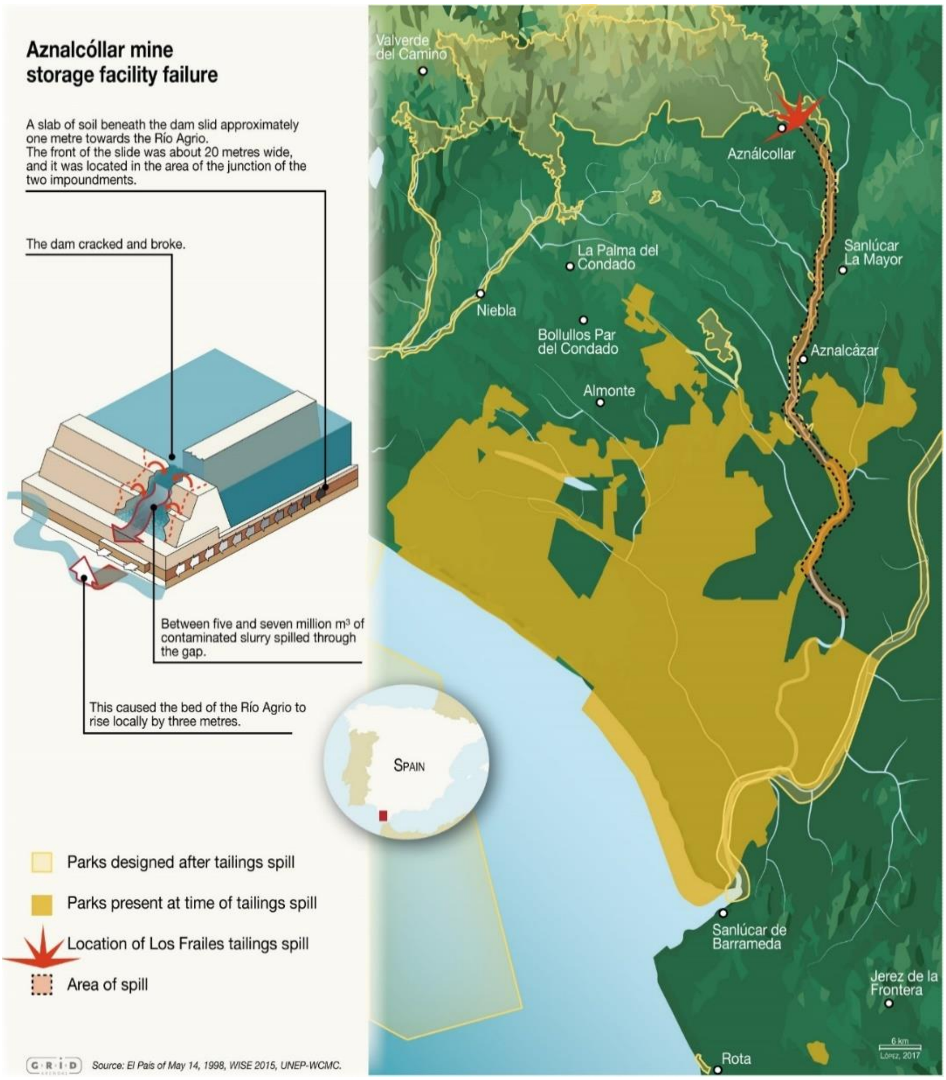

Figure 1.4. Aznalcóllar mine tailing collapse accident in 1998 (retrieved from Thygesen, 2017c).

Figure 1.5. News of mine spill in Aznalcóllar after 20 years.

1.2.2. Mine tailings soils

Mine tailings soils are formed from the mine wastes they store. Apart from extremely high metal(loid) levels, they are generally characterized by high salinity, extreme pH values (from acid to alkaline), low availability of organic matter and nutrients, and lack of physical structure, which leads to low water retention capacity and aeration (Wong, 2003; Conesa et al., 2006; Mendez and Maier, 2008). Among others, this causes lack of vegetation and that tailing surfaces remain bare, which favor the spread of PTEs to adjacent areas such as forests, farms, cities and waterbodies (e.g., streams, rivers, lakes, groundwater) (Conesa and Schulin, 2010; Navarro-Cano et al., 2018). The difficulties for the establishment of plants are more accentuated in arid and semiarid climate conditions due to factors such as extreme temperatures, low precipitation or high winds that make vegetation colonization much more difficult (Mendez and Maier, 2008). Consequently, mine tailings are highly prone to being eroded by wind and water (Fig. 1.6), spreading PTEs to surrounding areas, which affects ecosystem sustainability and pose major risks for environmental and human health in the medium and long term (Rajapaksha et al., 2004; Conesa and Jiménez-Cárceles, 2007; Moreno-Brotons et al., 2010; Azarbad et al., 2013; Gutiérrez et al., 2016; Wang et al., 2019).

Figure 1.6. Erosion effects on abandoned mine tailings.

The high metal(loid) concentrations of mine tailing's soils can lead to ecotoxicity problems both *in situ* and *ex situ*, due to waste dispersion by erosion. In fact, one of the major risks of metal(loid)s is their environmental persistence and the toxic effects they can exert on living organisms (Adriano, 2001; Frouz et al., 2011; González et al., 2011; Lu et al., 2014; Xie et al., 2016; Zhang and Van Gestel, 2019). The ecotoxicity risks of metal(loid)-polluted systems are often related to the (bio)availability and mechanism of toxicity of the metal(loid)s present (Peijnenburg and Jager, 2003; Peijnenburg et al., 2007; van Gestel, 2008). Peijnenburg et al. (2007) defines bioavailability as "*the fraction of the* *total amount of a chemical present in a specific environmental compartment that, within a given time span, is either available or can be made available for uptake by (micro)organisms from either the direct surrounding of the organisms or by ingestion of food*". Metal(loid) (bio)availability is closely related to inherent soil properties (e.g., pH, salinity, organic matter content, texture) (Bagherifam et al., 2019; Zhang and Van Gestel, 2019; Zhang et al., 2019), but also to external factors such as the environmental conditions (e.g., temperature, soil moisture content) (Holmstrup et al., 2010; González-Alcaraz and van Gestel, 2015).

The hostile conditions imposed by tailing wastes not only hinder plant colonization, but also soil biodiversity and species abundance (Moynahan et al., 2002; Scharfstein and Gaurf, 2013; Rodríguez Martín et al., 2014; Martinez et al., 2018; Risueño et al., 2020a), often leading to low-biologically active tailing soils (soils with low capacity to shelter living organisms and, consequently, to support biological processes). Thereby, the functionality of mine tailings soils can be restricted (capability to carry out different abiotic and biotic processes), as well as their capacity to provide ecosystem services (e.g., climate regulation, biodiversity preservation or pollution control) (Barrios, 2007; Niemeyer et al., 2012; Brown et al., 2014; Adhikari and Hartemink, 2016; Fazekaš et al., 2019).

1.3. General overview of remediation techniques for mine tailings

Different remediation techniques have been described to manage metal(loid) mine tailings. Conventional techniques often include engineering solutions such as removal operations, soil washing, dam building or on-site isolation by sealing (Mendez and Maier, 2008; Huang et al., 2012; Kossoff et al., 2014; Gil-Loaiza et al, 2016; Palansooriya et al., 2020). These latest techniques are based on the application of physical, chemical and/or biological methods for tailings stabilization (Tordoff et al., 2000) (Fig. 1.7). Physical stabilization usually consists of covering mine waste with innocuous materials (e.g., waste rocks, gravels) to reduce wind and water erosion, while chemical stabilization uses chemical agents (e.g., lignin sulfonate, resinous adhesive) to generate a surface crust resistant to erosion processes in mine tailings (Mendez and Maier, 2008). On the other hand, biological conventional methods are typically based on the afforestation of mine tailings after covering mine wastes with, for example, topsoil from non-polluted sites.

These engineering interventions must be imperative under high and immediate risk of tailings structural collapse and/or acid drainage situations.

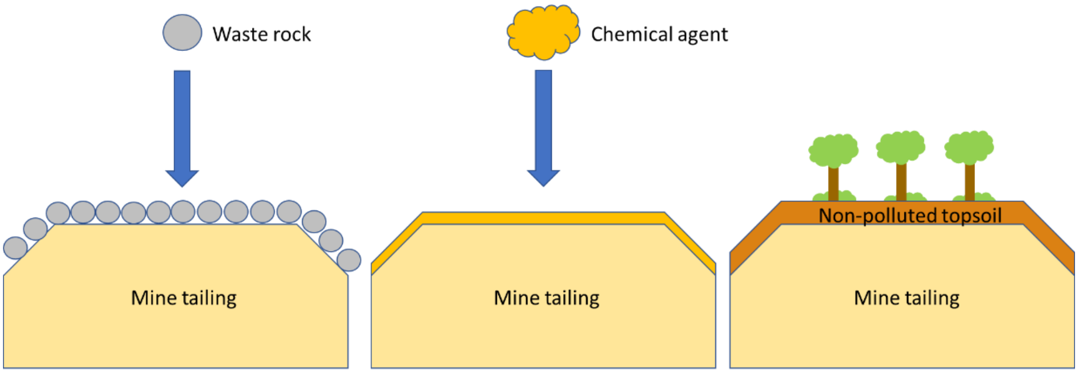

Figure 1.7. Conventional physical, chemical and biological stabilization of mine tailings.

However, these conventional solutions have numerous drawbacks. They are generally expensive and/or technically difficult to implement (e.g., geostructural restrictions, topographical access difficulties) (Tordoff et al., 2000; Mendez and Maier, 2008). Furthermore, transporting large volumes of (hazardous) wastes, as well as having an appropriate dumping site for their re-allocation, is not an easy task (Mendez and Maier, 2008; Conesa and Schulin, 2010; Lottermoser, 2010). In addition, in drylands such as (semi)arid regions, engineering options for the restoration of mine tailings based on covering materials and afforestation for isolating the mine wastes are much more questioned, due to the difficulty of plants to obtain nutritional and water resources from shallow surface layers together with the harsh conditions imposed by the dry climate (Huang et al., 2012). For these reasons, many authors have studied different options of soil remediation based on phytotechnologies or phytomanagement techniques (Anderson et al., 2009; Scharfstein and Gaurf, 2013; Chen et al., 2018; Zama et al., 2018; Palansooriya et al., 2020; Santos et al., 2020). These options are considered especially suitable when mine tailings do not present an immediate risk, either because of their location (e.g., far from population centers) and/or because of their lower risk of structural collapse, but they remain a constant source of PTEs dispersion in the short, medium and long term (time bomb), negatively affecting not only the environment but also human health and well-being.

1.4. Phytomanagement and phytostabilization

According to Robinson et al. (2009), phytomanagement describes a set of technologies that include the manipulation of the soil-plant system (through the use of soil amendments and/or tolerant plant species) to control the fluxes of pollutants in the environment. It comprises a set of cheaper and environmentally friendly alternative options that can be carried out without a great use of heavy machinery (Mendez and Maier, 2008; Robinson et al., 2009; Conesa and Schulin, 2010; Navarro-Cano et al., 2018; Wei et al., 2021), which has been proven to be useful for the restoration of ecosystems functions (Burges et al., 2018). In ancient mining areas, where old mine facilities are often embedded in natural landscapes and are considered part of the cultural heritage, phytomanagement techniques could be suitable options to preserve the sites and, at the same time, to satisfy environmental regulations (Rieuwerts et al., 2009; Navarro-Cano et al., 2018).

Phytomanagement techniques comprise several methods that focus on different approaches (Robinson et al., 2009; Conesa et al., 2012; Ali et al., 2013; Burges et al., 2018): i) phytoextraction, the use of plants to translocate pollutants from soil to the easily harvestable shoots (Kumar et al., 1995); ii) phytofiltration, the use of plant roots to extract, concentrate or precipitate pollutants from aqueous solutions (Kumar et al., 1995); iii) phytovolatilization, the use of plants to transform soil pollutants into their gaseous state (Raskin et al., 1997); iv) phytostabilization, the use of plants to reduce soil pollutants mobility and (bio)availability via root uptake, precipitation or reduction to prevent their entry into the food chain as well as their migration to groundwater (Salt et al., 1995) (Fig. 1.8). The effectiveness of these methods as remediation options has been described by several authors (Reboreda and Caçador, 2008; Robinson et al., 2009; Ali et al., 2013; Abdullah et al., 2016; Moreno-Barriga et al., 2017b; Burges et al., 2018), who also detailed that the suitability of each method depends on the polluted site conditions. For example, the use of hyperaccumulators plants in mine tailings soils as phytoextraction strategy normally has negligible effects due to the high metal(loid) levels present (Robinson et al., 2009). In this sense, more authors are paying attention to phytostabilization methods as suitable options to remediate mine tailings soils, especially when the risks of structural collapse are low and/or they are far from population centers (Beesley et al., 2011; Scharfstein and Gaurf, 2013; Párraga-Aguado et al., 2014; Navarro-Cano et al., 2018).

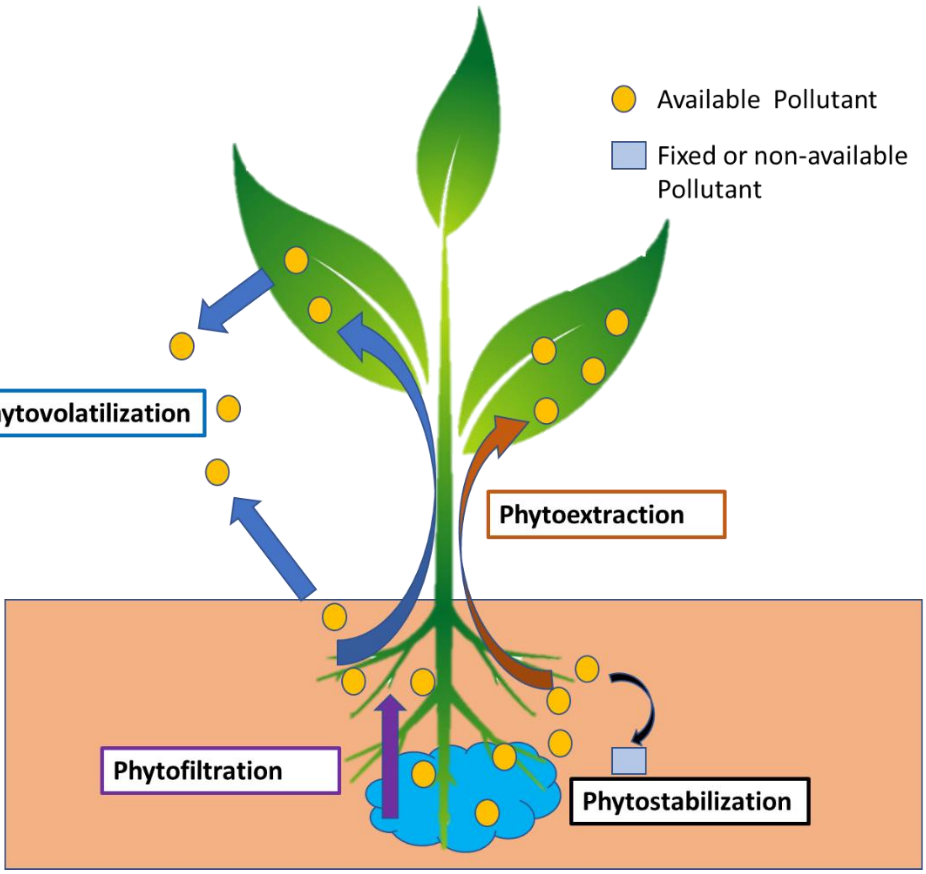

Figure 1.8. Examples of phytomanagement techniques applied to soils affected by inorganic pollutants (e.g., metal(loid)s).

Within phytomanagement, aided phytostabilization (afforestation following soil amendments application) is especially recommended for metal(loid) mine tailings areas where a suitable option can be to immobilize metal(loid)s on site (Oustriere et al., 2016) (Zornoza et al., 2015) and, in addition, integrate tailings into the surrounding landscape (Tordoff et al., 2000; Mendez and Maier, 2008; Navarro-Cano et al., 2018). Aided phytostabilization seeks to reduce metal(loid)s mobility and (bio)availability, but also the establishment of a permanent vegetation cover which minimizes erosion problems and pollutants dispersion. Moreover, plant development on mine tailings can stimulate soil microbial activity that, at the same time, improves soil conditions and favors plant growth (Mendez and Maier, 2008; Li et al., 2011; Párraga-Aguado et al., 2013; Antoniadis et al., 2017; Risueño et al., 2020b). These plants and soil microorganisms can immobilize metal(loid)s throw different processes such as adsorption onto rhizosphere surface and/or plant accumulation complexation (with organic matter), precipitation in inorganic forms (e.g., sulfides, carbonates, or phosphates) or in organic compounds (e.g., oxalates) (Wenzel, 2009; Antoniadis et al., 2017). This phytostabilization approach has been developed in metal(loid) mine tailings areas on many occasions (Zornoza et al., 2015; Gil-Loaiza et al., 2016; Palansooriya et al., 2020).

1.5. Passive restoration

An alternative phytostabilization approach is passive restoration, which relies on the capability of native plants to colonize and grow spontaneously in disturbed places (Prach and Hobbs, 2008; Prach and Tolvanen, 2016). This method is particularly interesting when mine tailings are embedded in vegetated areas that can spread propagules and seeds to tailings (Navarro-Cano et al., 2018).

Under field conditions, metal(loid) mine tailings wastes undergo hydrogeochemical stabilization due to the action of atmospheric factors such as rain, which implies a decrease in the capacity to provide extreme acidity, salinity, and soluble pollutants (Huang et al., 2012; You et al., 2018). Early microbial colonizers, adapted to extreme conditions, may grow in the raw materials, so contributing to the initial alteration phases (Colin et al., 2019). The amelioration of the initial extreme conditions favors the colonization by adapted native plants, which improve microclimate conditions through shading and promote soil improvement (Navarro-Cano et al., 2018; Oreja et al., 2020). This is followed by a concomitant evolution of soil microorganisms and vegetation, leading to the development of ecological linkages between root zone microorganisms and aboveground plant communities (Huang et al., 2012). These vegetation patches (fertility islands) can have different shape, cover and species composition (Párraga-Aguado et al., 2013; Navarro-Cano et al., 2018), and their soils show higher contents of soil organic carbon and nitrogen and higher microbial enzyme activity than the adjacent surrounding barren areas (Párraga-Aguado et al., 2013, 2014; Navarro-Cano et al., 2018; Risueño et al., 2020b). So, these fertility islands act as soil microbial hotspots (small soil volumes with higher microbial abundance, activity and/or diversity compared to the average soil conditions leading to faster and intensive functioning process rates) (Kuzyakov and Blagodatskaya, 2015). In fertility islands the so-called nurse species play a major role. Nurse species are stress-resistant pioneer plants with functional traits that allow them not only to colonize bare soils but also to facilitate the growth of less stress-tolerant species, hence promoting the development of fertility islands with facilitation-driven plant communities (Navarro-Cano et al., 2018). These authors identified relationships between soil bacterial community shifts and predominance of nurse plants with different functional traits (trees, shrubs, dwarf shrubs and perennial grasses). This colonization process is sometimes favored by the existence of specific edaphic niches with more hospitable conditions such as lower salinity or softer structure that facilitate roots penetration (Párraga-Aguado et al., 2013).

Due to the difficulties for the establishment and survival of vegetation in these extreme environments (Huang et al., 2012), favoring the natural recruitment of native species might help to create nucleation spots and fertility islands to trigger vegetation expansion within mine tailings. Among others, this could be favored by the addition of soil amendments.

1.6. An overview of soil amendments for remediation of metal(loid)-polluted soils

The use of amendments to immobilize metal(loid)s and improve the conditions of polluted soils, such as those in mine tailings, has been widely studied (Zeng et al., 2015; Zornoza et al., 2015; Wu et al., 2016; Lebrun et al., 2020; Palansooriya et al., 2020). Metal(loid)s mobilization or immobilization is driven by soil properties (e.g., pH, salinity, redox status, organic matter content) and soil chemical processes (e.g., sorption, desorption, precipitation, interaction with organic matter) (Palansooriya et al., 2020). Inorganic and organic soil amendments have been applied for this purpose.

1.6.1. Inorganic amendments

Among the different types of inorganic amendments, carbonates, oxides and hydroxides of Ca and Mg have been widely used to raise the pH of acidic soils and thus decrease metal solubility (Palansooriya et al., 2020). Liming materials have been also used to raise the pH with different degrees of success (Fernández-Caliani and Barba-Brioso, 2010; Simón et al., 2010; González-Alcaraz et al., 2013; Zornoza et al., 2016). Coal fly ash, which comes from burning pulverized coal in electrical generation plants (Ram and Masto, 2014), is also used as an amendment that counteracts soil acidity and immobilize metals (Houben et al., 2012). It can also improve soil water holding capacity, bulk density and provide nutrients to soils (Palansooriya et al., 2020). However, caution must be taken since coal fly ashes might present high metal(loid) concentrations, which might pose environmental risks (Ram and Masto, 2014).

Metal oxides, as hydroxides, oxyhydroxides and hydrous oxides of Al, Fe, and Mn, have also been employed to immobilize metal(loid)s in polluted soils (Zeng et al., 2017). Under oxidized conditions they have a strong sorption potential and immobilization effects (Rinklebe et al., 2016). Clay minerals are also used, however, the adsorption of pollutants is closely related with the type of clay mineral (García-Sanchez et al., 2002).

1.6.2. Organic amendments

The objective of using organic amendments is not only to decrease metal(loid) mobility/(bio)availability, but also to provide organic matter and nutrients to the soil as well as to improve its structure and increase its water holding capacity (Wu et al., 2016; Moreno-Barriga et al., 2017a; Palansooriya et al., 2020). Different types of organic wastes, or by-products manufactured from them, are commonly used as organic amendments (Sohi et al., 2010; Wu et al., 2016; Palansooriya et al., 2020). This is the case of biosolids that are derived from sewage sludge treated in wastewater plants. Biosolids have high levels of organic matter and nutrients, but they might also contain pathogens depending on the sewage sludge of origin (Palansooriya et al., 2020). One type of organic amendment that has been utilized in mine tailings soils is animal wastes (e.g., cow dung, pig or chicken manure) (Zornoza et al., 2015; Palansooriya et al., 2020). Animal wastes are commonly used by farmers as fertilizers due to their high nutrient content. However, they might contain additional pollutants, such as metal(loid)s, antibiotics or microbial pathogens (Palansooriya et al., 2020).

Another type of widely used organic amendment is urban solid refuse (USR) (Clemente et al., 2015; Párraga-Aguado et al., 2017; Abou Jaoude et al., 2019; Conesa and Párraga-Aguado, 2021). In fact, the increased generation of USR during the last decades and its management and possible recycling is one of the priorities within the European Union's environmental policy as part of the circular economy strategy (European Commission, 2008). Its composition is highly variable since, among others, it depends on the climate, standard of living, time of the year and waste collection system (Hernández et al., 2015; Abou Jaoude et al., 2019). USR is generally characterized by high levels of labile organic matter and nutrients that stimulate plants and microbial activity (Ros, 2000). However it may also contain diverse pollutants (e.g., metal(loid)s, pharmaceutical products, microbial pathogens) and/or show some characteristics (e.g., extreme salinity) that could negatively affect the soil (Hernández et al., 2015). For this reason, prior to be applied, USR must comply with some legal requirements according to its use (Alvarenga et al., 2007). In this sense, composting processes could eliminate some of the pollutants present in USR (e.g., microbial pathogens). Composting consists of a three-stage process (mesophilic, thermophilic, and maturation) where microbial and enzyme activities stabilize organic waste obtaining humic substances, mineral ions, water and CO_2 (Zeng et al., 2015). Although compost characteristics are directly related to the properties of the original material, composts are normally characterized by showing high concentrations of labile organic matter and nutrients easily available to plants and soil (micro)organisms (Hernández et al., 2015; Wu et al., 2016; Abou Jaoude et al., 2019).

Among the organic amendments applied to improve metal(loid)-polluted soils, biochar is one of the most promising (Chen et al., 2018). It consists of a product of organic materials pyrolysis in the absence of oxygen (Sohi et al., 2010) that favors pH increase, water retention, carbon sequestration, nutrient replenishment, and microbial activity (Lehmann and Joseph, 2012). Since its properties are closely related to the characteristics of the source product, biochar can also have high content of metal(loid)s (Zornoza et al., 2016; Fazekaš et al., 2019; Palansooriya et al., 2020; Godlewska et al., 2021). This must be considered before its field application. Nevertheless, biochar has been proposed as an effective amendment to improve mine tailings soils by immobilizing metals, reducing their mobility and availability (Beesley et al., 2011; Wu et al., 2017; Nie et al., 2018; Palansooriya et al., 2020). However, because biochar contains high levels of recalcitrant organic matter, it is poor in labile organic carbon and readily available nutrients and its use at high dose in large areas is not always feasible given its high economic cost (Rodríguez-Vila et al., 2014; Ghosh et al., 2015; Wu et al., 2016, 2017). Among others, these limitations can be solved by mixing biochar with composted raw organic materials that are richer in more labile organic matter. In particular, mixing biochar with composted USR has shown good results when applied to metal(loid)-polluted soils (Karami et al., 2011; Wu et al., 2017).

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CHAPTER 2

Study area

2.1. Location and general characteristics

The former metal mining district of Cartagena-La Union (≈50 km^2) is located in southeast Spain (Cartagena, Murcia Region; 30S689151E, 4164433N) (Fig. 2.1). It is a mountainous area (maximum height ≈400 m a.s.l.) belonging to the Betic Ranges, which runs through the mining district from west to east and parallel to the Mediterranean Sea.

Figure 2.1. Location map of the Cartagena-La Union mining district.

The area presents a Mediterranean semiarid climate with a mean annual precipitation ≈200-300 mm, a mean annual temperature ≈18 °C, and a mean annual evapotranspiration rate ≈1000 mm (Fig. 2.2). Winters are characterized by mild temperatures, without significant frost, while summers are very hot, often exceeding temperatures of 40 ºC. Wind episodes are a frequent phenomenon in the area throughout the year (Sánchez-Bisquert, 2017), while most rain events are registered in spring and autumn, frequently in the form of heavy storms. The formation of fog and dew provide surplus water to the vegetation (Conesa, 2005; Jiménez-Cárceles, 2006).

The typical watercourses in the area are dry rivers, that is, temporary streams, locally called ramblas, whose water regime is closely related to the rainfall events. Hence, the ramblas usually only carry water during (or after) heavy rain episodes and remain dry most of the year. Despite precipitation scarcity, the high energy that is concentrated during storm events confers ramblas a major role in transporting sediments and water from the mining area to the surroundings including the Campo de Cartagena, the Mar Menor lagoon and the Mediterranean Sea (Conesa and Schulin, 2010) (Fig 2.3).

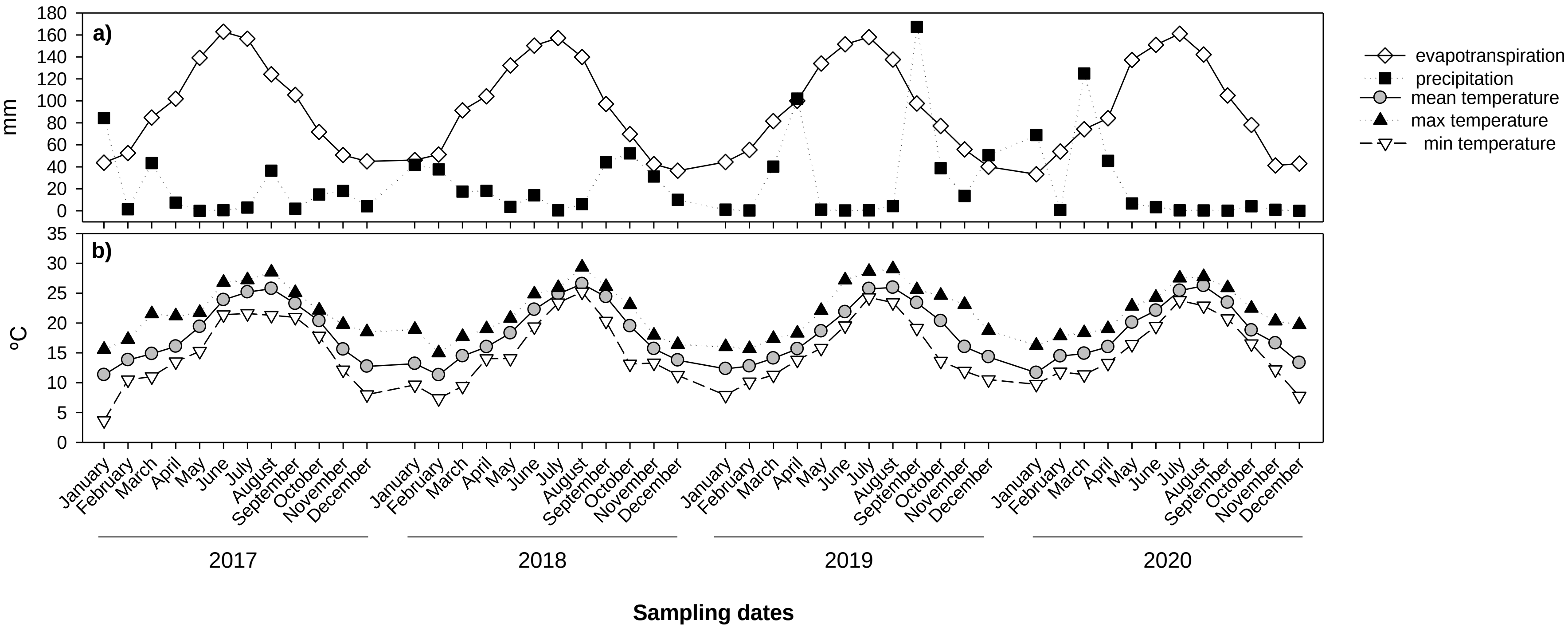

Figure 2.2. Climate data (mean monthly values) from Los Belones (Cartagena) meteorological station. Location: N 37º 36' 40,14'' W 0º 48' 13,65''. Period: 2017-2020. a) Evapotranspiration (mm) measure according to Allen et al. (1998), and precipitation (mm). b) Mean, maximum and minimum temperature (ºC).

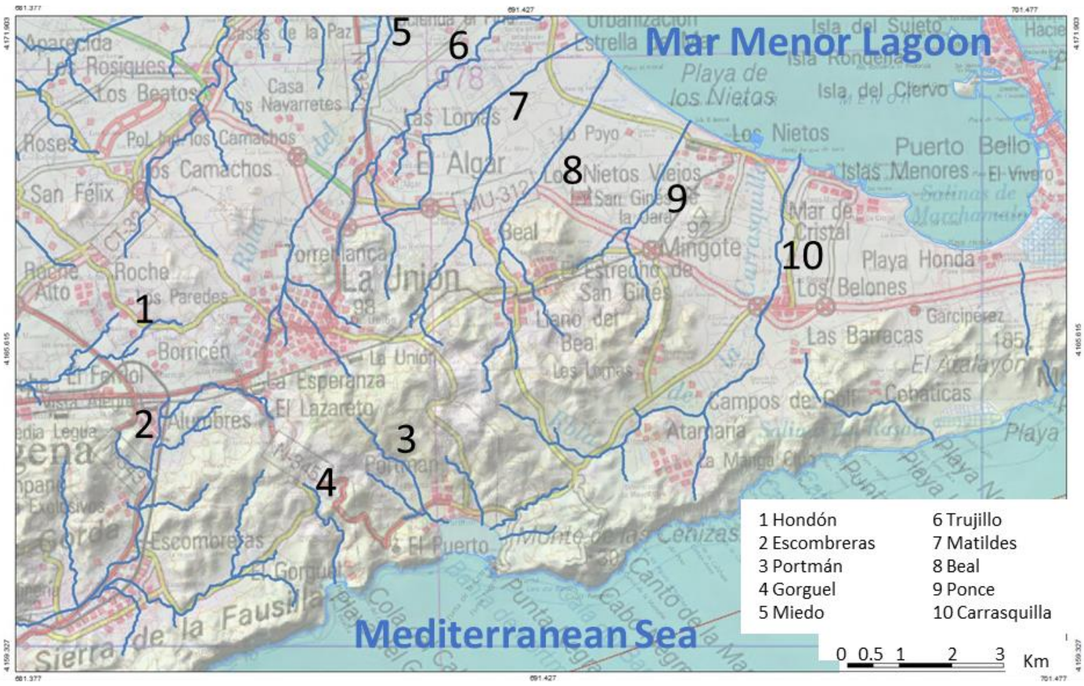

Figure 2.3. Watercourses in the mining district of Cartagena-La Unión (CHS, 2021).

The mining district is in an area rich in biodiversity and priority habitats and that hosts some protected natural areas. The most important is the Natural Park of Calblanque, Monte de las Cenizas y Peña del Águila (BORM, 1995) (Fig. 2.4), which was also catalogued as Site of Community Importance in the Nature 2000 Network. The native vegetation mainly consists in xerophytic Mediterranean shrubs, dwarf shrubs and perennial grasses (e.g., *Anthyllis cytisoides*, *Atriplex halimus*, *Helichrysum decumbens*, *Lygeum spartum*, *Pistacia lentiscus*, *Rosmarinus officinalis*, *Stipa tenacissima*) and small forests of pine trees (*Pinus halepensis*) from afforestation. Several endemic plant species appear in the area such as *Limonium carthaginense* and *Teucrium carthaginense*, and others that are threatened and protected by regional laws (e.g., *Chamaerops humilis*, *Lafuentea rotundifolia*, *Phyllirea angustifolia*, *Rhamnus alaternus*) (BORM, 2003). Of particular interest is *Tetraclinis articulata*, a Cupressaceae tree whose distribution area in Europe is restricted to this mountain range (Alcaraz, 2017), and that is included in the red list of threatened species (Sánchez Gómez et al., 2011).

Figure 2.4. Natural Park of Calblanque, Monte de las Cenizas y Peña del Águila.

The landscape in the mining district and its surroundings have been strongly transformed by the intense use of the territory. To the north of the mining district is the agricultural plain of the Campo de Cartagena (Fig. 2.5), one of the main horticultural production areas in Europe with ≈50,000 ha of irrigated land (Díaz-García, 2021). To the northeast is located the Mar Menor, the largest coastal saline lagoon of the Mediterranean basin (Conesa and Jiménez-Cárceles, 2007). It is separated from the Mediterranean Sea by a narrow sandy belt called La Manga (Fig. 2.5). Both the Mar Menor lagoon and La Manga have experienced an intense urban development in the last decades due to the high tourist activity in the area. In addition, the Mar Menor undergoes a severe eutrophication process due to the nutrient-enriched waters it receives from the agricultural fields of the Campo de Cartagena (Álvarez-Rogel et al., 2020).

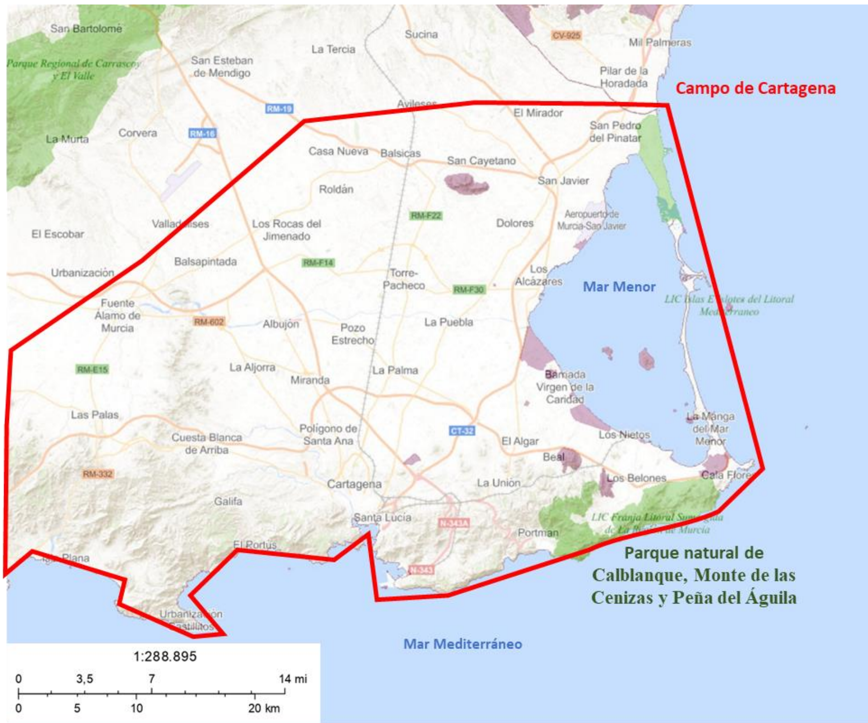

Figure 2.5. Campo de Cartagena and Mar Menor lagoon (IGME, 2021).

2.2. History and impacts of the mining activities

The territory has a long history of mining exploitation for more than 2000 years. Ore deposits were exploited by different civilizations in the past, since the Iberian period to the Phoenicians and Carthaginians, following by Romans. The principal metals extracted were Pb, Zn and Fe obtained from sulfide minerals such as galena (PbS), blende (ZnS) and pyrite (FeS_2), but also from carbonates such as cerussite ($PbCO_3$), and iron oxides, hydroxides, sulphates, and others (Oen et al., 1975; Conesa et al., 2008b; Conesa and Schulin, 2010). Until the second half of the 20th century mining activities were mainly based on small underground exploitations. However, from this period onwards, the development of modern metallurgy, as well as smelting and flotation techniques in refineries, allowed the extraction and processing of higher ore quantities. Between 1950s and 1991 huge amounts of metal ores were processed leading to the production of thousands of millions of tons of mine wastes. This led to the impacts of mining activities becoming much more intense in the area (Fig. 2.6). Some authors estimated that the quantity of rocks extracted during this time was comparable to that excavated during the previous period of more than 2000 years (Manteca and Ovejero, 1992). Mining increased until the 1980s when different problems in the mining sector such as the fall of the metal prices, the tightening of the mining activity laws or the scarcity of mineral reserves in the area, as well as the greater concern of environmental problems in the public opinion, caused a strong crisis in the mining sector of the region (Conesa et al., 2008b). Finally, in 1991, the last mining exploitations stopped their activity, and the last mines were closed (Conesa and Schulin, 2010).

Figure 2.6. Mining impacts in the mine district.

The drastic changes suffered in the landscape remain nowadays in the area due to the general absence of restoration projects following the cessation of the mining activities. The excavation of huge amounts of materials in open cast mines led to the existence of steep slopes in large quarries that generally reached the groundwater level, with vertical rock walls hostile for vegetation growth (Fig. 2.7) (Conesa and Schulin, 2010). The oxidation of underground sulfide materials after excavations led to pH drops in some areas and water acidification processes (Alcolea, 2015), which increase the solubility of metals and the environmental risks (Adriano, 2001).

The main types of mining wastes and abandoned structures that can be found in the area were classified by García-García (2004) as mine spoils, mine tailings, mine wastes in sea, gravimetric spoils, underground spoils, gossans, high size wastes and smelting wastes (Table 2.1; Fig. 2.7). Moreover, mining activity required the construction of some buildings and/or structures (e.g., chimneys, lifts, bridges), which also remain in the area and could be an interesting cultural heritage with some restoration (Fig. 2.7).

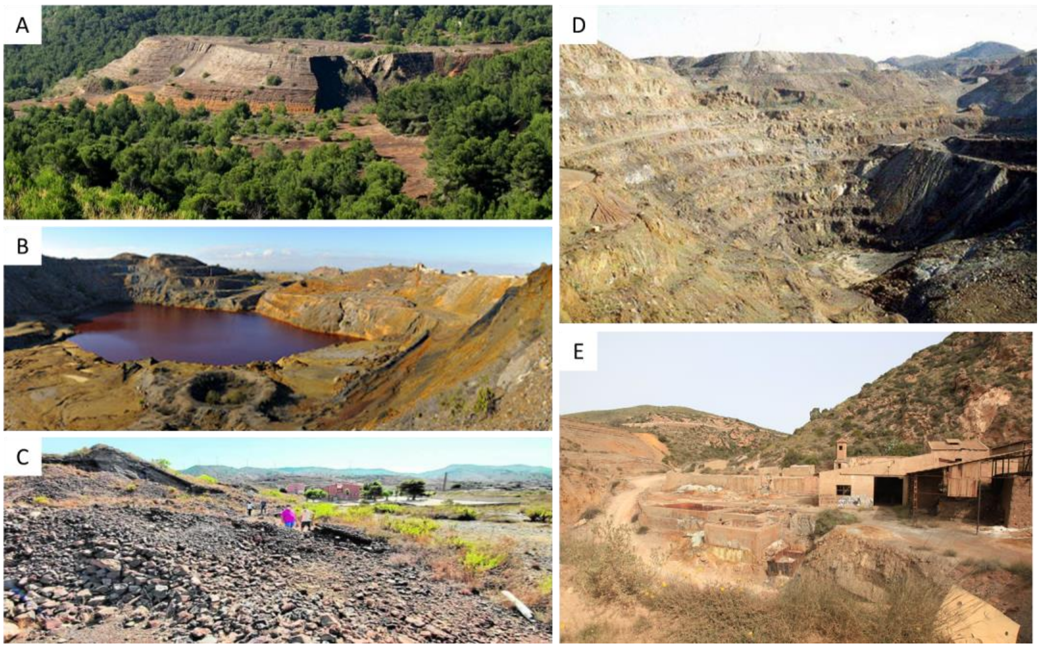

Figure 2.7. Wastes and structures in the mining district of Cartagena-La Unión: A) mine tailing; B) open pit; C) high size wastes; D) quarry; E) mine buildings.

As shown, mine tailings (average 20 m height and surface ≈40,000-80,000 m^2) are one of the main structures containing mine wastes (Fig. 2.7). Most of them were abandoned without any restoration when the mines were progressively closed. Nowadays, 89 mine tailings that store a total volume of ≈23 Mm^3 of mine wastes remain on site (Table 2.1; Fig. 2.8). Although landslides accidents have been not common (the last occurred in 1972), abandoned tailings can be considered the most hazardous structures from the point of view of their high metal(loid) levels and risks of dispersion. So far scarce attempts have been made to restore these structures and many of them remain bare until today, becoming a source of potentially toxic elements (PTEs) to the surroundings including villages, watercourses, beaches, agricultural lands and natural areas such as the Mar Menor lagoon (Conesa and Schulin, 2010; Robles-Arenas and Candela, 2010; Sánchez-Bisquert et al., 2017; Blondet et al., 2019). Some of these mine tailings have been spontaneously colonized by the surrounding native vegetation, shaping a patchy structure with ≈20-30% cover of the total tailings surface (Fig. 2.9).

Table 2.1. Types of mine waste, number of structures, total volume of wastes and total area affected in the mining district of Cartagena-La Unión (García-García, 2004).

Type of wastes	No. of structures	Volume (Mm^3)	Area (km^2)
Mine spoils	32	136	4.21
Mine tailings	89	23	2.18
Mine wastes in sea	3	25	0.83
Gravimetric spoils	119	3.73	0.65
Underground spoils	176	3.01	0.48
Gossans	11	6.93	0.26
High size wastes	1	0.59	0.06
Smelting wastes	19	0.66	0.13
Excavation materials from wells	1902	0.51	0.02
Total	**2352**	**199**	**8.82**

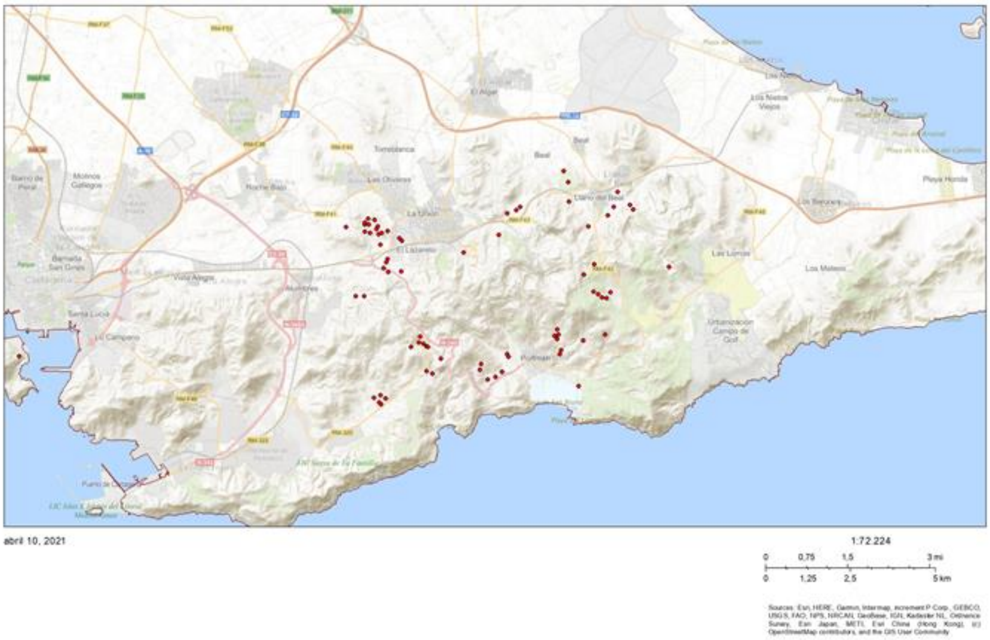

Figure 2.8. Mine tailings distribution in the mining district of Cartagena-La Unión (IGME, 2021).

Figure 2.9. Mine tailing partially covered by the vegetation.

As explained in Chapter 1, metal(loid) mine tailings are inhospitable environments for biota, but their characteristics and potential risks vary depending on the type of mine wastes they store (Conesa et al., 2008a). Acidic mine tailings can reach pH values ≈3, which confer them high metals solubility. This extreme acidity can be reached when some sulfide minerals such as pyrite are oxidized upon air exposition. Usually, these tailings also show high salinity and are prone to generate acid drainage water. Neutral/basic mine tailings show pH values ≈7 or even higher, which reduces metals solubility but, at the same time, may increase the solubility of some metalloids such as As. In neutral tailings salinity is generally lower and acidic drainage water is not generated. The formation of secondary minerals such as gypsum in mine tailings is a common process when sulphates from sulfide oxidation are combined with Ca. On many occasions, different types of materials can be found in the same tailing due to the heterogeneity of wastes that were stored (Fig. 2.10) (Pellegrini et al., 2016).

Figure 2.10. Layered mine tailing.

Among the places that have suffered the greatest impacts from mining activities is the Bay of Portmán, the most polluted bay on the Mediterranean coast (Fig. 2.11) (Martinez-Frias, 1997). Between 1957 and 1990, more than 57 million tons of mine waste were spilled into the bay as result of the activity of the multinational company Peñarroya

España S.A., which was the most important company in the area during this intensive mining period. This caused an environmental disaster with harmful consequences as the clogging of the bay, with the corresponding displacement of the coastline of about 700 m towards the Mediterranean Sea (Fig. 2.11), or the spread of metal(loid) pollution to 10 km^2 of the continental shelf (Mestre et al., 2017; Pérez-Sirvent et al., 2018; Gambi et al., 2020).

Figure 2.11. Mining impacts in Portmán bay.

Another area highly affected by the dispersion of polluted mine wastes is the Mar Menor lagoon (Conesa and Jiménez-Cárceles, 2007; Romero et al., 2020). Both the location of the mine tailings near the head of the watercourses (Fig 2.3) and the torrential rain regime of the area have facilitated and continues to facilitate the transport of mine wastes to distant areas (Martín-Crespo et al., 2020). Several works have described the presence of high levels of metal(loid)s in the sediments of different watercourses or in the beaches and coastal salt marshes of the Mar Menor (Robles-Arenas et al., 2006; Jiménez-Cárceles et al., 2008; Navarro et al., 2008), which have accumulated over decades (Fig. 2.12). The soils of some of these wetlands reach concentrations of Zn, As and Pb of more than 62,000 mg kg^{-1}, 700 mg kg^{-1} and 16,800 mg kg^{-1}, respectively (Jiménez-Cárceles et al., 2008; María-Cervantes et al., 2009; Conesa et al., 2011). Moreover, several authors have described the accumulation of metal(loid)s in different living organisms in the Mar Menor lagoon (Marín-Guirao et al., 2005a; b; María-Cervantes et al., 2009; Robles-Arenas et al., 2006; Conesa and Jiménez-Cárceles, 2007; León and Bellido, 2016; Martín-Crespo et al., 2020).

Figure 2.12. Lo Poyo salt marsh in the Mar Menor lagoon.

Pollution dispersion from the mining area by water and wind erosion also reaches cities, recreative areas and even croplands (Conesa et al., 2010; Moreno-Brotons et al., 2010; Jiménez-Martínez et al., 2016; González-Alcaraz and van Gestel, 2016; Sánchez-Bisquert et al., 2017; Blondet et al., 2019; Martín-Crespo et al., 2020). Nevertheless, due to the basic pH of soils, the solubility of metals is usually low and the uptake by crops is generally limited (Conesa et al., 2010). However, chronic exposure to polluted airborne pollutants from tailings can lead to health risks for the surrounding populations (Conesa and Schulin, 2010; Sánchez-Bisquert et al., 2017; Blondet et al., 2019).

2.3. Current situation relative to the restoration of the mining area

At present, the mine waste problematic in the former mining district of Cartagena-La Unión remains unsolved. Scarce attempts to remediate or restore the different areas affected by mining pollution have been developed (Conesa and Schulin, 2010). Since 2017, there has been a significant increase of popular movements in the mining area. Local organizations such as the Association of People Affected by Mine Wastes from El Llano del Beal or the Portman Neighborhood League demand that the Regional Government implement urgent actions to stop and/or reduce the health risks of local populations.

One of the most famous restoration plans in the mining district area concerns the Portmán Bay, which was designed to respond to the historic request of the local population to restore the village bay (Banos-González et al., 2017). In particular, the restoration plan consisted of dredging the mine wastes from the bay to recover its original shoreline. The dredged mine wastes would be transported to an old quarry in the area where they would be sealed. Moreover, the partial restoration of the bay beach was planned as well as the construction of a recreational and fishing port (Sánchez, 2019). The works began in 2016 but, after the ruling handed down by the National High Court, the restoration project was stopped in 2019 (Sánchez, 2019). The standstill of the works was due to the conflict between two companies related to the public restoration tender (Díaz, 2019). In addition, some technical failures were detected in the restoration plan related to the treatment of mine wastes, which could pose a risk to the environment and the health of the local population (Lorente and García, 2019; Díaz, 2019; Sánchez, 2019). The current situation has led to a feeling of dissatisfaction in Portman's residents (Ojeda, 2021).

In relation to the Mar Menor lagoon, different Regional regulations have recently been developed to protect its ecological and socioeconomic value (BORM, 2018, 2019, 2020). In order to avoid the spread of metal(loid) pollution, these regulations stablish the necessity of determining which mining structures and soils polluted by mine wastes imply a high environmental risk for the lagoon and their surroundings. In this sense, the current intervention strategy from the Regional Government, to be implemented between 2018 and 2028, seeks to establish a framework of preventive policies that includes actions aimed at the restoration and prevention of sites affected by metal mining (Environmental Recovery Plan for Soils Affected by Mining; PRASAM, 2018-2028). This plan focusses on promoting knowledge of the current situation of mining district, improving in the management of sites affected by mine wastes, and promoting the environmental and landscape recovery of the affected areas. During the execution period of the plan, it will be carried out the integral restoration of some specific areas selected as those with the greatest environmental risk, structural failures, and pollution dispersal problems. PRASAM also intends to include the history and culture of the mining district into the tourist services of the area, adapting for example some old mining facilities for touristic use. This approach could be a good option to put in value the history and cultural heritage of the area and improve its socio-economic situation (Conesa et al., 2008b).

2.4. References

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CHAPTER 3

Background and objectives

3.1. Background

The present PhD Thesis has been developed within the framework of the research line of the R&D Group Environmental Soil Science, Chemistry and Agricultural Technology, of the Universidad Politécnica de Cartagena (UPCT), on soils affected by metal mining activities in the former mining district of Cartagena-La Unión and nearby areas, and the study of phytomanagement options for remediation.

Between 2002 and 2007, a series of R&D projects were carried out funded by the Spanish Ministry of Education, Culture and Sport (REN2001-2142/GLO), the Spanish Ministry of Science and Technology (CGL2004-05807/BOS) and the Fundación Séneca of the Murcia Region (08739/PI08). These projects were mainly developed in the topographically lower areas of the territory, close to the Mar Menor lagoon, to where the sediments polluted with metal(loid)s have been and continue to be transported by erosion from the mining area. The results of these projects revealed the presence of high concentrations of metal(loid)s in salt marsh soils, the first shoreline sediments of the lagoon and in some molluscs and plants in the area (Álvarez-Rogel et al., 2004; Carrasco et al., 2006; Jiménez-Cárceles et al., 2008; María-Cervantes et al., 2009, 2010, 2011; Conesa et al., 2011). After this, the works developed under experimental greenhouse conditions for the R&D projects CGL2007-64915 (2008-2010, Spanish Ministry of Science and Technology) and CGL2010-20214 (2011-2014, Spanish Ministry of Economy and Competitiveness) demonstrated that the effects of soil amendments (liming) on metal(loid) dynamics are modulated by the presence/absence of vegetation and the oxidation status of the soil. Therefore, it is paramount to know the specific conditions of the soil-water-plant system in order to employ the most appropriate management strategies (González-Alcaraz et al., 2011, 2013a, 2013b. 2013c; González-Alcaraz and Álvarez-Rogel, 2013).

During the following years, the R&D project CTM2011-23958 (2012-2015, Spanish Ministry of Economy and Competitiveness) was carried out in and around the mine tailings themselves. It provided detailed information on: i) the soil gradients that condition the establishment of pioneer vegetation in mine wastes (Conesa et al., 2011; Párraga-Aguado et al., 2013); ii) the ecological functionality in relation to different types of plant species and its importance for future revegetation plans (Parraga-Aguado et al., 2014b); iii) the importance of facilitation/competition mechanisms in phytomanagement techniques in terms of phytostabilization (Parraga-Aguado et al., 2014c); iv) the validity of isotopic tools for the description of ecophysiological processes in plant species growing in mine tailings (Parraga-Aguado et al., 2014a, 2014b, 2014c); v) the need to determine the biogeochemical cycles of the metal(loid)s present in the system to properly evaluate the potential transfer risks in the food chain (Parraga-Aguado et al., 2014a). More recently, the R&D project CGL2017-82264-R (2018-2020, Spanish Ministry of Economy and Competitiveness) has evaluated the microbial composition of mine tailings soils and its relationship with soil functionality (Risueño et al., 2020a, 2020b). Regarding the toxic effects of soils affected by mine wastes, ecotoxicity bioassays with different soil invertebrate species were also carry out. The results revealed that: i) soils with acidic pH, high salinity, and available metal(loid)s are more toxic to soil invertebrates (González-Alcaraz and van Gestel, 2015; González-Alcaraz et al., 2015); ii) certain changes in air temperature and soil moisture conditions can alter soil invertebrates' performance, but also soil biogeochemical behaviour, leading to changes in soil metal(loid) bioavailability and invertebrates' metal(loid) bioaccumulation, increasing the toxicity risks of metal(loid)-polluted soils to soil invertebrates (González-Alcaraz and van Gestel, 2015, 2016a, 2016b; González-Alcaraz et al., 2015, 2018); iii) these effects do not follow a specific pattern over successive invertebrate generations (Barmentlo et al., 2017).

On the other hand, the R&D Group carried out characterization studies of urban solid refuse (USR), its composting and its addition for the improvement of degraded agricultural and mining soils. These works were mainly developed through contracts with the recycling company Pedro Segura S.L. (2010-2012) and the research line established with the Cartagena City Council (2016-2017). Furthermore, the use of USR and biochar as amendments to improve the edaphic conditions of areas affected by metal(loid) mine wastes has also been studied in recent R&D projects (Nationals: CGL2013-49009-C3-1-R, 2014-2017, and CGL2014-54029-R, 2015-2017; Regional: 19248/PI/14, 2015-2018) (Álvarez-Rogel et al., 2018; Martínez-Oró et al., 2019; Conesa and Párraga-Aguado, 2021a; 2021b). Moreover, the short-term response of microbial communities' composition in a mine tailing soil amended with biochar and manure compost has been assessed (Risueño et al., 2021).

To gain more insight about the functional and ecotoxicological aspects of mine tailings soils, to highlight the importance of vegetation patches for phytomanagement, and the usefulness of biochar and USR as suitable amendments to trigger spontaneous vegetation colonization, the R&D project CGL2016-80981-R (*Functionality and resilience of soils* *polluted by mining wastes under climate change conditions in Mediterranean environments: ecotoxicological aspects and the use biochar for remediation*) was developed between 2017 and 2020 funded by the Spanish Ministry of Science, Innovation and Universities. Part of the results of this project are reflected in this PhD Thesis.

3.2. Objectives

The **general objective** of the PhD Thesis was to deepen the knowledge of physical, physicochemical, functional and ecotoxicological aspects in soils of abandoned metal(loid) mine tailings from Mediterranean semiarid environments and their relationship with spontaneous plant colonization, and whether the addition of organic amendments contributes to improving these ecosystems by promoting the recovery of soil functionality and triggering the spontaneous colonization of vegetation.

To achieve this general objective, three **specific objectives** were raised:

1. To evaluate in which degree soil conditions can be modified following spontaneous vegetation colonization in abandoned metal(loid) mine tailings, and to provide evidence about the interest of this colonization for the phytomanagement of these structures.

2. To assess to what degree spontaneous plant colonization of abandoned metal(loid) mine tailings led to functional soil improvement and to identify, if possible, a critical level indicating that this functionality was moving towards that of the natural vegetated soils from the surrounding areas.

3. To assess the effectiveness of an organic amendment composed of biochar from pruning trees and compost from USR to ameliorate the conditions of barren metal(loid) acidic mine tailings soils and if these effects persist seasonally over a year, and whether the organic amendment favors spontaneous plant colonization.

The PhD Thesis has been structured as follows:

Part I. General aspects and methodology.

- Chapter 1. Introduction.
- Chapter 2. Study area.
- Chapter 3. Background and objectives.
- Chapter 4. Materials and methods.

Part II. Results and discussion.

- Chapter 5. Evidence supporting the value of spontaneous vegetation for phytomanagement of soil ecosystem functions in abandoned metal(loid) mine tailings.
- Chapter 6. The relationships between functional and physicochemical soil parameters in metal(loid) mine tailings from Mediterranean semiarid areas support the value of spontaneous vegetation colonization for phytomanagement.
- Chapter 7. Biochar and urban solid refuse ameliorate the inhospitality of acidic mine tailings and foster effective spontaneous plant colonization under semiarid climate.

Part III. Conclusions.

- Chapter 8. General conclusions and management recommendations.

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CHAPTER 4

Materials and methods

This chapter includes a detailed description of the field sampling and monitoring carried out and the soil analytical methods applied, and is organized as follows: 1) description of selected metal(loid) mine tailings and study environments within them and in the surrounding forests; 2) vegetation inventory methods and calculation of ecological indexes; 3) field work campaign in summer 2017 including vegetation monitoring and sampling and evaluation of physical, physicochemical and biological soil indicators; 4) field work campaign in spring 2018 including vegetation monitoring and sampling and evaluation of soil functional aspects; 5) organic amendment addition experiment in mine tailing soils; 6) soil analysis methods.

4.1. Description of mine tailings and study environments

The study was conducted in two mine tailings ≈2000 m apart built by mid-60's to store wastes from mines exploiting galena ore (IGME, 2002), and in the surrounding forest areas (Fig. 4.1).

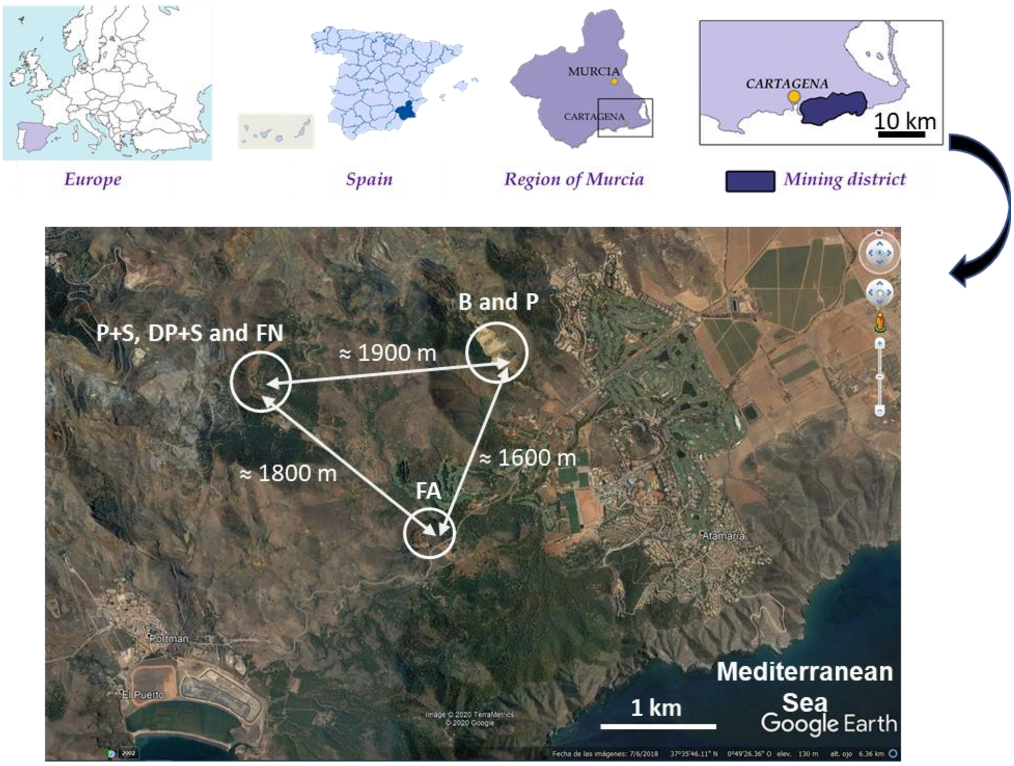

Figure 4.1. Location map of the study area (SE Spain) and environments. B: bare soils; P: small groups of *Pinus halepensis* trees; P+S: isolated *P. halepensis* trees + plants under the canopy; DP+S: dense patches with *P. halepensis* trees + plants under the canopy; FN: forest next to the mine tailings; FA: forest away from the mine tailings.

Both tailings were abandoned ≈40 years ago and have been partially colonized by native vegetation in a patchy structure with covers between ≈20% and ≈50%. In tailing 1 (total surface area ≈6.4 ha) the plots were located in a zone of ≈0.9 ha, and in tailing 2 (total surface area ≈1.5 ha) in two areas of ≈0.1 and ≈0.3 ha, respectively (Figs. 4.2 and 4.3). Micro-topographical depressed areas within the tailings with visual evidence of water accumulation were avoided. The tailings are located at a similar altitude (≈170-200 m a.s.l.) and embedded in small valleys NW to SE facing. One environment devoid of vegetation and five different types of vegetated environments (according to their physiognomy and plant composition) were selected in April-May 2017. Because we aimed to evaluate whether soil functionality in mine tailing's vegetation patches was comparable to that of surrounding pine forests, *Pinus halepensis* was present in all the vegetated study environments, but with different species under canopy. The six environments studied were:

A) Four inside the mine tailings (Figs. 4.2 and 4.3):

1. Bare soils (B).

2. Patches with small groups of *P. halepensis* trees ≈2.5-5 m high growing scattered (P).

3. Patches formed by isolated *P. halepensis* trees >≈4-5 m high growing scattered with shrubs and herbs under the canopy (P+S).

4. Dense patches including several *P. halepensis* trees >≈4-5 m high and shrubs and herbs under the canopy (DP+S).

B) Two outside the mine tailings (Figs. 4.3 and 4.4):

5. Forest located next to the mine tailings with *P. halepensis* trees >≈5 m high and shrubs and herbs under the canopy (FN).

6. Forest located away from the mine tailings (≈1600-1800 m) with *P. halepensis* trees >≈5 m high and shrubs and herbs under the canopy (FA).

Four plots (2 m x 2 m) were established within each study environment.

In addition, in bare soils, four additional plots were established for the soil amendment addition experiment (AB).

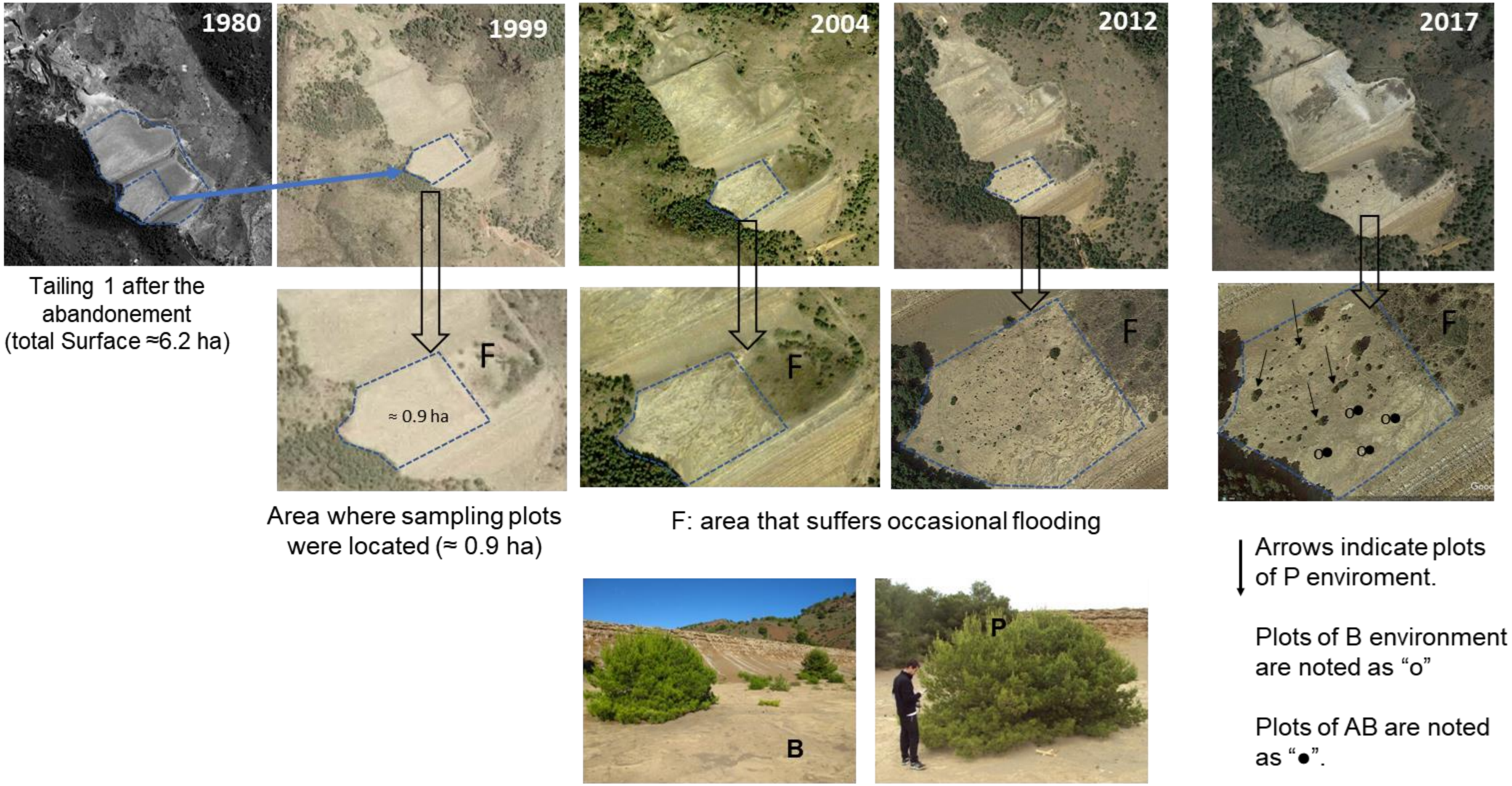

Figure 4.2. Views of mine tailing 1 where B (bare soils) and P (patches with small groups of *Pinus halepensis* trees growing scattered) environments were located. Images from Fototeca Digital del Centro Nacional de Información Geográfica (http://fototeca.cnig.es/) and Google Earth.

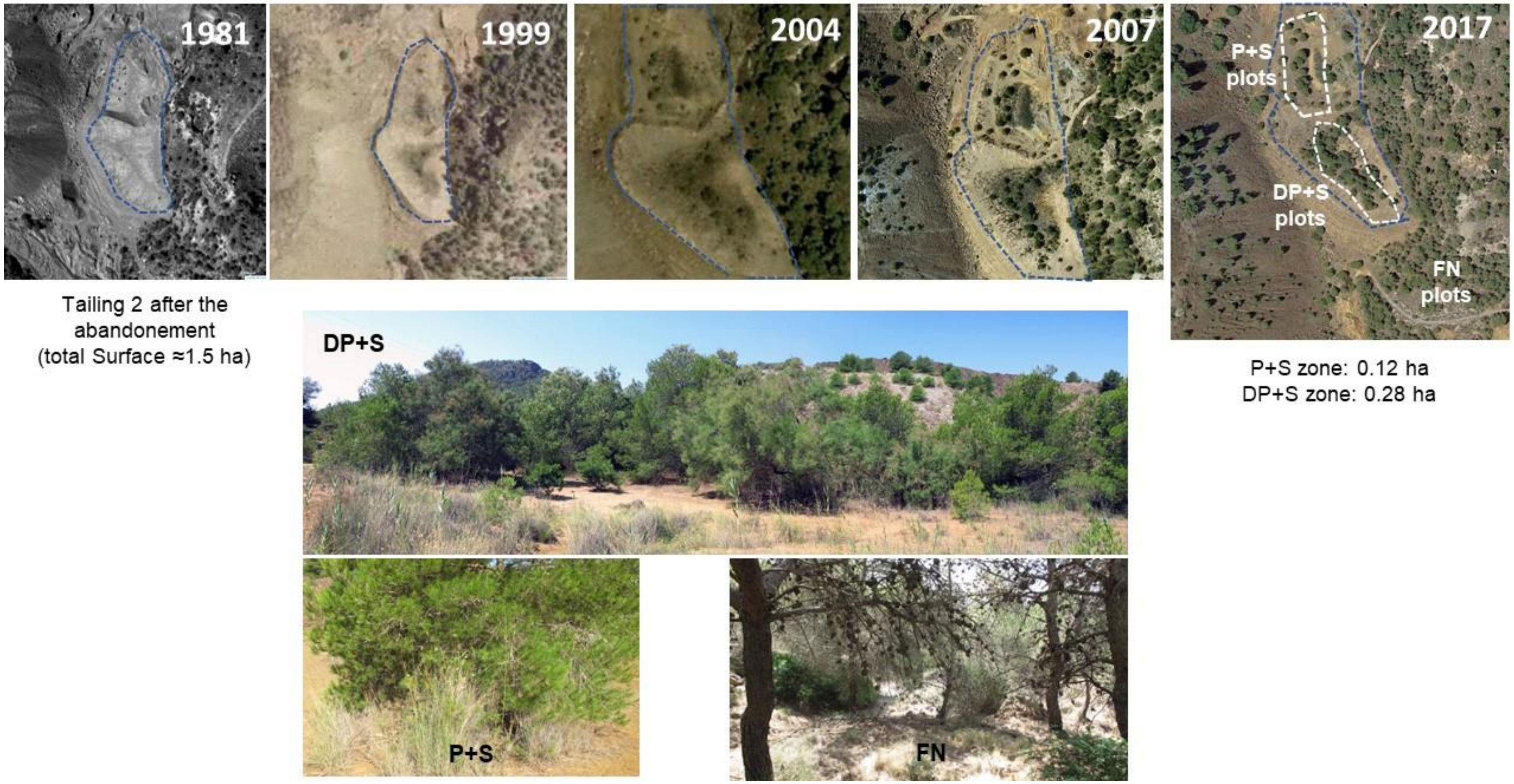

Figure 4.3. Views of mine tailing 2 where P+S (patches formed by isolated *Pinus halepensis* trees growing scattered with shrubs and herbs under the canopy) and DP+S (dense patches including several *P. halepensis* trees and shrubs and herbs under the canopy) environments were located, and location of FN environment (forest located next to the mine tailings with *P. halepensis* trees and shrubs and herbs under the canopy) outside the tailing. Images from Fototeca Digital del Centro Nacional de Información Geográfica (http://fototeca.cnig.es/) and Google Earth.

Figure 4.4. View of the forest located away from the mine tailings (FA environment).

4.2. Methods for vegetation inventory and calculation of ecological indexes

For each study environment and plot established, the plant cover (visual estimation) and the number of species and individuals per species were recorded (n = 4). Moreover, the family, life form (Table 4.1), functional group (Paula et al., 2009; Colin et al., 2019), and functional role in relation with mine tailing colonization (pioneer or nurse) (Navarro-Cano et al., 2018) were identified for each plant species. With the obtained data the Margalef index –R– (richness of the plant community; Margalef, 1958), the Shannon-Weaver index –H'– (heterogeneity of the plant community; Shannon and Weaver, 1963) and Pielou evenness index – J´ were calculated as follows:

$$R = \frac{(S-1)}{lnN} \quad \text{(Equation 1)}$$

$$H' = -\sum_{i=1}^{S} p_{i} \times log_2 p_i \quad \text{(Equation 2)}$$

$$J' = \frac{H'}{log_2 S} \quad \text{(Equation 3)}$$

where S is the number of species in the plot, N is the total number of individuals in the plot, p_i is the relative frequency of the species i in the plot, and Σp_i equals 1.

Table 4.1. Description of the vegetation life forms (Ellenberg and Müller-Dombois, 1967).

Life form	**Description**
Therophytes	Annual plants
Geophytes	Plants with periodic reduction of the complete shoot system to storage organs that are imbedded in the soil
Hemicryptophytes	Plants with periodic shoot reduction to a remnant shoot system that lies relatively flat on the ground surface
Chameophytes	Plants whose mature branches or shoots remains perennially within 25-50 cm above ground surface, or plants that grow taller than 25-50 cm, but whose shoots die back periodically to that height limit
Phanerophytes	Plants that can grow taller than 25-50 m, or whose shoots do not die periodically to that height limit
Microphanerophytes	Plants that grow <2 m tall, or whose shoots do not die periodically to that height limit
Nanophanerophytes	Plants that grow between 2-5 m tall, or whose shoots do not die periodically to that height limit
Macrophanerophytes	Plants that grow between 5-50 m tall, or whose shoots do not die periodically to that height limit

4.3. Field work campaign in summer 2017

4.3.1. Vegetation

Vegetation was inventoried in summer 2017 (June 2017) following the methodology described in Section 4.2 (plant species, number of individuals per species, family, life form, functional role as pioneer/nurse plant in the colonization process, Margalef index, and Shannon-Weaver index).

4.3.2. Soil sampling

In summer 2017, five soil subsamples were randomly collected per plot with a shovel (upper ≈15 cm) and mixed to get a representative, composite sample per plot. Aliquots of these samples were air-dried prior to soil structure and colour description, and the rest of the sample was sieved (2 mm) and stored at room temperature. In parallel, additional composite soil samples were extracted with sterilized spoons (upper ≈15 cm), placed in Falcon tubes, taken to the laboratory on ice inside a portable cooler and stored at -20 °C for water soluble determinations and microbiological analyses. In addition, one undisturbed soil core (98 cm^3) was collected within each plot for soil bulk density determination.

4.3.3. Soil physical and physicochemical indicators

Undisturbed soil cores were used to calculate the soil bulk density (n = 4 per study environment). Soil structure and color pattern were described in soil dried but not sieved samples.

In the dried sieved samples particle size distribution, cation exchange capacity (CEC) and NH_4^+- soluble metal(loid)s were analyzed (n = 4 per study environment). After this, soil aliquots were grounded in an Agatha mortar for measuring total organic carbon (TOC), total nitrogen (TN), total metal(loid)s (T-As, T-Cd, T-Fe, T-Mn, T-Pb, and T-Zn) and total CaO_3 (n = 4 per study environment). An aliquot of the frozen sample was used to perform water soluble extracts (1:2.5 soil:water suspensions; 2 h shaking) after thawing at room temperature (n = 4 per study environment). The parameters measured in the extracts were pH, electrical conductivity (EC) and water soluble salts (Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, SO_4^{2-}), water soluble metal(loid)s (W-As, W-Cd, W-Fe, W-Mn, W-Pb, and W-Zn) and water soluble organic carbon (WSOC).

4.3.4. Soil biological indicators

Microbial biomass carbon (MBC) and enzyme activities (dehydrogenase and β-glucosidase) were evaluated in aliquots of the frozen samples after thawing at room temperature (n = 4 per study environment). Ecotoxicity bioassays with the soil invertebrate *Enchytraeus crypticus* were performed in dried sieved samples (n = 4 per study environment). Soil respiration (CO_2 emission), organic matter decomposition (tea bag index, TBI) and feeding activity of soil dwelling organisms (bait lamina sticks) were estimated *in situ* in each plot.

4.4. Field work campaign in spring 2018

4.4.1. Vegetation

Vegetation was inventoried in spring 2018 (April 2018) following the methodology described in Section 4.2 (plant cover, plant species, number of individuals per species, family, life form, functional group, functional role as pioneer/nurse plant in the colonization process, Margalef richness index, Shannon-Weaver heterogeneity index, and Pielou evenness index).

4.4.2. Soil sampling

In spring 2018, five soil subsamples were randomly collected per plot with a shovel (upper ≈15 cm) and mixed to get a representative, composite sample per plot. Aliquots of these samples were air-dried, sieved (2 mm mesh) and stored at room temperature. In parallel, additional composite soil samples were extracted with sterilized spoons (upper ≈15 cm), placed in Falcon tubes, and taken to the laboratory on ice inside a portable cooler for water-soluble determinations and microbiological analyses. Some Falcon tubes were stored at -20 °C and the rest were frozen with liquid N and stored at -80 °C.

4.4.3. Soil physical and physicochemical indicators

In the dried sieved samples particle size distribution was analyzed (n = 4 per study environment). After this, soil aliquots were grounded in an Agatha mortar for measuring TOC, TN, total metal(loid)s (T-As, T-Cd, T-Fe, T-Mn, T-Pb, and T-Zn) and total CaO_3 (n = 4 per study environment). An aliquot of the frozen sample was used to perform water soluble extracts (1:2.5 soil:water suspensions; 2 h shaking) after thawing at room temperature (n = 4 per study environment). The parameters measured in the extracts were pH, EC, water soluble salts (Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, SO_4^{2-}), water soluble metal(loid)s (W-As, W-Cd, W-Fe, W-Mn, W-Pb, and W-Zn), WSOC, and water soluble organic nitrogen (WSON).

4.4.4. Soil microbiological indicators

MBC and β-glucosidase activity were evaluated in aliquots of the samples stored at -20 °C after thawing at room temperature (n = 4 per study environment). The metabolic activity and functional diversity of soil microorganisms (bacteria) was evaluated by means of the community-level physiological profile (CLPP) technique after thawing the aliquots frozen at -80 °C.

4.5. Field experiment of organic amendment addition in mine tailing soils

At the same time that the plots for each study environment were established (April-May 2017; see Section 4.1), an organic amendment addition experiment was initiated. For that, four additional plots (2 m x 2 m) were selected next to the plots of the B environment (bare soil plots). These four additional plots (AB), and the four B plots, were manually ploughed in the upper ≈15 cm (Fig. 4.5). Then, the four AB plots were amended at 3% dry weight with a 3:1 mixture of biochar and composted urban solid refuse –USR– (38.7 and 13.0 t ha^{-1}, respectively). The 3:1 proportion used was chosen based on data reported by other authors (e.g., Wu et al., 2016 and references cited therein). The 3% dose was based on previous experience (Párraga-Aguado et al., 2017; Álvarez-Rogel et al., 2018; Martínez-Oró et al., 2019) and laboratory trials to rise the pH of acidic barren soils to ≈7.5.

4.5.1. Organic amendment characteristics

The biochar used was a commercial biochar manufactured by the company Proininso S.A. (Málaga, Spain) from the pyrolysis of oak (forest woody biomass pyrolyzed by reactor/gasifier at >900 ºC and 0% oxygen content). The company is involved in the International Biochar Initiative and its biochar has previously been applied to restore mining soils (Rodríguez-Vila et al., 2014). The composted USR was provided by the municipal solid waste treatment plant of the Cartagena city (Spain). The main characteristics of both organic materials are given in Table 4.2. The biochar was alkaline, nearly decarbonated and poorly saline, with K^+ as the most abundant soluble ion. The USR was neutral, carbonated and saline, with high concentrations of soluble Cl^-, SO_4^{2-},

Na^+ and Ca^{2+}. The biochar had higher content of TOC but lower of total TN than the USR (≈4-fold higher and ≈3-fold lower, respectively). The TOC:TN ratio was ≈118 and ≈9 in the biochar and USR, respectively. WSOC and water total soluble nitrogen (WTSN) concentrations were higher in the USR (32-fold and 437-fold, respectively). These characteristics indicated much more recalcitrant organic matter in the biochar and higher content of labile organic compounds in the USR. Total metal(loid) concentrations were higher in the USR except for Mn (in mg kg^{-1}).

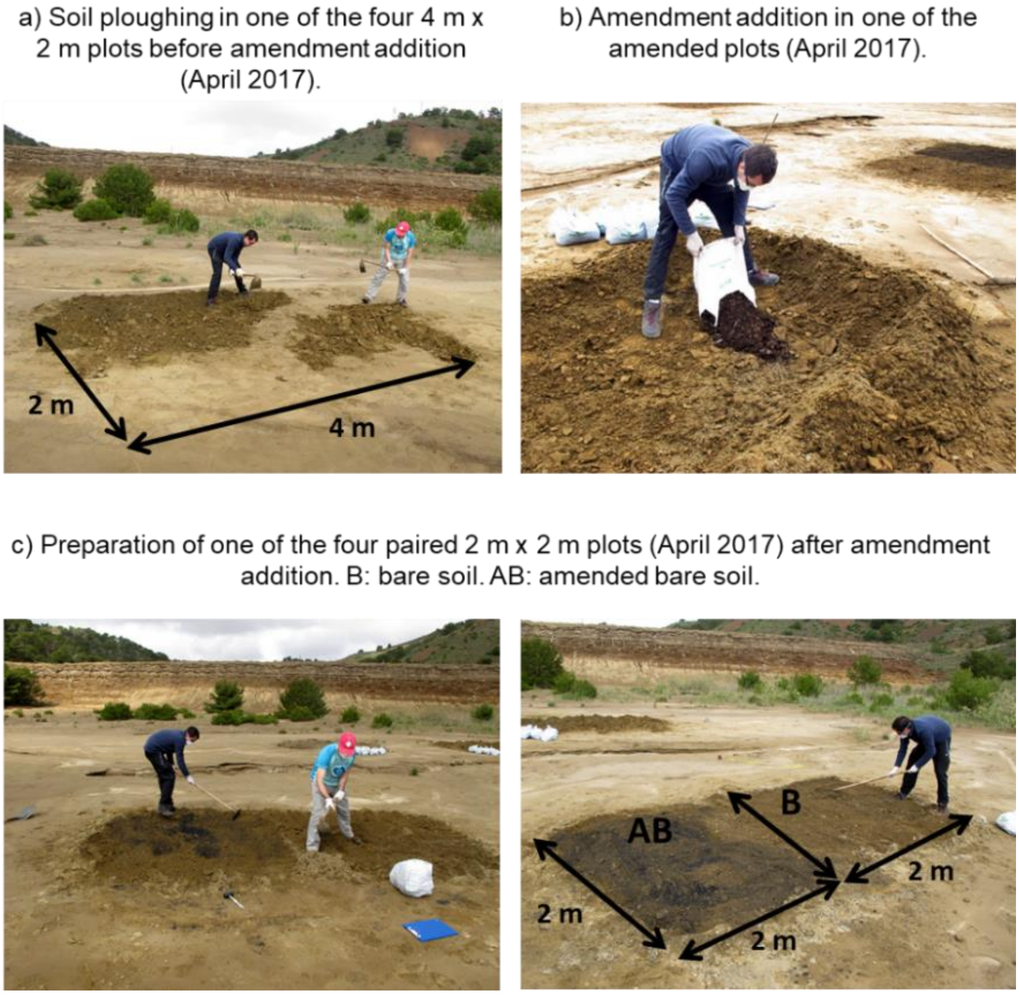

Figure 4.5. Preparation of the experimental plots for amendment addition.

Table 4.2. Characterization of biochar and urban solid refuse (USR) (average ± SE, n = 4). EC (electrical conductivity). TOC (total organic carbon). TN (total nitrogen). WSOC (water soluble organic carbon). WTSN (water total soluble nitrogen). T-metal(loid)s (total metal(loid) concentration). n.d. (not detected). All the values are expressed per dry matter.

Parameter	Biochar	USR
$CaCO_3$ (g kg^{-1}) [1]	3.03 ± 2.9	148 ± 2
TOC (g kg^{-1})	829 ± 0.4	206 ± 2
TN (g kg^{-1})	7.0 ± 0.05	22.2 ± 0.2
TOC:TN	118	9.27
pH [2]	9.90 ± 0.02	7.43 ± 0.05
EC (dS m^{-1}) [2]	2.65 ± 0.12	9.42 ± 0.10
Cl^- (g kg^{-1}) [2]	0.129±0.01	156±6.18
SO_4^{2-} (g kg^{-1}) [2]	0.108±0.006	136±6.54
Na^+ (g kg^{-1}) [2]	0.231±0.014	139±2.43
K^+ (g kg^{-1}) [2]	4.72±0.269	120±1.33
Ca^{2+}(g kg^{-1}) [2]	0.188±0.026	74.5±0.90
Mg^{2+}(g kg^{-1}) [2]	0.07±0.017	19.6±0.32
WSOC (mg kg^{-1}) [2]	788 ± 55	25266 ± 331
WTSN (mg kg^{-1}) [2]	8.66 ± 0.20	3787 ± 30
T-As (mg kg^{-1})	n.d.	31.2 ± 0.2
T-Cd (mg kg^{-1})	n.d.	57.6 ± 3.4
T-Fe (mg kg^{-1})	2516 ± 33	19872 ± 152
T-Mn (mg kg^{-1})	964 ± 13	471 ± 12
T-Pb (mg kg^{-1})	n.d.	617 ± 25
T-Zn (mg kg^{-1})	100 ± 1.4	1160 ± 13

[1] Bernard's calcimeter with 4N HCl (Muller and Gastner, 1971; Hulseman, 1996).

[2] Extraction in soil:water suspensions (1:5 w:v) (US Salinity Laboratory Staff, 1954) and concentrations measured as described in section 4.6

4.5.2. Soil sampling and field monitoring

Two months after ploughing and amendment application (June 2017), five soil subsamples were randomly collected from each plot (upper ≈15 cm) and placed in the same plastic bag to constitute a representative, composite sample per treatment for an initial evaluation (n = 4) (Table 4.3). Samples were extracted with a shovel and taken to the laboratory where they were air-dried, sieved (2 mm mesh) and stored at room temperature prior to laboratory analyses. Particle size distribution, total $CaCO_3$, CEC, TOC, TN and total metal(loid)s (T-As, T-Cd, T-Fe, T-Mn, T-Pb, and T-Zn) were determined. Ecotoxicity bioassays were also performed (Table 4.3).

Additionally, a seasonal monitoring program including soil sampling and analysis and *in situ* collection of soil and vegetation data were carried out (Table 4.3). In June 2017 (summer 2017), November 2017 (autumn 2017), January 2018 (winter 2018) and April 2018 (spring 2018) new composite soil samples were extracted with sterilized spoons (upper ≈15 cm), put in sterile Falcon tubes and taken to the laboratory on ice inside a portable cooler. Material in Falcon tubes was not sieved or dried. Some tubes were stored at -20 °C to measure TOC, MBC and dehydrogenase activity, and to perform soil:water suspensions to analyze pH, EC, water soluble salts (Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, SO_4^{2-}), water soluble metal(loid)s (W-As, W-Cd, W-Fe, W-Mn, W-Pb, and W-Zn), and WSOC (Table 4.3). The rest of the material was frozen with liquid nitrogen and stored at -80 °C to evaluate the community-level physiological profile (Table 4.3). In parallel, the decomposition of organic matter (TBI) and the feeding activity of soil dwelling organisms (bait lamina sticks) were evaluated seasonally *in situ* (Table 4.3).

At the end of the described seasonal monitoring in spring 2018, new composite soil samples were collected to carry out new ecotoxicity bioassays (Table 4.3). Undisturbed soil cores (98 cm^3) were also collected to measure bulk density and water retention capacity (Table 4.3). Additionally, soil CO_2 emission (soil respiration) was measured *in situ*. Soil bulk density and water retention capacity were not measured at the beginning because the soil was recently ploughed.

Plant species colonizing the plots were recorded throughout the study period. The number of individuals and the cover percentage (visual estimation) from each species were annotated at each seasonal sampling time. Additionally, vegetation data were newly recorded two years after finishing the seasonal sampling program (spring 2020).

Table 4.3. Monitoring program throughout the assay of soil amendment addition. The seasons in which each parameter was evaluated are indicated with an "X". CEC (cation exchange capacity). TOC (total organic carbon). TN (total nitrogen). T-metal(loid)s (total metal(loid) concentration). EC (electrical conductivity). W-metal(loid)s (water soluble metal(loid) concentration). WSOC (water soluble organic carbon). MBC (microbial biomass carbon). DH (dehydrogenase activity). CLPP (community-level physiological profile). TBI (tea bag index).

Parameter	**Season and year**			
	Summer 2017	**Autumn 2017**	**Winter 2018**	**Spring 2018**
Particle size distribution	X			
Total $CaCO_3$	X			
CEC	X			
TOC	X	X	X	X
TN	X			X
T-metal(loid)s	X			
pH	X	X	X	X
EC	X	X	X	X
Water soluble salts	X	X	X	X
W-metal(loid)s	X	X	X	X
WSOC	X	X	X	X
MBC	X	X	X	X
DH	X	X	X	X
CLPP	X	X		X
Bulk density				X
Water retention capacity				X
Ecotoxicity	X			X
Soil moisture	X	X	X	X
Soil temperature	X	X	X	X
TBI	X	X	X	X
Feeding activity	X	X	X	X
CO_2 emission				X

4.6. Sample analysis methods

4.6.1. Physical parameters

In undisturbed soil samples:

- **Soil bulk density**. Undisturbed soil cores were dried at 65 °C until constant weight to calculate the soil bulk density as the weight:volume ratio (Burke et al., 1986). Units of measure in g cm^{-3}.
- **Water retention capacity**. After dried, the cores were used to calculate the water retention capacity at 33 kPa (field capacity) in a 15-bar ceramic plate extractor (Soilmoisture Equipment Corp.). Units of measure in cm^3 cm^{-3}.

In soil air-dried samples:

- **Soil structure** (FAO, 2006) and **color pattern** (Munsell®, 1994) were described in soil dried samples before sieving.

In air-dried and sieved samples (2 mm):

- **Particle size distribution** according to the Bouyoucos densimeter method (Gee and Bauder, 1986).

4.6.2. Physicochemical parameters

In air-dried and sieved samples (2 mm):

- **Cation exchange capacity (CEC)** with 1N CH_3COONH_4 (Chapman, 1965). The CEC extracts were filtered through nylon membrane syringe filters (0.45 µm, WICOM) and the concentrations of **NH_4^+-As, NH_4^+-Cd, NH_4^+-Cu, NH_4^+-Pb and NH_4^+-Zn** analyzed with an ICP-Ms (Agilent 7500A). NH_4^+-metal(loid)s were considered available elements. Units of measure in $cmol_c$ kg^{-1}.

In aliquots of soil samples grounded in an Agatha mortar:

- **Total $CaCO_3$ content**, **total nitrogen (TN) and total organic carbon (TOC)** content with an elemental analyzer (LECO CHN628) (ISO, 1995). Units of measure in g kg^{-1}.
- **Total metal(loid)s composition (T-As, T-Cd, T-Fe, T-Mn, T-Pb, and T-Zn)** by X-ray fluorescence (Bruker S4 Pioneer). Units of measure in mg kg^{-1}.

In soil samples stored at -20 °C:

An aliquot was used to perform water soluble extracts (1:2.5 soil:water suspensions; 2h shaking) after thawing at room temperature. The required water volume was adjusted based on the field soil moisture content. After soil:water extracts were filtered through nylon membrane syringe filters (0.45 µm, WICOM), the following parameters were measured:

- **pH** was measured with a Crison Basic 20 pH-meter.
- **Electrical conductivity (EC)** was measured with a Crison Basic 30 conductivity-meter. Units of measure in dS m^{-1}.
- **Water soluble organic carbon (WSOC)** concentrations were measured with a TOC analyser (TOC-VCSH Shimadzu). Units of measure in mg kg^{-1}.
- **Water soluble salts (Na^+, Ca^{2+}, Mg^{2+}, K^+, Cl^-, SO_4^{2-}, NO_3^- and NH_4^+)** concentrations were measured with an ion chromatography (Metrohm 861). Units of measure in mEq L^{-1}.
- **Water soluble organic nitrogen (WSON)**. Water total soluble nitrogen (WTSN) concentrations were analyzed with a TOC analyzer (TOC-VCSH Shimadzu). Then, N-NO_3^- and N-NH_4^+ concentrations were pooled together and subtracted from the WTSN to obtain the dissolved organic nitrogen (WSON) fraction. Units of measure in mg kg^{-1}.
- **Water soluble metal(loid) concentration (W-As, W-Cd, W-Cu, W-Pb and W-Zn)** were analysed by ICP-Ms (Agilent 7500A). W-metal(loid)s were also considered available elements. Units of measure in mg kg^{-1}.

4.6.3. Biological parameters

In air-dried and sieved samples (2 mm):

- **Ecotoxicity bioassays** with the soil invertebrate *Enchytraeus crypticus* (phylum Annelida, class Oligochaeta, family Enchytraeidae) were performed according to the ISO 16387 and OECD 220 guidelines (ISO, 2004; OECD, 2004). This model invertebrate species was selected due to its main role in soil functioning processes (e.g., organic matter/nutrient cycling, soil bioturbation, soil structure improvement) and its use as bioindicator of stress conditions (e.g., presence of metal(loid)s in soil)

(Didden and Römbke, 2001; Castro-Ferreira et al., 2012). Ten adult enchytraeids (≈1 cm long and with visible clitellum) were exposed to soil previously moistened at 50% of its maximum water holding capacity (ISO, 1998) and incubated for 21 d in an acclimatized room at 20 ºC and 12h:12h light:dark photoperiod. Bioassays with *E. crypticus* were also performed in the standard, clean soil Lufa 2.2 (Speyer, Germany) to check for the correct performance of soil invertebrates. During the incubation period soil water loss was replenished with demineralized water and food (oatmeal) was provided weekly. After that, the number of surviving adults (i.e., survival) and juveniles produced (i.e., reproduction) were counted. For more details on the procedure see González-Alcaraz et al. (2015).

For each study environment, *E. crypticus* reproduction was related to the sum of toxic units –TU– (ΣTU) based on soil water soluble metal(loid) concentrations (Weltje, 1998). TUs for As, Cd, Cu, Pb and Zn were calculated by dividing the W-metal(loid) concentrations (1:2.5 soil:water extracts; Section 4.6.2) by EC50 values (metal(loid) concentration causing 50% reduction in reproduction) recorded from the bibliography (Table 4.4).

Table 4.4. EC50 values for the toxicity of As, Cd, Cu, Pb and Zn to enchytraeids or earthworms (in case no data on enchytraeids were available) based on water soluble meta(loid) concentrations used for toxic units' calculation. EC50s are expressed as mg kg^{-1}.

Metal(loid)	Organisms	EC50	Reference
As	*Eisenia andrei*	2.61[a]	Romero-Freire et al. (2015)
Cd	*Enchytraeus crypticus*	0.30[b]	Castro-Ferreira et al. (2012) Ardestani and van Gestel (2013)
Cu	*Enchytraeus crypticus*	6.91	Posthuma et al. (1997)
Pb	*Enchytraeus crypticus*	0.46	Luo et al. (2014)
Zn	*Enchytraeus crypticus*	3.90	Posthuma et al. (1997)

[a] EC50 for As was estimated as the average value of the EC50s provided for different soils with distinct properties.

[b] EC50 for Cd was estimated following Equation 4.

$$EC50\ (Water\ extractable\ Cd) = EC50\ (Total\ Cd) \times \frac{Water\ extractable\ Cd}{Total\ Cd} \qquad \text{(Equation 4)}$$

We used the EC50 based on total Cd concentration (35 mg kg^{-1}) shown by Castro-Ferreira et al. (2012), and the total (4.37 and 18.2 mg kg^{-1}) and water soluble (0.04 and 0.15 mg kg^{-1}) Cd concentrations in Lufa 2.2 soil reported by Ardestani and van Gestel (2013).

<u>In samples stored at -20 °C:</u>

- **Microbial biomass carbon (MBC)** was estimated following the fumigation-extraction method (Vance et al., 1987; Wu et al., 1990). Once thawed, soil samples were fumigated with $CHCl_3$, incubated for 24 h at room temperature and organic carbon extracted with 0.5 M K_2SO_4 and measured with a TOC analyzer (TOC-VCSH Shimadzu). Units of measure in mg C kg^{-1} dry soil.
- **Dehydrogenase activity (DH)** was determined according to García et al. (1997). Soil samples were incubated with iodonitrotetrazolium chloride at room temperature (20 ºC) for 20 h and the iodonitrotetrazolium formazan (INTF) formed was measured by spectrophotometry at 490 nm (Thermo Fisher Scientifc Multiskan GO). Units of measure in µg INTF g^{-1} dry soil h^{-1}.
- **β-glucosidase activity (β-glu)** was determined according to the modification of Ravit et al. (2003) proposed by Reboreda and Caçador (2008). Thawed soil samples were incubated with p-nitrophenyl-b-D-glucopyranoside (≥99% purity, Sigma-Aldrich) at 37 °C for 60 min and the released p-nitrophenol (pNF) was measured by spectrophotometry at 410 nm (Thermo Fisher Scientifc Multiskan GO). Units of measure in µmol pNF g^{-1} dry soil h^{-1}.

<u>In soil samples stored at -80 °C:</u>

- **Community-level physiological profile (CLPP).** The Biolog EcoPlate™ system was used (Biolog, Hayward, CA, USA). Each Biolog EcoPlate contains 31 different substrate wells of common C sources present in soil and a blank well, each replicated three times. The different substrates were classified within six C source groups according to Sala et al. (2006): amines and amides, amino acids, carbohydrates, carboxylic acids, phenolic acids, and polymers (Table 4.5).

  Three grams of thawed soil were shaken (orbital shaker) with 27 mL of sterile deionized water and 20 sterile glass beads for 10 min at 200 rpm and room temperature (Samarajeewa et al., 2017). Soil:water suspensions were diluted 100-fold to attain a cellular density of approximately 106 cell mL^{-1} (Preston-Mafham et al., 2002). Afterwards, 100 µL of the diluted suspensions were inoculated into the Biolog EcoPlates (one plate per plot) and incubated for 192 h in the dark at 20 °C. Substrate utilization rate was determined by measuring the color development due to the reduction of a tetrazolium violet redox dye. Color intensity was recorded every 24 h by spectrophotometry at 590 nm (Biolog MicroStation System, Hayward, CA, USA). Absorbance values were corrected using the blank well at the corresponding measurement time. Substrate utilization was considered positive when the corrected absorbance value was >0.06 (Preston-Mafham et al., 2002; Classen et al., 2003; Dumontet et al., 2017; Deary et al., 2018).

The data collected for the whole incubation period (0-192 h) was used to calculate the average well-color development – AWCD (average of absorbance values for all the substrates consumed that provides a measurement of the total metabolic activity) and the substrate average well-color development – SAWCD (average of substrate consumption by C source groups) (Garland, 1997; Sofo et al., 2010). In addition, the corrected absorbance values >0.25 were used to derive the following ecological diversity indices: substrate richness – S (number of different substrates consumed), Shannon-Weaver index – H´ (diversity of substrates consumed), and Pielou index – J´ (equitability/dominance of activities across all substrates consumed) (Garland, 1997; Sofo et al., 2010; Gryta et al., 2014).

Table 4.5. Carbon source classification of the different substrates present in the Biolog EcoPlate™ system (Sala et al., 2006).

Carbon source group	**Substrate**
Amines and amides	Phenylethylamine
	Putrescine
Amino acids	L-arginine
	L-asparagine
	L-phenylalanine
	L-serine
	L-threonine
	Glycyl-L-glutamic acid
Carbohydrates	α-D-lactose
	β-methyl-D-glucoside
	D-cellobiose
	D-galactonic acid γ-lactone
	D-mannitol
	D-xylose
	D,L-α-glycerol phosphate
	Glucose-1-phosphate
	i-erythritol
	N-acetyl-D-glucosamine
Carboxylic acids	α-Ketobutyric acid
	γ-hydroxybutyric acid
	D-galacturonic acid
	D-glucosaminic acid
	D-malic acid
	Itaconic acid
	Piruvic acid methyl ester
Polymers	α-cyclodextrin
	Glycogen
	Tween 40
	Tween 80
Phenolic compounds	2-hydroxy benzoic acid
	4-hydroxy benzoic acid

4.6.4. Parameters measured under field conditions

- **Soil temperature** was regularly measured by inserting digital thermometers at ≈5 cm depth (n = 3 per plot of study environment).
- **Soil moisture** content was regularly evaluated by collecting soil aliquots that were weighted before and after drying at 70 ºC until constant weight (n = 2 per plot of study environment).
- **Organic matter decomposition** was evaluated by means of the tea bag index (TBI) method (Keuskamp et al., 2013). Lipton rooibos tea (EAN: 82 22700 18843 8) and Lipton green tea Sencha exclusive collection (EAN: 87 14100 77054 2) bags were used. At each sampling time, tea bags were buried at ≈10 cm depth (10 bags per tea type and plot; n = 4 per study environment) (Fig. 4.6) and regularly collected during ≈100 d to determine the remaining mass (after removing soil particles and drying at 70 °C for 48 h) and calculate the TBI (http://www.teatime4science.org/publications/).

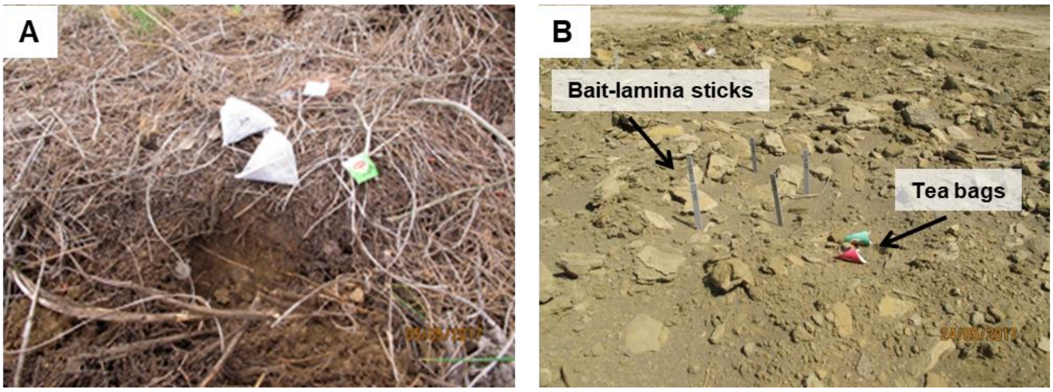

Figure 4.6. A) Tea bags before buried for tea bag index; B) Evaluation of organic matter decomposition and faunal feeding activity

- **Feeding activity of soil dwelling organisms** were estimated using bait-lamina sticks (Terra Protecta® GmbH, Berlin, Germany) (Kratz, 1998; ISO, 2016). Two groups of 5 baited sticks (i.e., n = 10 per plot of study environment) 10 cm long were vertically inserted into the soil (Figs. 4.6 and 4.7). Each stick had 16 holes filled with a mixture of cellulose and bran powder (70% and 30%, respectively) together with a small amount of activated carbon. After 20 d the number of holes partially and fully empty was recorded.

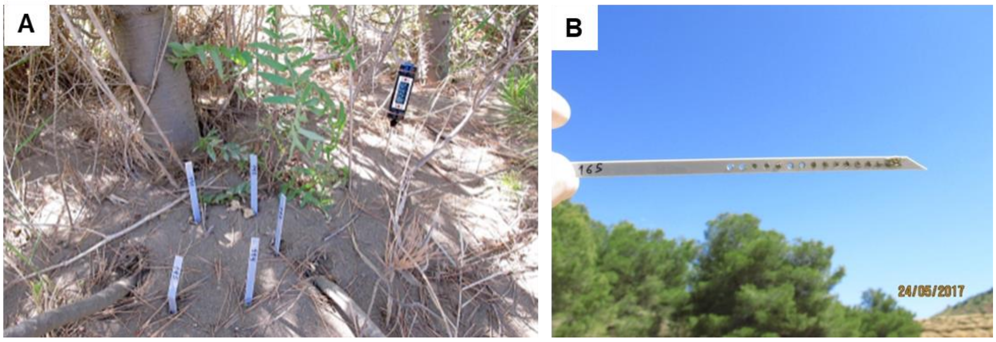

Figure 4.7. A) Bait lamina sticks buried for faunal feeding activity evaluation; B) Detail of eaten and uneaten holes in a bait-lamina stick.

- **Soil respiration** (integrated measure of the activity of roots, soil microorganisms and soil fauna) was evaluated by measuring the CO_2 emission from the soil with a SRC-1 Respiration System CIRAS-2 (n = 3 per plot of study environment) (Fig. 4.8). Units of measure in g m^{-2} h^{-1}.

Figure 4.8. Respiration System CIRAS-2 to measure soil CO_2 emission (spring 2018).

4.7. Statistical analyses

Data are shown as average±standard error. Statistical analyses were performed with IBM SPSS Statistics 19.0.0 (SPSS, 2010), PRIMER v6 software packages and the ‘CANOCO for Windows’ program v4.02 (Jogman et al., 1987; ter Braak and Smilauer, 1999). Differences were considered significant at $p<0.05$. Data were transformed when they did not fulfil the assumptions of normal distribution (Shapiro-Wilk’s test) and/or homogeneity of variances (Levene’s test). For further detailed information about the statistics analyses see Chapters 5, 6 and 7.

4.8. References

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PART II

RESULTS AND DISCUSSION

CHAPTER 5

Evidence supporting the value of spontaneous vegetation for phytomanagement of soil ecosystem functions in abandoned metal(loid) mine tailings

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Abstract

This work studies a set of soil indicators (physical, physico-chemical and biological), evaluated under field and laboratory conditions, in different stages of spontaneous vegetation colonization in abandoned metal(loid) mine tailings from Mediterranean semiarid areas. The results provide evidence about the interest of spontaneous colonization by native vegetation for the phytomanagement of abandoned metal(loid) mine tailings in terms of providing ecosystem functions. Bare soils (B), small groups of pine trees (P), scattered pine trees with shrubs and herbs under the canopy (P+S), and dense patches of pine trees with shrubs and herbs under the canopy (DP+S) were studied inside mine tailings abandoned ~20 years ago. Besides, pine forests next (FN) and away (FA) from the tailings were also studied. Pioneer and nurse plants were mainly found inside the tailings, although ecological indexes in P+S and DP+S were similar to FN and FA. Pedogenesis evidence such as structure development and increase in cation exchange capacity, organic C and N were found in tailing soils from B to DP+S. However, soil metal(loid)s did not follow the same variation pattern. For example (in mg kg^{-1}): P showed the maximum total Cu (≈277) and Zn (≈17,860), while P+S of As (≈1250) and Pb (≈14,570). B had the maximum water soluble Pb (≈4) and Zn (≈207), while FA of As (≈0.192) and Cu (≈0.149). Soil microbial biomass C, enzyme activity, CO_2 emission, organic matter decomposition and feeding activity of soil dwelling organisms indicated similar, or even higher, biological activity in P+S and DP+S than in FN and FA. In fact, FA showed the highest soil ecotoxicity risk (reduced enchytraeid reproduction). Therefore, mine tailing soils can be effectively modified following spontaneous vegetation colonization, achieving conditions with capacity to provide certain ecosystem functions. Hence, phytomanagement of these tailings should be preceded by a detailed knowledge of the existing spontaneously colonized sites, which should be preserved to take advantage of their potentiality.

Keywords: Mediterranean environments; Soil pollution; Mine wastes; Soil indicators; Soil biological activity; Soil ecotoxicity

5.1. Introduction

As explained in Chapter 1 (see Section 1.4), phytostabilizaton is a phytomanagement technique that involves the establishment of vegetation on contaminated sites to reduce the transfer of potentially toxic elements (PTEs) and to enhance the value of the land (Robinson et al., 2009). An important aspect to successfully implement this option is to acquire knowledge about the role of native vegetation that spontaneously colonizes tailings in improving soil conditions (Navarro Cano et al., 2018). This phenomenon is known as passive restoration (Prach and Hobbs, 2008; Gutiérrez et al., 2016; Prach and Tolvanen, 2016) and its study is considered of particular interest when mine tailings are embedded into natural areas (Navarro Cano et al., 2018).

Studying soil conditions in mine tailing sites spontaneously colonized in relation to tailings bare sites and to vegetated spots from surrounding areas out of the tailings can help to understand the role of passive restoration and to improve tailings phytomanagement strategies. In fact, it has been suggested that legislation should favor natural processes over technical reclamation in post-mining sites, except in those cases of well-justified public concern (Tropek et al., 2012). Plant growth in mine tailings can trigger a cascade effect on ecosystem functions and services such as soil organic carbon sequestration, nutrient cycling, reduction of available PTE concentration, reduction of erosion risks and biodiversity increase (Wang et al., 2014; Navarro-Cano et al., 2015; Burges et al., 2018). These are ecosystem functions considered key for the success of mine tailings phytomanagement (Mendez and Maier, 2008).

So far, most of the studies on this topic have been mainly focused on changes in some soil physicochemical and/or microbiological parameters (namely soil quality indicators or soil indicators; Bünemann et al., 2018) and their relationship with vegetation cover. However, it is feasible to move forward on this issue and address a more integrated, comprehensive approach of the soil benefits given by vegetation that spontaneously colonize mine tailings and the ecosystem functions provided. For that, a variety of soil indicators linked to ecosystem functions should be assessed under field and laboratory conditions in different stages of plant colonization.

This chapter includes the work planned to respond to the first specific objective of the PhD Thesis (see Section 3.1): To evaluate in which degree soil conditions can be modified following spontaneous vegetation colonization in abandoned metal(loid) mine tailings from semiarid drylands, and to provide evidence about the interest of this colonization for the phytomanagement of these structures. A set of physical, physicochemical and biological soil indicators were assessed, under laboratory and field conditions, and vegetation ecological indexes evaluated in metal(loid) mine tailings and surrounding forests from an abandoned mining area under Mediterranean semiarid climate in SE Spain. Our intent was not to establish cause-effect relationships between vegetation stages and soil conditions, nor do we assume that the study stages represent the unique successional trajectory of vegetation colonization in mine tailings. We discuss the results in terms of the capacity of the soil-plant system of spontaneously colonized sites to provide ecosystem functions typically target by phytoestabilization such as erosion reduction (inferred from plant cover increase and soil structure improvement), pollution attenuation and habitat provision (Navarro-Cano et al., 2018). We hypothesized that spontaneous vegetation colonization can promote pedogenesis and improve soil functioning in abandoned metal(loid) mine tailings to an acceptable level, relative to the surrounding forests, for providing soil-based ecosystem services.

5.2. Materials and methods

This chapter contains the data corresponding to the field work campaign carried out in summer 2017 in the selected study environments: bare soils (B); patches with small groups of *Pinus halepensis* trees ≈2.5-5 m high growing scattered (P); patches formed by isolated *P. halepensis* trees >≈4-5 m high growing scattered with shrubs and herbs under the canopy (P+S); dense patches including several *P. halepensis* trees >≈4-5 m high and shrubs and herbs under the canopy (DP+S); forest located next to the mine tailings with *P. halepensis* trees >≈5 m high and shrubs and herbs under the canopy (FN); forest located away from the mine tailings (≈1600-1800 m) with *P. halepensis* trees >≈5 m high and shrubs and herbs under the canopy (FA). In particular, the following parameters are displayed (for a complete description of the methodologies used see Chapter 4):

- Vegetation parameters. Plant species; number of individuals per species; family; life form; functional role as pioneer/nurse plant in the colonization process; Margalef richness index (R); Shannon-Weaver heterogeneity index (H’).
- Soil parameters. Bulk density; soil structure; color description; cation exchange capacity (CEC); NH_4^+ soluble metal(loid)s concentration (NH_4^+-metal(loid)s); total $CaCO_3$; total nitrogen (TN); total organic carbon (TOC); total metal(loid)s

concentration (T-metal(loid)s); pH; electrical conductivity (EC); water soluble organic carbon (WSOC); water soluble salts (Na^+, Ca^{2+}, Mg^{2+}, K^+, Cl^-, SO_4^{2-} and NO_3^-); water soluble metal(loid)s (W-metal(loid)s); microbial biomass carbon (MBC); dehydrogenase activity (DH); β-glucosidase activity (β-glu); ecotoxicity bioassays; soil respiration (CO_2 emission); organic matter decomposition (TBI); feeding activity of soil dwelling organisms; soil temperature; soil moisture.

Related to the statistical analyses, differences were considered significant at $p < 0.05$. Data were transformed when necessary to achieve normal distribution (Shapiro-Wilk's test) and/or homogeneity of variances (Levene's test). Different transformations were used, depending on the nature of the data, including logarithmic- and arcsine square root-transformations. One-way ANOVA followed by Tukey post-hoc test was used to check for differences among the study environments. Spearman's correlations were calculated to evaluate the relationships among all the assessed parameters. The relationships among the whole set of soil data were examined by Principal Component Analysis (PCA). This analysis can be applied to environmental variables in the 'role of species' (ter Braak and Smilauer, 1999). The analysis was centered and standardized by species (i.e., environmental variables) according to Jogman et al. (1987) and ter Braak and Smilauer (1999).

5.3. Results

5.3.1. Vegetation inventory and ecological indexes

A total of 48 plant species, distributed in 24 families, were recorded in the five vegetated study environments (Table 5.1). Four families accounted for ≈50% of the species present: Gramineae, Compositae, and Lamiaceae. Terophytes appeared in P and P+S, but they were absent in DP+S, FN and FA; hemicryptophytes and chamaephytes were the most abundant forms in DP+S and they were also important in FN together with the other life forms, except for terophytes; geophytes and phanerophytes dominated in FA (Figure 5.1a). Regarding the functional role in relation with the plant colonization process of barren mine tailings (Navarro-Cano et al., 2018), 18 plant species were considered pioneer and 10 nurse (Table 5.1). Pioneer species were more abundant in P+S (10), DP+S (7) and FN (6) than in P and FA, while nurse species appeared in similar number (4-5) in almost all the vegetated study environments (Figure 5.1a).

Apart from *P. halepensis* no other species was growing in all the sampling plots, but seven species appeared in three or more environments (Table 5.1). *Pistacea lentiscus* and *Rhamnus lycioides* appeared in P, P+S, DP+S and FN, *Sonchus tenerrimus*, *Piptatherum miliaceum* and *Phragmites australis* (all of them nurse or pioneer species) in P, P+S, DP+S and FN, while *Brachypodium retusum* (pioneer) and *Lygeum spartum* (nurse) appeared in all the vegetated environments except in P.

The Margalef (R) and Shannon-Weaver (H') indexes followed a similar trend (Figs. 5.1b and 5.1c). The P environment had significant lower values (0.79±0.30) and no differences were obtained among the other four vegetated study environments (between ≈1.8 and ≈2.6).

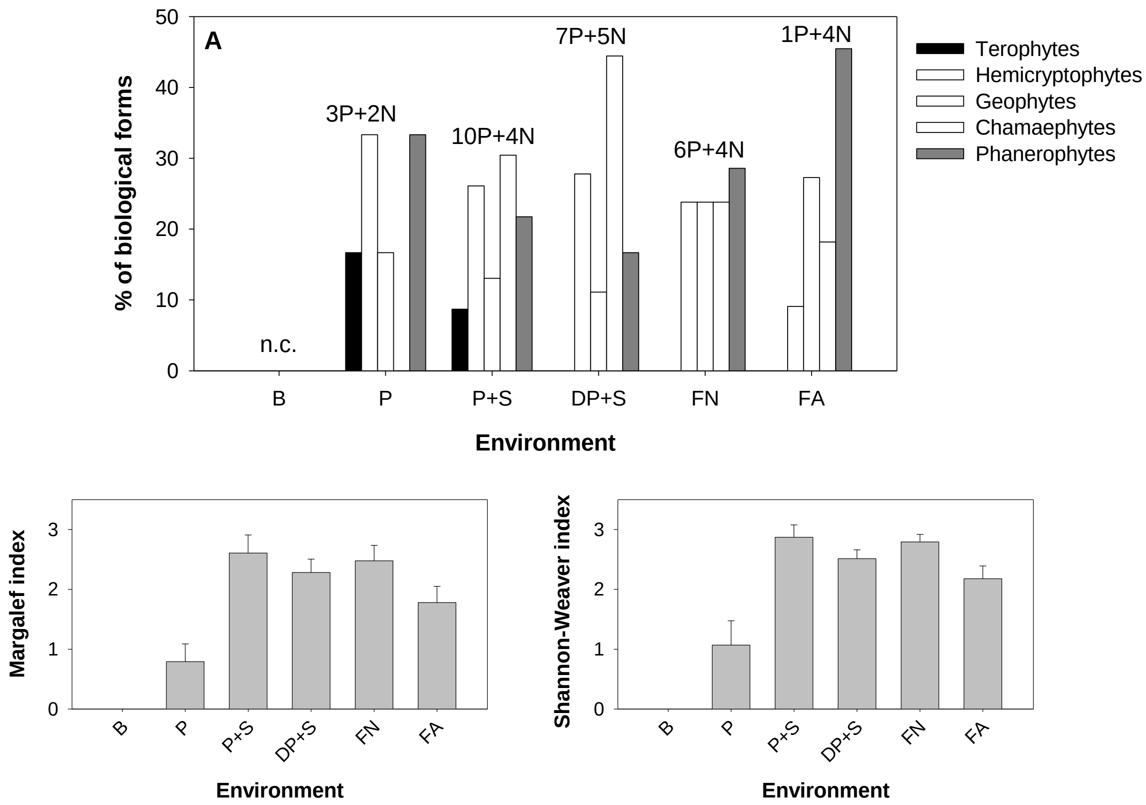

Figure 5.1. A. Biological forms of the plant species growing in the study environments. Above the columns it is indicated the number of pioneer (P) and nurse (N) species. B and C. Ecological indexes. Columns represent average values and bars on columns standard error (n=4). Different letters indicate significant differences among environments (one-way ANOVA with Tukey post hoc test, p<0.05). See Section 5.2 for meaning of the environments (B, P, P+S, DP+S, FN and FA). n.c. (not calculated).

Table 5.1. Family, life form and total number of individuals of the plant species recorded in the study environments. See Section 5.2 for meaning of the environments (B, P, P+S, DP+S, FN and FA). P (pioneer species)/N (nurse species)/n.d. (no defined) refers to the functional role in relation with the colonization of barren mine tailings according to Navarro-Cano et al. (2018).

				Total number of individuals					
Plant species	**Family**	**Life form**	**P/N species**	**B**	**P**	**P+S**	**DP+S**	**FN**	**FA**
Arenaria montana L.	Caryophyllaceae	Chameophyte	n.d.			2			
Arisarum vulgare Targ. Tozz	Araceae	Geophyte	n.d.						1
Asparagus acutifolius L.	Liliaceae	Hemicryptophyte	P				1		
Asparagus horridus L.	Liliaceae	Hemicryptophyte	P			1		2	
Asphodelus cerasiferus J. Gay	Xanthorrhoeaceae	Geophyte	n.d.					1	
Atractylis humilis L.	Compositae	Terophyte	n.d.					1	
Brachypodium distachyon (L.) Beauv.	Gramineae	Terophyte	P			2			
Brachypodium retusum (Pers.) Beauv.	Gramineae	Chameophyte	P			1	4	3	1
Bromus fasciculatus C.Presl	Poaceae	Terophyte	P			1			
Calicotome intermedia (C. Presl) Guss.	Leguminosae	Nanophanerophyte	P						4
Chamaerops humilis L.	Arecaceae	Nanophanerophyte	N			1			2
Cheirolophus intybaceus (Lam.) Dostál	Compositae	Hemicryptophyte	n.d.					1	
Convolvulus althaeoides L.	Convolvulaceae	Hemicryptophyte	P				1		
Dactylis glomerata L.	Gramineae	Hemicryptophyte	n.d.			1		1	
Eryngium campestre L.	Umbeliferae	Geophyte	n.d.			1		1	
Helianthemum syriacum (Jacq.) Dum. Cours.	Cistaceae	Chameophyte	P			1	2		
Helichrysum decumbens (Lag.) Camb	Compositae	Chameophyte	N				4		
Hippocrepis ciliata Willd.	Leguminosae	Terophyte	n.d.		1				
Hyparrhenia sinaica (Delile) Llauradó	Gramineae	Hemicryptophyte	N			4			

Plant species	Family	Life form	P/N species	Total number of individuals					
				B	**P**	**P+S**	**DP+S**	**FN**	**FA**
Lapiedra martinezii Lag.	Amaryllidaceae	Geophyte	n.d.					1	3
Leontodon taraxacoides (Willd.) Merat.	Compositae	Hemicryptophyte	P			4			
Limonium carthaginense (Rouy) Hubbard a Sandwith	Plumbaginaceae	Chameophyte	N				2		
Limonium cossonianum Kunthze, Revis.	Plumbaginaceae	Chameophyte	P					1	
Lygeum spartum L.	Gramineae	Geophyte	N			4	3	1	2
Nothoscordum inodorum (Aiton) G. Nicholson	Liliaceae	Geophyte	n.d.					1	
Phagnalon saxatile (L.) Cass.	Compositae	Chameophyte	P			2	2		
Phillyrea angustifolia L.	Oleaceae	Microphanerophyte	n.d.					3	
Phragmites australis (Cav.) Trin.	Gramineae	Geophyte	P		1	1	4		
Pinus halepensis Miller	Pinaceae	Macrophanerophyte	N		4	4	4	4	4
Piptatherum miliaceum (L.) Cosson	Gramineae	Hemicryptophyte	N		1	3	3		
Pistacia lentiscus L.	Anacardiaceae	Microphanerophyte	n.d.			3	4	1	
Polygala rupestris Pourr.	Polygalaceae	Chameophyte	n.d.			2	1		
Rhamnus lycioides L.	Rhamnaceae	Microphanerophyte	n.d.		1	1	3	4	
Rosmarinus officinalis L.	Lamiaceae	Nanophanerophyte	n.d.						2
Rubia peregrina L.	Rubiaceae	Chameophyte	n.d.				1		
Ruta angustifolia Pers.	Rutaceae	Chameophyte	P			1			
Salsola genistoides Juss. ex Poir.	Amaranthaceae	Nanophanerophyte	P					4	
Salsola oppositifolia Desf.	Amaranthaceae	Nanophanerophyte	N					1	
Satureja obovata Lag.	Lamiaceae	Chameophyte	n.d.						1

				Total number of individuals					
Plant species	**Family**	**Life form**	**P/N species**	**B**	**P**	**P+S**	**DP+S**	**FN**	**FA**
Sedum sediforme (Jacq) Pau	Crassulaceae	Chameophyte	n.d.			1			
Sonchus tenerrimus L.	Compositae	Hemicryptophyte	P		1	2	1	1	
Stipa tenacissima L.	Gramineae	Hemicryptophyte	N			1		3	1
Tetraclinis articulata (Vahl) Mast.	Cupressaceae	Phanerophyte	n.d.						2
Teucrium capitatum L.	Lamiaceae	Chameophyte	P					1	
Teucrium carthaginense Lange	Lamiaceae	Chameophyte	n.d.					3	
Thymelaea hirsuta (L.) Endl.	Thymelaeaceae	Nanophanerophyte	P			2			
Thymus hyemalis Lange	Lamiaceae	Chameophyte	N				1		
Zygophyllum fabago L.	Zygophyllaceae	Hemicryptophyte	P		1				

5.3.2. Soil moisture and temperature and physical soil indicators (texture, structure, color and bulk density)

Soil moisture content was ≈6-10% at the beginning of the study period (day 14, mid-May) in all the environments and decreased to ≈3-4% by the end of August (day 104) (Fig. 5.2a), except in P where it was always < ≈2%. In the vegetated environments soil temperature ranged from ≈18 to ≈28 ºC (Fig. 5.2b), while bare soils (B) showed ≈ 3 to 5 ºC higher throughout most of the study period.

The soils of all the study environments showed a coarse texture (sandy loam or loamy sand), with clay+silt contents between ≈14% (P) and ≈41% (FA) and sand contents between ≈59% (FA) and ≈86% (P) (Table 5.2).

In the mine tailings soil structure showed a clear change, from strong lamination by forming a platy structure in bare soils (B) to granular with particles aggregation around roots and plant remains in P, P+S and DP+S (Table 5.3; Fig. 5.3). It is of highlight the large content of filaments of fungi mycelium found in the P environment, which much contribute to agglutinate soil particles. Granular structure was also identified in the nearby (FN) and away (FA) forests, but in these two environments the existence of blocky aggregates indicated stronger structural development (Table 5.3; Fig. 5.3). Olive, yellowish or greyish colors typical of metal(loid) mine wastes were observed in tailing soils, but they were darker in P+S and DP+S (HUE 10YR) than in B and P (HUE 2.5Y in B) (Table 5.3). Brown colors characterized both forest soils, but they were darker (HUE 5YR) in FA than in FN (HUE 7.5YR) (Table 5.3).

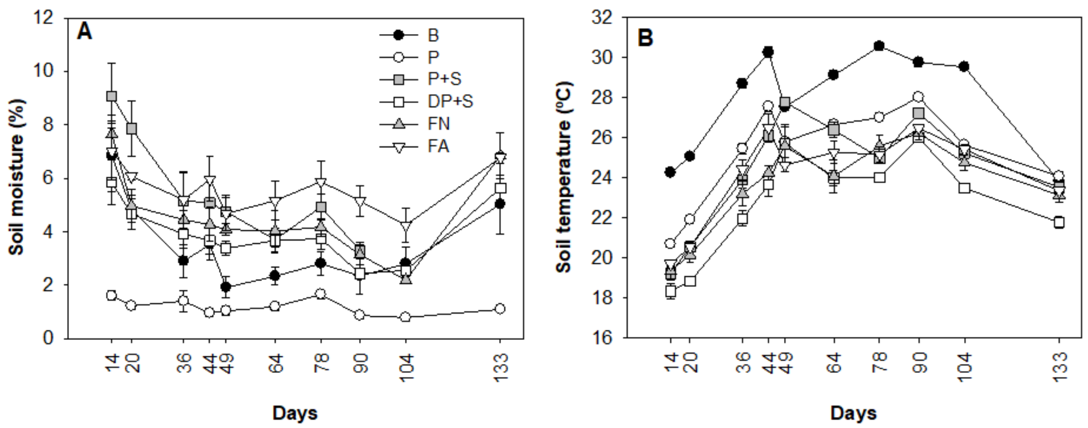

Figure 5.2. Soil moisture content and temperature throughout the sampling period in the study environments. Dots represent average values and bars standard error (n=4). See Section 5.2 for meaning of the environments (B, P, P+S, DP+S, FN and FA).

Table 5.2. Texture and particle size distribution of the study environments. See Section 5.2 for meaning of the environments (B, P, P+S, DP+S, FN and FA). Values are average ± standard error (n=4).

Environment	**Texture**	**Particle size distribution**		
		Clay (%)	**Silt (%)**	**Sand (%)**
B	Loamy fine sand	7.5 ± 1.3	22.5 ± 3.9	70.0 ± 5.1
P	Sandy loam	4.5 ± 0.5	9.5 ± 1.0	86.0 ± 1.2
P+S	Sandy loam	9.0 ± 2.6	19.0 ± 2.4	72.0 ± 4.2
DP+S	Sandy loam	5.5 ± 1.0	23.0 ± 2.6	71.5 ± 3.4
FN	Sandy loam	8.0 ± 0.0	22.0 ± 1.8	70.0 ± 1.8
FA	Sandy loam	19.0 ± 0.6	22.0 ± 0.8	59.0 ± 1.0

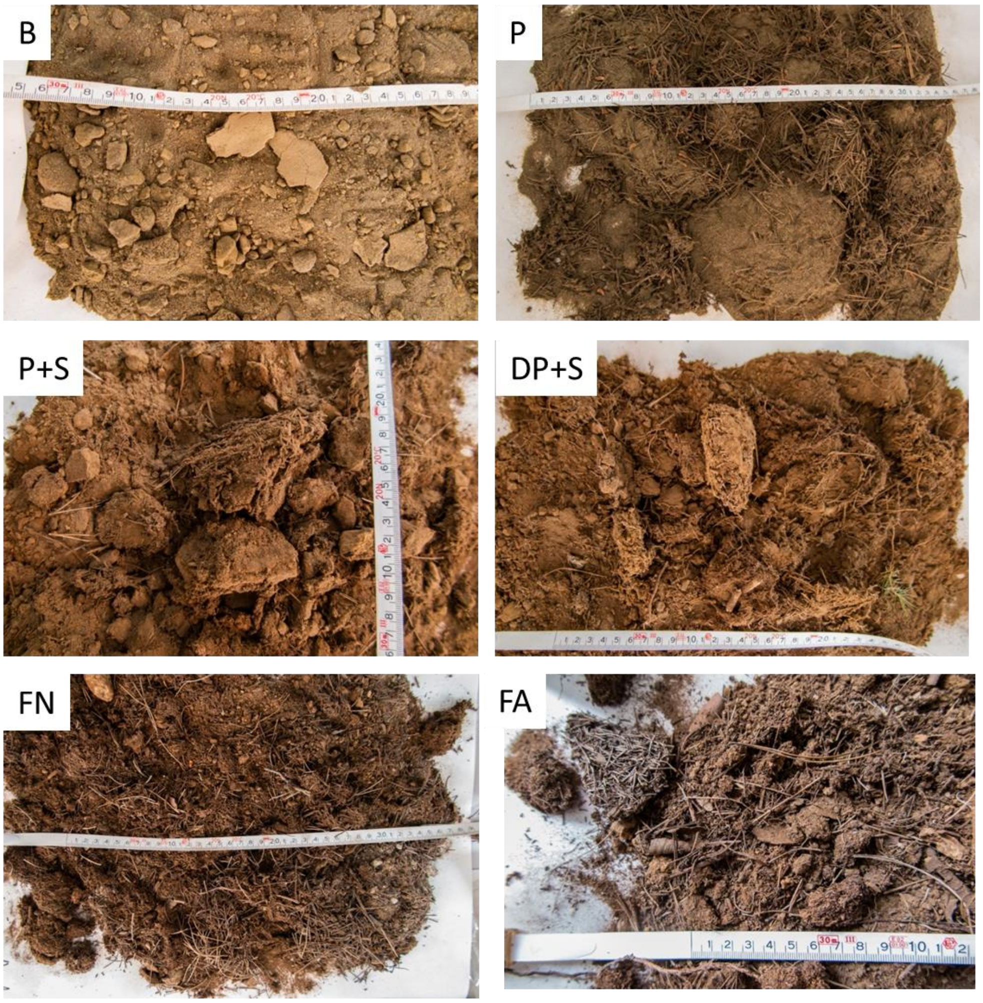

Figure 5.3. Structural soil development in the study environments. See Section 5.2 for meaning of the environments (B, P, P+S, DP+S, FN and FA).

Table 5.3. Soil structure and color pattern in the study environments. See Section 5.2 for meaning of the environments (B, P, P+S, DP+S, FN and FA).

Environment	Type, size and class of soil structure	Remarks	Soil color
B	Platy moderate to strong medium (2-5 mm) and coarse (5-10 mm); very fine lumps (<5 mm); and single grains.	Horizontal lamination very patent.	2.5Y 4-5/4 Olive brown to Light olive brown
P	Granular weak medium (10-20 mm) to coarse (20-50 mm) and single grains.	Particle aggregation around pine needles and organic remains.	2.5Y 4-5/3-4 Light olive brown to Olive brown
P+S	Granular weak and moderate medium (10-20 mm) and coarse (20-50 mm). Platy moderate medium (2-5 mm) and coarse (5-10 mm); fine (5-10 mm) and medium (10-20 mm) lumps.	Laminar material transition to granular-lumpy structure because of particle aggregation around roots and plant remains.	10YR 3-5/3-6 Dark brown to Yellowish brown
DP+S	Granular weak and moderate coarse (5-10 mm) and very coarse (>10 mm). Platy moderate medium (2-5 mm) and coarse (5-10 mm); and fine (5-10 mm) and medium (10-20 mm) lumps.	Laminar material transition to granular-lumpy structure because of particle aggregation around roots and plant remains.	10YR 3-4/2-3 Very dark greyish brown to Brown
FN	Blocky weak fine (1-2 mm) and medium (2-5 mm). Granular moderate coarse (5-10 mm).	Many particles around plant roots and plant remains.	7.5YR 3-4/2-3 Dark Brown to Brown
FA	Blocky weak and moderate fine (1-2 mm) and medium (2-5 mm). Granular weak medium (2-5 mm) and coarse (>10 mm).	Angular blocks in close contact with plant roots and stones.	5YR 3/2 Dark reddish brown

Soil bulk density showed a progressive decrease from B (1.48±0.03 g cm^{-3}) to FA (0.47±0.09 g cm^{-3}); the B environment had significant higher values than most of the other study environments (Fig. 5.4a).

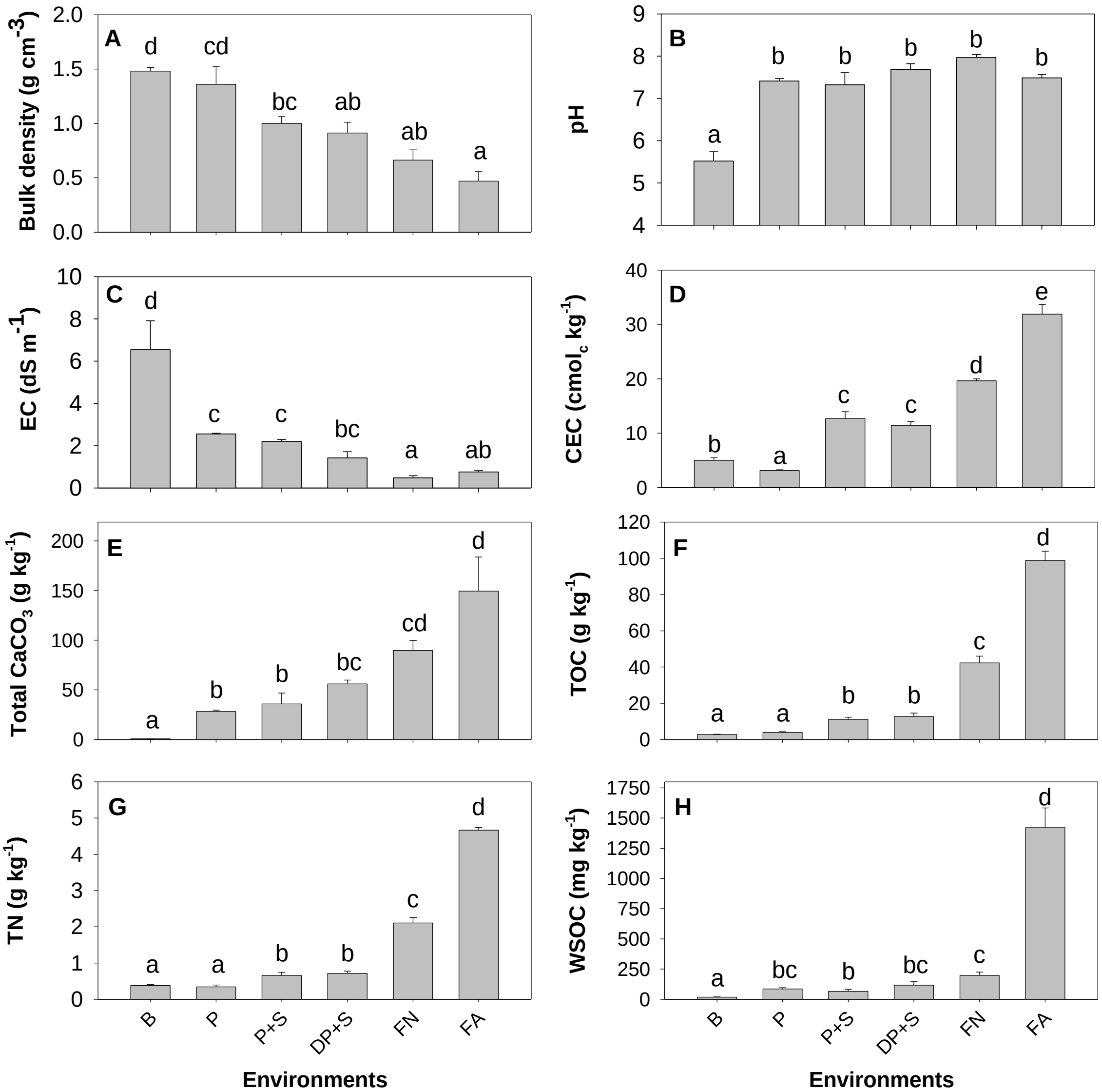

Figure 5.4. Bulk density, pH, electrical conductivity (EC), cation exchange capacity (CEC), total $CaCO_3$, total organic carbon (TOC), total nitrogen (TN) and water soluble organic carbon (WSOC) in the study environments. Columns represent average values and bars on columns standard error (n=4). Different letters indicate significant differences among environments (one-way ANOVA with Tukey post hoc test, p<0.05). See Section 5.2 for meaning of the environments (B, P, P+S, DP+S, FN and FA).

5.3.3. Physico-chemical soil indicators (pH, EC, water soluble salts, CEC, total $CaCO_3$, TOC, TN and WSOC)

The soil pH was significantly lower (≈5.5 vs. ≈7.3 to ≈8.0) and the EC significantly higher (≈6.5 vs. ≈0.5 to ≈2.5 dS m^{-1}) in bare soils (B) than in the other study environments (Figs. 5.4b and 5.4c). Soil salinity was dominated by SO_4^{2-}, the most abundant ion and the most strongly correlated with EC values (r=0.954, p<0.001), although the correlations between the other salts and EC were also positive (r≥0.437, p≤0.033) except for K^+ (the most abundant ion in FA), which was negatively correlated with EC (r=-0.568, p=0.004). Na^+ and Mg^{2+} were positively correlated with Cl^-, NO_3^- and SO_4^{2-} (r≥0.563, p≤0.004), and Ca^{2+} with SO_4^{2-} (r=0.821, p=0.000). K^+ was negatively correlated with SO_4^{2-} (r=-0.510, p=0.011) (Fig. 5.5).

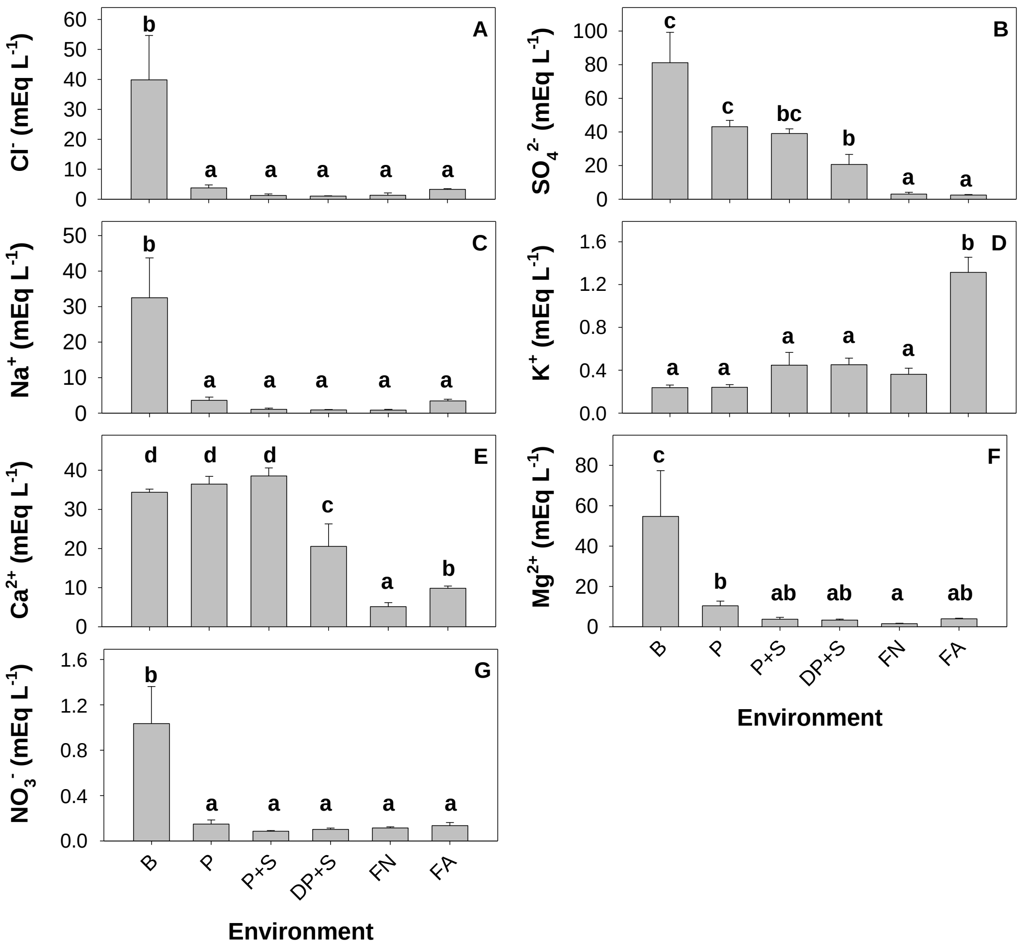

Figure 5.5. Water soluble salts (1:2.5 soil:water extracts) in the study environments. Columns represent average values and bars on columns standard error (n=4). Different letters indicate significant differences among environments (one-way ANOVA with Tukey post hoc test, p<0.05). See Section 5.2 for meaning of the environments (B, P, P+S, DP+S, FN and FA).

The CEC, total $CaCO_3$, TOC, TN and WSOC followed a tendency to increase from bare soils (B) to forest away soils (FA), the latter one showing significantly higher values than the other study environments (Figs. 5.4d to 5.4h).

5.3.4. Soil metal(loid) concentrations (As, Cd, Cu, Pb and Zn)

Different to the physico-chemical soil indicators described in the previous section, metal(loid) concentrations did not follow a similar variation pattern from B to FA. Total As (T-As), Cu (T-Cu), Pb (T-Pb) and Zn (T-Zn) reached higher concentrations in tailing soils (Figs. 5.6a, 5.6g, 5.6j and 5.6m), while total Cd (T-Cd) was < 20 mg kg^{-1} in all the study environments (Fig. 5.6d). The highest T-Cu (277±9 mg kg^{-1}) and T-Zn (17,857±684 mg kg^{-1}) concentrations were found in P, and the highest T-As (1250±164 mg kg^{-1}) and T-Pb (14,570±804 mg kg^{-1}) in P+S. FA had significantly lower T-As (69±11 mg kg^{-1}), T-Pb (1343±242 mg kg^{-1}) and T-Zn (758±71 mg kg^{-1}) concentrations than the other study environments.

Regarding NH_4^+ soluble metal(loid)s, the highest concentrations of NH_4^+-As were found in FA (0.19±0.05 mg kg^{-1}) (Fig. 5.6b). The P environment showed the highest concentrations of NH_4^+-Cd (0.91±0.75 mg kg^{-1}), NH_4^+-Cu (1.27±0.13 mg kg^{-1}) and NH_4^+-Zn (500±100 mg kg^{-1}), and B of NH_4^+-Pb (452±113 mg kg^{-1}) (Figs. 5.6e, 5.6h, 5.6k and 5.6n). Regarding water soluble metal(oid)s (Figs. 5.6c, 5.6f, 5.6i, 5.6l and 5.6o), bare soils (B) had, by far, the highest concentrations of W-Cd (3.16±1.23 mg kg^{-1}), W-Pb (3.75±1.57 mg kg^{-1}) and W-Zn (207±50 mg kg^{-1}), but this environment showed the lowest W-As (0.001±0.0004 mg kg^{-1}). It is of highlight that the highest concentrations of W-As (0.192±0.050 mg kg^{-1}) and W-Cu (0.149±0.023 mg kg^{-1}) were found in FA.

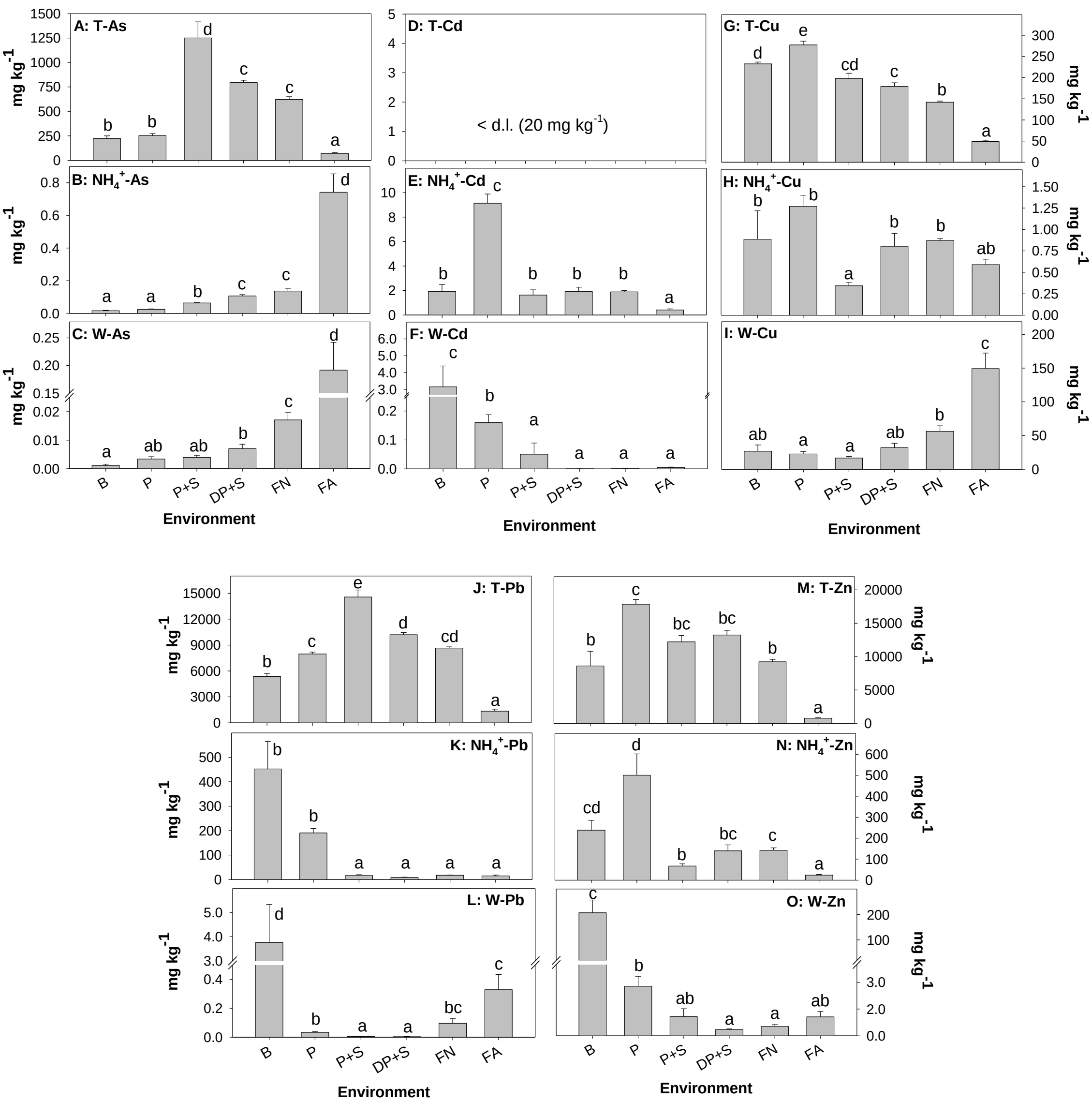

Figure 5.6. Total (T-Me), NH_4^+ soluble (NH_4^+-Me) and water soluble (W-Me) metal(loid) concentrations in the study environments. Columns represent average values and bars on columns standard error (n=4). Different letters indicate significant differences among environments (one-way ANOVA with Tukey post hoc test, $p<0.05$). See Section 5.2 for meaning of the environments (B, P, P+S, DP+S, FN and FA).

5.3.5. Biological soil indicators (MBC, dehydrogenase activity, β-glucosidase activity, CO_2 emissions, TBI, bait-lamina and ecotoxicity)

The biological soil indicators measured followed different variation patterns in the study environments (Figs. 5.7 and 5.10). MBC tended to increase from B (4.85±0.91 mg C kg^{-1}) to FA (1523±520 mg C kg^{-1}) and the soils inside the mine tailings showed significantly lower concentrations than the soils from the surrounding forests (Fig. 5.7a).

Dehydrogenase activity was negligible in B (0.02±0.01 μg INTF g^{-1} h^{-1}) and progressively increased from P+S (0.42±0.12 μg INTF g^{-1} h^{-1}) to FA (3.28±0.32 μg INTF g^{-1} h^{-1}) (Fig. 5.7b). An unexpected high activity of this enzyme was reached in the P environment (4.75±0.32 μg INTF g^{-1} h^{-1}). β-glucosidase activity was no detected in bare soils (B) and tended to increase from P (0.47±0.21 μmol PNF g^{-1} h^{-1}) to FA (3.75±0.5 μmol PNF g^{-1} h^{-1}) (Fig. 5.7c).

The highest soil CO_2 emissions were recorded in FA (0.74±0.18 g CO_2 m^2 h^{-1}) (Fig. 5.7d). In the remaining study environments, soil CO_2 emissions showed a progressive increase from B (0.042±3.2 10^{-3} g CO_2 m^2 h^{-1}) to P+S (0.57±0.06 g CO_2 m^2 h^{-1}) and dropped from this last environment to FN (0.18±0.05 g CO_2 m^2 h^{-1}) (Fig. 5.7d).

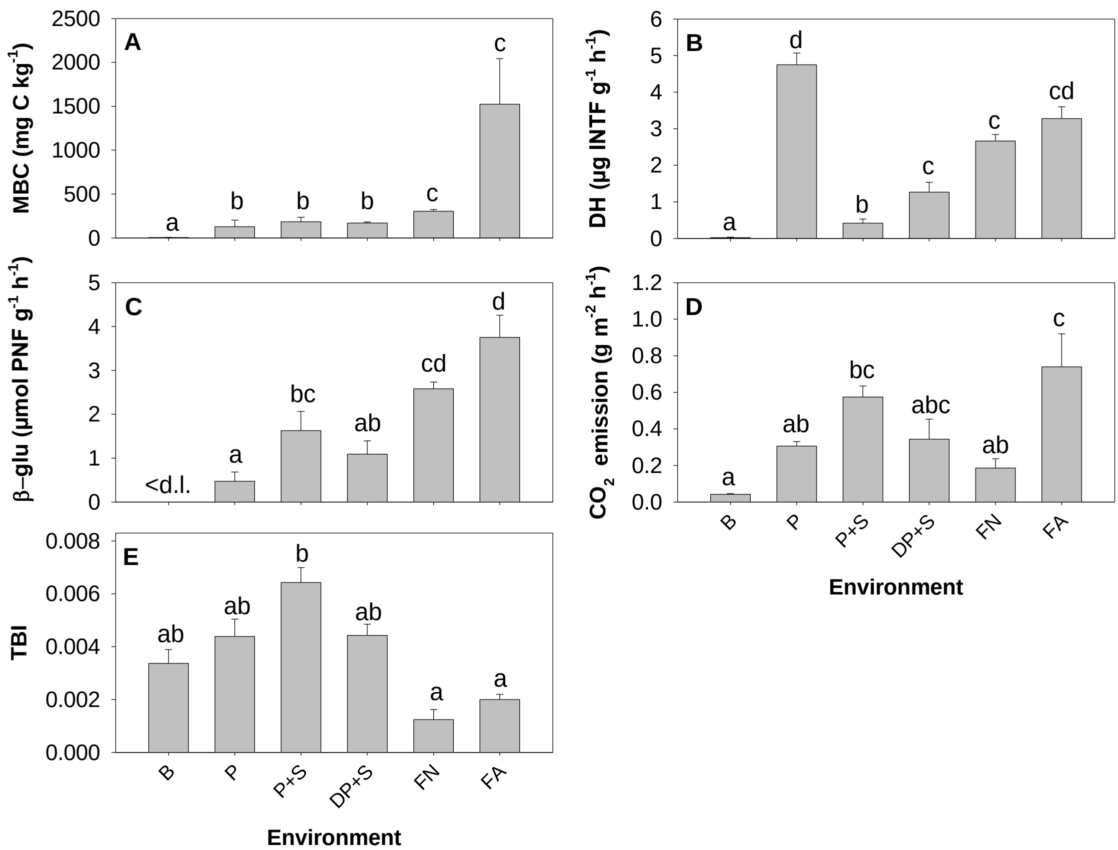

Figure 5.7. Microbial biomass carbon (MBC), dehydrogenase activity (DH), β-glucosidase activity (β-glu), CO_2 emission and tea bag index (TBI) in the study environments. Columns represent average values and bars on columns standard error (n=4). Different letters indicate significant differences among environments (one-way ANOVA with Tukey post hoc test, $p<0.05$). See Section 5.2 for meaning of the environments (B, P, P+S, DP+S, FN and FA). d.l. (detection limit): β-glucosidase activity (0.05 μmol PNF g^{-1} h^{-1}).

TBI showed greater values in mine tailing soils than in forest soils, although significant differences between both groups were only obtained for P+S vs. FN and FA (Fig. 5.7e). TBI had a similar variation pattern than soil CO_2 emissions within mine tailings, with a progressive increase from B to P+S and then decreased in DP+S (Fig. 5.7e). After 110 d buried, between ≈40% and ≈60% of mass in green tea bags was lost in B, P+S and DP+S, but for P, FN and FA between ≈70% and ≈80% of initial mass remained in the tea bags (Fig. 5.8a). For rooibos tea (Fig. 5.8b), less than ≈15% of initial mass was lost after 110 d buried regardless of the study environment.

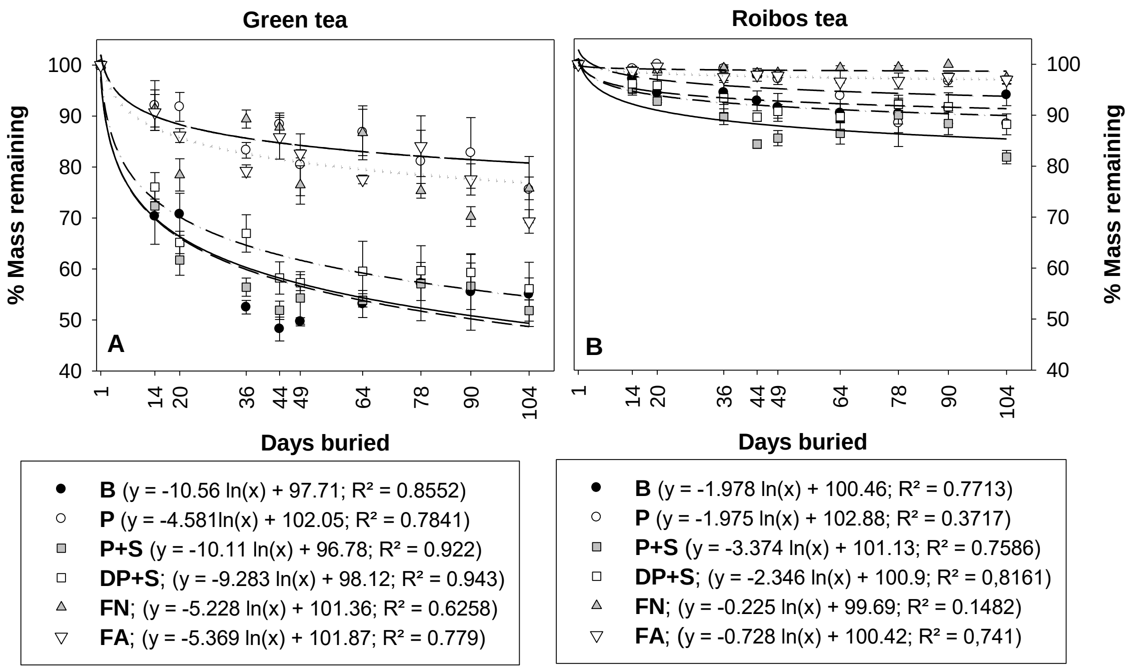

Figure 5.8. Evolution of weight loss in Green and Roibos tea bags in the study environments. Dots represent average values and bars standard error (n=4). See Section 5.2 for meaning of the environments (B, P, P+S, DP+S, FN and FA).

The feeding activity of soil dwelling organisms was generally low in all the study environments, with average values of eaten baits per stick between ≈2 and ≈6 (Fig. 5.9). Within the mine tailings, B (49) and P (62) showed greater total number of fed holes than P+S (34) and DP+S (11). In the surrounding forests, FA (67) showed more fed holes than FN (12.5).

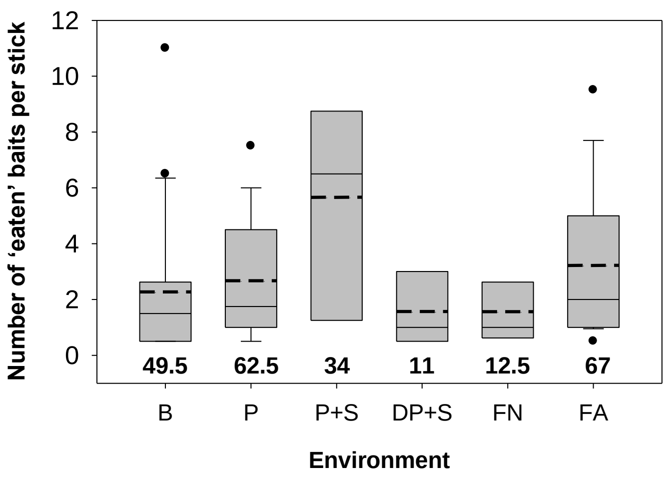

Figure 5.9. Box plots for the feeding activity of soil dwelling organisms in the study environments. Solid lines within boxes indicate the median value and dotted lines the mean. Boxes include data within the 25th and 75th percentiles; whisker lines refer to the 5th and 95th percentiles. Numbers below boxes indicates the total number of holes fed upon in each environment. See Section 5.2 for meaning of the environments (B, P, P+S, DP+S, FN and FA).

Regarding ecotoxicity bioassays, the control performance of *E. crypticus* in Lufa 2.2 soil met the validity criteria established by the ISO and OECD guidelines (ISO, 2004; OECD, 2004): adult survival ≥80%, number of juveniles produced ≥50, and coefficient of variation for reproduction ≤50% within replicates (data not shown). In all the study environments adult survival ranked between ≈60% and ≈90% and no significant differences were observed among environments (Fig. 5.10a). Unlike survival, the reproduction of *E. crypticus* was affected by the study environment (Fig. 5.10b). The number of juveniles produced was significantly lower in B (≈4) than in the other environments, followed by P and FA (≈27-41). The environment P+S, DP+S and FN showed significant higher number of juveniles (≈150-205), without differences among them. Bare soils (B) showed ≈50 fold higher levels of water soluble metal(loid)s (expressed as ΣTU) than the other study environments (Fig. 5.10c), followed by P and FA with ≈13-2 fold higher levels than P+S, DP+S and FN.

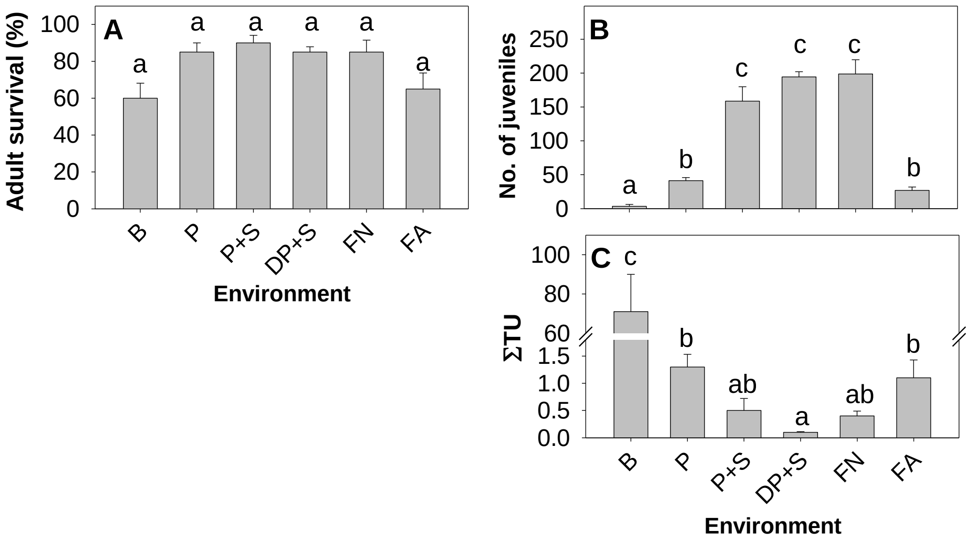

Figure 5.10. A and B. Survival and reproduction of *Enchytraeus crypticus* after 21 days of exposure to the study environments. C. Sum of toxic units (ΣTU) for As, Cd, Cu, Pb and Zn based on water soluble concentrations in soil (1:2.5 water:soil extracts) and EC50 values (concentration causing 50% reduction in reproduction) recorded from the bibliography. Columns represent average values and bars on columns standard error (n=4). Different letters indicate significant differences among environments (one-way ANOVA with Tukey post hoc test, $p<0.05$). See Section 5.2 for meaning of the environments (B, P, P+S, DP+S, FN and FA).

5.3.6. Environmental gradient

The results of the PCA analysis (68.9% of total variance explained by the first two axes) compiled the relationships among the whole set of soil indicators (Fig. 5.11). The main gradient (X-axis, total variance explained 45.8%) identified forests soils located outside the mine tailings (FN and FA) on the right side, and soils located within the mine tailings (B, P, P+S and DP+S) on the left side. In agreement with the data showed in Figs. 5.4, 5.6 and 5.7, FA soils were depicted as those with better physical, physico-chemical and biological conditions (the lowest bulk density and the highest CEC, $CaCO_3$, TOC, TN, WOSC, MBC, β-glucosidase activity and CO_2 emissions) but also with the highest NH_4^+-As, W-As and W-Cu. The secondary gradient (Y-axis, total variance explained 23.1%) affected the soils located within the mine tailings, with bare soils (B) highlighted on the positive side as the most saline sites with the lowest pH values and the highest concentrations of NH_4^+-Pb and W-Cd, W-Pb and W-Zn (i.e., the most inhospitable environment). Denser vegetated sites within the mine tailings (P+S and DP+S) with higher TOC, TN, MBC, enzyme activities, CO_2 emissions and TBI (Figs. 5.4 and 5.7) were depicted far from bare soils (B), on the negative side of the Y-axis, and they were characterized by the highest T-As and T-Pb concentrations and the highest *E. crypticus* survival and reproduction. FN soils were located nearer to P+S and DP+S than to FA, despite belonging to a forest outside the mine tailings. The P environment had an intermediate position regarding the secondary gradient, between bare soils (B) and denser vegetated sites (P+S and DP+S) within the mine tailings.

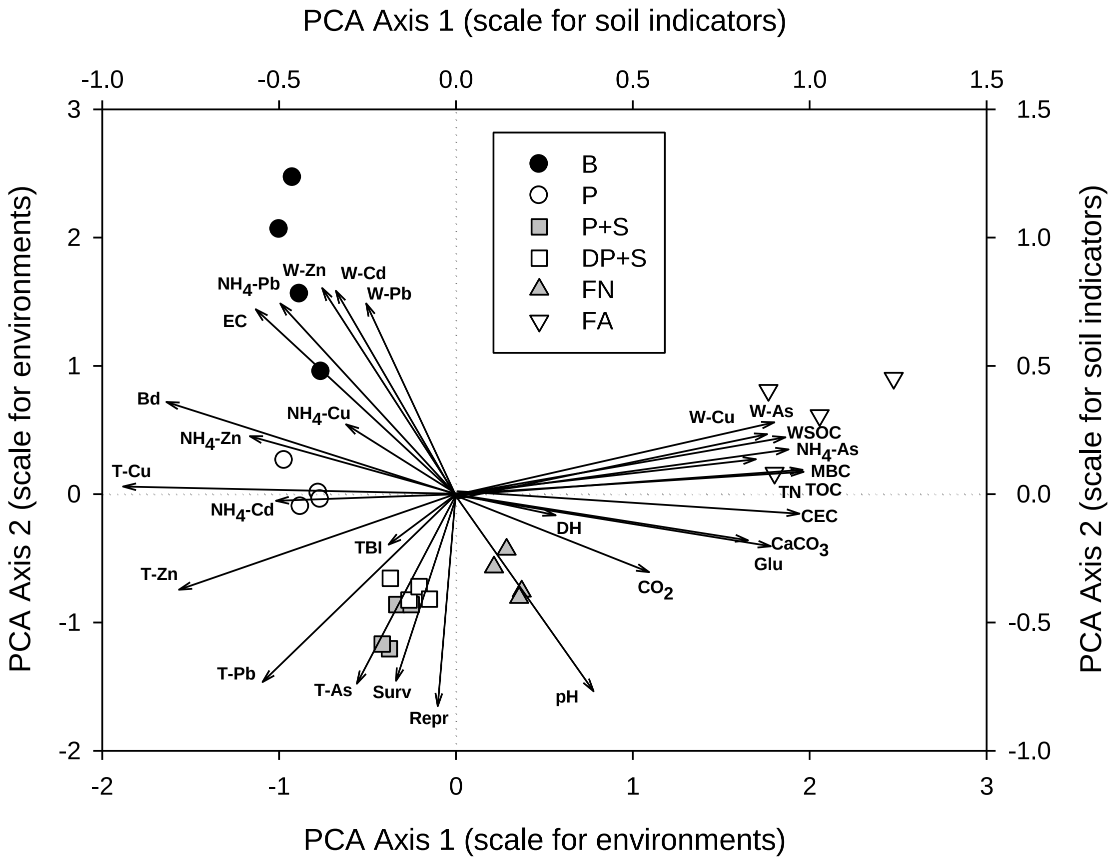

Figure 5.11. Results of the Principal Component Analysis (PCA). Cumulative percentage of variance explained by the first two axes: 68.9%. Bd: bulk density; EC: electrical conductivity; CEC: cation exchange capacity; TOC: total organic carbon; TN: total nitrogen; WSOC: water soluble organic carbon; T-metal(loid): total metal(loid); NH_4-metal(loid): NH_4^+ soluble metal(loid); W-metal(loid): water soluble metal(loid); MBC: microbial biomass carbon; DH: dehydrogenase activity; Glu: β-glucosidase activity; CO_2: soil CO_2 emissions; TBI: tea bag index; Surv: *E. crypticus* survival; Repr: *E. crypticus* reproduction. See Section 5.2 for meaning of the environments (B, P, P+S, DP+S, FN and FA).

5.4. Discussion

We explored the relationships between spontaneous vegetation colonization of metal(loid) mine tailings from Mediterranean semiarid areas and a set of soil indicators. Our results suggest that mine tailing soils can be effectively modified following spontaneous colonization, achieving a state with better capacity to provide ecosystem functions. The latter is of great interest for the phytomanagement of land destroyed by mining activities, a process that has been considered comparable to the earlier stages of primary succession (Huang et al., 2012).

5.4.1. Pedo-genetic development, physical and physico-chemical soil conditions and metal(loid) behavior

The variation pattern of the soil indicators measured illustrates how mine wastes stored in abandoned barren tailings can improve their edaphic attributes following spontaneous vegetation colonization, without total metal(loid) levels being a major limiting factor for plant establishment and soil functions improvement. In vegetated sites the substrate changed from just being a compacted platy material to porous granular aggregates around roots and plant remains, with similar physical features to the surrounding forest soils (Table 5.3). The latter influences a number of interconnected soil parameters including physical, physico-chemical and biological. Platy cemented layers contribute to immobilize metals by precipitation of stable mineral phases. However, they also act as hydraulic barriers that reduce pore water transport, drainage and metals and salts leaching. Moreover, these structures are barriers inhibiting air diffusion into the soil (Pellegrini et al., 2016 and references cites therein), which hinders soil biota activity. Huang et al. (2012) stated that these changes are driven by drying-wetting cycles and associated variations in physico-chemical conditions (e.g., pH, salinity) but not in total metal(loid) concentrations, and described three main processes implied in soil formation in mine tailings: 1) hydro-geochemical stabilization, which implies a decrease in the capacity to provide extreme acidity, salinity and soluble metals; 2) establishment of a microbial community; 3) development of ecological linkages between root zones and plant communities. In our study, a higher salinity (EC) and a lower pH were outstanding parameters of bare soils (B) (Figs. 5.4 and 5.11), mainly attributable to the oxidation of mine wastes sulfide to sulfate (García-Lorenzo et al., 2012), the main ion contributing to EC (Fig. 5.5). Moreover, lower pH led to greater availability of Pb, Cd and Zn in B, as shown by the high concentrations of NH_4^+-Pb, W-Pb, W-Zn and W-Cd (negative correlations pH vs. NH_4^+-Pb, W-Pb, W-Zn and W-Cd; $r \leq -0.718$, $p \leq 0.001$), even if the highest total metal(loid)s concentrations were not reached in this environment (Figs. 5.4 and 5.11). Plants may also contribute to reduce metal(loid) soil availability by up taking or immobilizing them in the rhizosphere. Several studies in this mining area have analyzed the content of different metal(loid)s in the tissues of a variety of plant species and in their corresponding rhizospheric soils. Although a consistent pattern of decreasing soil metal(loid) concentrations beneath the plant canopy has not been found, low translocation factors to aerial parts have been described (Martínez-Sánchez et al., 2012; Párraga-Aguado et al., 2014a; Navarro-Cano et al., 2018), which indicates a low risks of metal(loid) transfer to the food web via consumption of aerial plant parts.

Although initial microtopographic or microclimatic differences within a tailing, or between tailings, might influence early stages of pedogenesis and plant colonization, the results showed the important role of colonizer vegetation in modifying conditions under the canopy. In fact, the B and P environments were located in the same tailing only 25 m apart, so it is reasonable to assume that initial microtopography and microclimatic conditions were similar just after the abandonment of the mine tailing. However, temperature and moisture data showed that vegetated soils (P) were drier and colder than unvegetated ones (B). A lower soil temperature in vegetated sites has been related with the shadow effects of the plants canopy (Oreja et al, 2020). A lower soil moisture can be attributable to two main factors: i) the coarser texture in P (Table 5.2) due to the accumulation of sand particles blown up by wind that may form small pedestals beneath plants canopy (Navarro-Cano et al., 2018), which, together with a higher porosity (as shown by a lower bulk density), increase drainage capacity; ii) soil water uptake by plants. The CEC, total $CaCO_3$, TOC, TN, MBC and WSOC were positively correlated among them ($r \geq 0.728$, $p \leq 0.041$) and negatively correlated with EC and bulk density ($r \leq -0.796$, $p \leq 0.001$) (Fig. 5.11). A decrease in bulk density is a clear evidence of the development of soil physical structure, a process triggered by increases in organic matter content and microbial activity that are linked to biogeochemical cycles (e.g., organic matter turnover). Litter incorporation and surface aggregate stability has been considered the most strongly associated attributes with soil multifunctionality in drylands (Eldridge, 2019). In fact, soil structure is of paramount importance to deliver ecosystem functions since the volume of pores, chambers, channels and cracks provides a suitable environment for soil biota and the growth of plant roots (Morgado et al., 2018; Rabot el at 2018). This agrees with the increase of $CaCO_3$ observed in vegetated tailing environments (P, P+S, DP+S) relative to bare (B), since this compound can form through dissolution of CO_2 emitted during organic matter decomposition and roots respiration into HCO_3^-, and precipitation with pore water Ca^{2+} (De Soto et al., 2019). Moreover, structure also contributes to the incorporation of plant remains, water and air storage and improves hydraulic conditions favoring solute movement including salts and soluble metal(loid) lixiviation due to better drainage conditions (Pellegrini et al., 2016).

In agreement with previous works (Párraga-Aguado et al., 2013), lower total metal(loid) concentrations in soils away from the mine tailings (FA) were expected. According to García-Lorenzo et al. (2012), B, P, P+S and DP+S can be considered as environments with primary contamination, FN with secondary contamination, and FA with tertiary contamination. However, changes in total metal(loid)s did not match with the variations in available metal(loid) concentrations. Metal(loid) availability depends on the geochemical behavior and this is related not only to pH but also to soil components (Simón et al., 2010, 2015). Palumbo-Roe et al. (2009) compared two mining soils with similar total Pb concentrations and indicated that Pb dissolution was 10-fold higher when it was controlled by carbonates than by complexation to oxy-hydroxides. Simón et al. (2010) also indicated that $CaCO_3$ was less relevant for Pb immobilization than Fe-oxides. In agreement with the previous findings, in our study, a decrease in W-Pb was observed when total Fe concentrations increased (negative correlation between both parameters, $r=-0.759$, $p<0.01$; Table 5.4), while W-Pb and total $CaCO_3$ were uncorrelated. FA soils showed higher W-Pb than the other study environments, except for bare soils (B), even though they had by far the lowest total metal(loid) concentrations (Figs. 5.6 and 5.11).

Although the lowest T-As was found in FA soils, NH_4^+-As and W-As (available forms) in this environment were consistently higher than in all the others including bare soils (B) (Figs. 5.6 and 5.11). The previous results can be explained by the geochemical behavior of As, which is controlled by factors such as redox conditions, pH and organic matter presence. Simón et al. (2010, 2015) demonstrated that an increase in labile organic matter can led to an increase in water soluble As and that As fixing is limited under conditions of high pH and $CaCO_3$ content. Under these conditions the oxyanion $HAsO_4^{2-}$ is the dominant specie, which displays low adsorption (or desorption) on solid surfaces and low co-precipitation with Fe oxy-hydroxides (Simón et al., 2015; González et al., 2012). In fact, in our study, NH_4^+-As and W-As were strongly correlated with WSOC ($r>0.860$, p<0.001) and with pH and $CaCO_3$ (r>0.675, p<0.001). The former authors noted that Fe-oxides were very efficient for reducing the mobility and availability of As in the absence of $CaCO_3$. Hence, the low availability of As in B, P, P+S and DP+S could be attributable to the substantial content of this metalloid bounded to amorphous and crystalline Fe-oxides in this kind of mine wastes (Párraga-Aguado et al., 2015).

Table 5.4. Fe total concentrations in the study environments. B: bare soils; P: small groups of *Pinus halepensis* trees; P+S: isolated *P. halepensis* trees + plants under the canopy; DP+S: dense patches with *P. halepensis* trees + plants under the canopy; FN: forest next to the mine tailings; FA: forest away from the mine tailings. Values are average ± standard error (n=4).

Environment	**Fe (g 100 g^{-1})**
B	15.3 ± 0.34
P	15.8 ± 0.26
P+S	29.2 ± 1.26
DP+S	22.5 ± 0.37
FN	17.4 ± 0.56
FA	2.7 ± 0.17

As observed for other metal(loid)s, increases in T-Cu did not imply increases in NH_4^+-Cu and W-Cu (Figs. 5.6 and 5.11). In fact, W-Cu was strongly correlated with WSOC (r=0.783, p<0.001) and the highest concentration of this metal in water extracts was found in FA soils. Experimental studies have shown that Cu forms complexes with organic compounds, leading to increasing concentrations of this element in soil pore water (Pardo et al., 2016; Párraga-Aguado et al., 2017).

The concentrations of W-Zn and W-Cd consistently dropped from B to FA and were negatively correlated with pH and $CaCO_3$ content (r<-0.625, p<0.01) (Figs. 5.6 and 5.11). Our results agree with the leaching experiments performed by Simón et al. (2010), in which decreases in porewater Zn and Cd concentrations were observed when $CaCO_3$ increased. However, NH_4^+-Zn and NH_4^+-Cd did not linearly decrease from B to FA. In fact, the P environment showed the largest concentrations of both NH_4^+- soluble metals (Fig. 5.6), which might reflect the variability in the composition and properties of the wastes stored in mine tailings (Conesa and Schulin, 2010).

5.4.2. Vegetation colonization and soil biological activity, ecotoxicity and functionality

When plants colonize mine tailings they provide organic matter, which is gradually buried and leads to an increase of biological activity, reducing the influence of climate as a driver factor of pedogenesis and increasing the role of biological processes (Sun et al., 2018). In our study, the latter is shown by the positive correlations among soil CO_2 emissions, MBC, enzyme activities and TBI ($r=0.454$, $p=0.026$) and between MBC and enzyme activities ($r= 0.849$, $p<0.01$). At this stage, the rhizosphere becomes a crucial microenvironment not only for providing organic carbon through organic remains and roots exudates, but also as physical support for microorganisms to adhere to and perform key processes necessary for element transformations (Caffery and Kemp, 1990; Hinsinger et al., 2009). Risueño et al. (2020) found a synergistic interaction between native microbial communities and spontaneous vegetation colonizing mine tailings soils and stated that the presence of plants stimulated the existence of bacterial groups involved in soil biogeochemical cycles. In relation with this issue, plant species assemblages with contrasting life forms (e.g., terophytes, cryptophytes, chamaephytes, phanerophytes) have more capacity to be self-sustainable, promote soil microbial diversity and improve key ecosystem functions such as primary productivity, nutrient retention and resilience against disturbance (Hooper et al., 2005). In addition, more heterogeneous plant litter composition can favor diversity of decomposers and this stimulates microbial processes related to organic matter turnover which, in turn, has favorable feedback effects to plant growth and belowground ecosystem functions (Hättenschwiler et al., 2005; Meier and Bowman, 2008).

Differently to Ramsey et al. (2005), we did not find a linear decrease in soil CO_2 emissions when increasing soil metal(loid) concentrations (Figs. 5.6 and 5.7). By contrast, our data showed high soil CO_2 emissions in mine tailings environments (Fig. 5.7), which were significantly correlated with TBI values ($r=0.454$, $p<0.026$), providing evidence of soil heterotrophic microbial activity even with low MBC and β-glucosidase activity. The latter can be attributable to the ecosystem redundancy capacity, which allows functioning maintenance due to the redundant functions of the tolerant microbial populations (Bradfor and Fierer, 2012).

In agreement with other studies (Kuramae et al., 2011; Azarbad et al., 2013), the results for soil CO_2 emissions, TBI, feeding activity and ecotoxicity demonstrate that even in strongly metal(loid)-affected sites such as mine tailings it is possible to reach acceptable soil functions. Other authors have also pointed that microbial activity in metal-polluted bare soils is not only disrupted by the toxic effects to microorganisms, but also by the scarce organic matter and nutrient contents due to the absence of vegetation cover (Konopka et al., 1999). Zhan and Sun (2012) showed that microbial diversity and structure in rhizosphere and bulk soils from mine tailings were mainly influenced by the presence of pioneer plants and the environmental conditions, especially nutrient elements and Fe contents, alongside with plant community colonization.

Although soil feeding activity was detected in all the study environments, it was scarce, as shown by the low number of fed holes, even in the forests outside the mine tailings (FN and FA) (Fig. 5.9). This could be related to the low moisture content and the high temperature of soils throughout the study period (Fig. 5.2), which could have hindered fauna activity. This agrees with González-Alcaraz et al. (2019) who found that soil enchytraeids lost their capacity to avoid metal(loid)-contaminated soils under water stress situations, and with Simpson et al. (2012) who showed that the feeding activity of soil organisms (determined by bait-lamina sticks) was compromised under drought stress. Within the mine tailings, higher number of baits were consumed in simpler (B and P) than in more complex (P+S and DP+S) environments (Fig. 5.9). This could be attributable to the scarcity of resources in the two former environments and the preference of soil dwelling organisms for local debris in the latter ones, as observed in previous works (André et al., 2009). The results for the environment DP+S were similar to those for FN (Fig. 5.9), in accordance to other soil indicators measured, which supports our hypothesis that mine tailings soils might achieve acceptable functions following spontaneous vegetation colonization.

In our study, the ecological indexes data showed that the richness and heterogeneity of the vegetation cover growing in the mine tailings environments was similar to that in the nearby (FN) and away (FA) forests, except for P (Fig. 5.1). However, the prevalent biological forms and colonizing strategies of the species growing within and outside the mine tailings were different. Our results agree with Shu et al. (2005) and Ginocchio et al. (2017) who showed that Poaceae and Asteraceae were the most represented plant families spontaneously growing in mine tailings soils. Wind dispersion of the high amount of seeds they produce confer to species of these families high immigration and colonization capacity of stressful environments like mine tailings (Shu et al., 2005). The maximum number of pioneer species was reached in P+S and DP+S environments, although they were also abundant in FN due to the vicinity of the mine tailings, and the minimum in FA (Fig. 5.1). Nurse plants, however, were present at similar levels in the mine tailings and forest environments. Pioneer species are efficient in colonizing barren areas, but nurse species, in addition, form facilitation-driven communities with the capacity to drive a cascade of beneficial ecosystem functions (Navarro-Cano el at., 2018). In this sense, phytomanagement should promote the establishment of a variety of nurse plants with different life forms in mine tailings, which can trigger a higher long-term stability of these environments by increasing their resilience against disturbances, such as prolonged dry spells or heavy rains, leading to a better provision of ecosystem functions. For instance, Párraga-Aguado et al. (2014b) related different isotopic $\delta^{18}O$ composition in *P. miliaceum*, *P. halepensis*, *Helichysum decumbens* and *Tetraclinis articulata* (four species with different life forms found in the study environments) with contrasting water use strategies, which favor better use of the scarce hydric resources in semiarid drylands. Moreover, the presence of vegetation increased soil moisture and reduced temperature beneath their canopy (Fig. 5.2), which reduces the climatic stress and has a positive feedback in plant cover by stimulating seeds recruitment (Navarro-Cano et al., 2019; Oreja et al., 2020).

Our results agree with studies showing that erosion can contribute to spread polluted particles to sites away from mine tailings (Sánchez-Bisquert et al., 2017), and that these soils, despite having lower total metal(loid) concentrations, can be more toxic than soils located just in the tailings (González-Alcaraz and van Gestel, 2015, 2016). The latter highlights again the crucial role of phytostabilization by spontaneous vegetation colonization in mine tailings at two levels: by acting as phytobarriers for reducing airborne spread of pollutants and by promoting soil structural development and particle retention (Sánchez-López et al., 2015; Vangronsveld et al., 2009); and by decreasing available metal(loid) concentrations and toxicity (Robinson et al., 2009). A more stable vegetation cover and a better soil structure can reduce PTE availability and dispersion contributing to environmental and human health preservation, one of the main ecosystem functions triggered by the vegetation-soil feedbacks.

In our study, the environment DP+S seems to be the most hospitable tailing site (Fig. 5.11). However, we did not find a progressive linear increase of vegetation indexes encompassing obvious increases for all the soil indicators evaluated. Rather than, we found that after vegetation cover reached a certain status (P+S), the soil seemed to develop minimum conditions to provide valuable functions. The results of the PCA analysis illustrate how within the mine tailings there are inhospitable sites without vegetation cover (B), highly toxic to soil biota (*E. crypticus* reproduction negatively correlated with ΣTU; $r=-0.772$, $p=0.000$), but also vegetated sites (P+S and DP+S) that can provide suitable habitats for soil organisms, regardless of the total metal(loid)s concentrations. Habitat provision is a key ecosystem function for increasing soil biodiversity and other related functions (Bach et al., 2020). It is interesting to note that ecotoxicity indicators had a minor role in the main gradient defined by the PCA's X-Axis. The latter supports that once a combination of improved soil properties allows to achieve a certain state, the role of each individual parameter seems to be not as relevant as in the first stages of the soil development process. According to the previous considerations, ecotoxicity seems to be a key indicator within the mine tailings environments, but its contribution to maintain soil functions is less relevant in the forests, even if they are enriched in some available metal(loid)s (As and Cu). However, further research is needed to establish acceptable boundaries for this and other indicators influencing ecosystem functions.

5.5. Conclusions

Our findings show that spontaneous vegetation colonization of abandoned metal(loid) mine tailings from Mediterranean semiarid areas induce the improvement of physical, physico-chemical and biological soil indicators.

In agreement with previous studies, our results point the key role of pioneer and nurse plant species in the colonization process of barren tailings and the subsequent development of soil functions. Once the vegetation growing in the tailings reaches a certain status, the improvement of soil conditions allows to achieve acceptable soil functions regardless of the metal(loid) levels. When the latter occurs, the role of each individual parameter seems to be not as relevant as in the first stages of the soil development process. A concomitant improvement of vegetation cover and soil conditions in the mine tailings ensures the provision of valuable ecosystem functions such as nutrient retention, habitat provision and pollutant dispersion reduction. In fact, even if low total metal(loid)s were found in the forest away from the mine tailings, the presence of high levels of available As and Cu and of ecotoxicity risk in this environment emphasizes the main role of the vegetation growing in tailings as phytobarrier for reducing the spread of pollutants and preserve environmental and human health. Hence, our findings support the value of passive restoration as a suitable option for the regeneration of abandoned metal(loid)s mine tailings from semiarid drylands. This does not imply that spontaneous vegetation colonization has the capacity, by itself, to fully restore ecosystem functions and eliminate environmental hazards of abandoned mine tailings. Rather than, our results show that spontaneous colonization by native vegetation should be seriously considered as a valuable complementary alternative to others. Moreover, the management of these structures, either by conventional techniques and/or phytomanagement, should be preceded by a detailed knowledge of the already existing spontaneously colonized sites (i.e., fertility islands), which should be preserved to take advantage of their high potentiality.

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CHAPTER 6

The relationships between functional and physicochemical soil parameters in metal(loid) mine tailings from Mediterranean semiarid areas support the value of spontaneous vegetation colonization for phytomanagement

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Abstract

Spontaneous growth of native vegetation in abandoned metal(loid) mine tailings can be valuable for phytomanagement restoration projects. This study aimed to assess the degree to which spontaneous plant colonization of abandoned metal(loid) mine tailings from Mediterranean semiarid areas led to functional soil improvement, and to identify, if possible, a critical level indicating that this functionality was moving towards that of the vegetated soils from the surroundings. Vegetation ecological indexes, plant life forms and species functional roles, together with physicochemical and functional soils parameters, were studied in metal(loid) mine tailings abandoned ≈40 years ago and surrounding forests in SE Spain. Vegetation patches showed only small differences in physicochemical parameters related to soil abiotic stress conditions (pH, salinity and metals), regardless of the vegetation. However, vegetation patches with greater species diversity and richness and presence of plants with contrasted life forms and functional traits that facilitate the growth of less stress-tolerant species showed an increase of the soil microbial functionality (higher microbial biomass C, β-glucosidase activity, bacterial metabolic activity and functional diversity). Moreover, these vegetation patches showed a functional soil status comparable to that of the forests outside the mine tailings. In this sense, the present study showed the value of preserving these vegetation patches since they may act as nucleation spots favoring positive plant-soil feedbacks that may help to accelerate the functional recovery of these degraded areas. Furthermore, strategies to promote the creation of new vegetation patches including a variety of species with contrasted life forms and functional traits should be considered in phytomanagement restoration projects for abandoned metal(loid) mine tailings.

Keywords: Drylands; Mine wastes; Fertility islands; Community-level physiological profile; Microbial functional diversity; Soil functioning

6.1. Introduction

As explained in Chapter 1 (see Section 1.2), the hostile conditions offered by mine tailings waste often hinder plant colonization and lead to low-biologically active tailing soils, which restricts their functionality and the provision of ecosystem services (Niemeyer et al., 2012; Brown et al., 2014). Conventional remediation techniques such mine wastes removal and washing, or the use of capping materials and afforestation for *in situ* wastes isolation, are expensive and often technically difficult to implement (Mendez and Maier, 2008). Alternative options, useful for ecosystem functions restoration, include a set of cheaper and environmentally friendly alternatives such as phytomanagement by phytostabilization (Mendez and Maier, 2008; Robinson et al., 2009; Antoniadis et al., 2017; Burges et al., 2018). The implementation of this option can take advantage from the study of the native vegetation that spontaneously colonizes mine tailings and the soils beneath (i.e., passive restoration) (Prach and Hobbs, 2008; Gutiérrez et al., 2016; Prach and Tolvanen, 2016).

This chapter includes the work planned to respond to the second specific objective of the PhD Thesis (see Section 3.1): To assess to what degree spontaneous plant colonization of abandoned metal(loid) mine tailings from semiarid Mediterranean areas led to functional soil improvement. Furthermore, the objective was also to identify, if possible, a critical level indicating that this functionality was moving towards that of the natural vegetated soils from the surrounding areas. We did not intend to establish cause-effect relationships relative to successional trajectories of vegetation and soil microorganisms, but to assess the benefits of preserving these spontaneous vegetated patches as potential nucleation spots favoring positive feedbacks between vegetation and soils for ecosystem restoration (Corbin and Holl, 2012). Moreover, the gained knowledge will help to highlight the value of restoration strategies based on promoting the creation of new vegetation patches in mine tailings phytomanagement programs. For this purpose, we carried out an evaluation of the vegetation and the analysis of a set of microbiological soil parameters in tailings vegetated patches with different physiognomy and plant composition and surrounding forests, within an abandoned mining area in southeast Spain, and related to the physicochemical soil conditions.

6.2. Materials and methods

This chapter contains the data corresponding to the field work campaign carried out in spring 2018 in the selected study environments: bare soils (B); patches with small groups of *Pinus halepensis* trees ≈2.5-5 m high growing scattered (P); patches formed by isolated *P. halepensis trees* >≈4-5 m high growing scattered with shrubs and herbs under the canopy (P+S); dense patches including several *P. halepensis* trees >≈4-5 m high and shrubs and herbs under the canopy (DP+S); forest located next to the mine tailings with *P. halepensis* trees >≈5 m high and shrubs and herbs under the canopy (FN); forest located away from the mine tailings (≈1600-1800 m) with *P. halepensis* trees >≈5 m high and shrubs and herbs under the canopy (FA). In particular, the following parameters are displayed (for a complete description of the methodologies used see Chapter 4):

- Vegetation parameters. Plant cover; number of species; number of individuals per species; family; life form; functional group; functional role as pioneer/nurse plant in the colonization process; Margalef richness index (R); Shannon-Weaver heterogeneity index (H′); Pielou evenness index (J′).
- Soil parameters. Particle size distribution; total $CaCO_3$; total organic carbon (TOC); total nitrogen (TN); total metal(loid)s concentration (T-metal(loid)s); pH; electrical conductivity (EC); water soluble metal(loid)s (W-metal(loid)s); water soluble organic carbon (WSOC); water soluble organic nitrogen (WSON); microbial biomass carbon (MBC); β-glucosidase activity (β-glu); community-level physiological profile (average well-color development – AWCD, substrate average well-color development – SAWCD, substrate richness index – S, Shannon-Weaver heterogeneity index – H', and Pielou evenness index – J′).

Related to the statistical analyses, Detrended Correspondence Analysis (DCA) was applied to plant species cover in the study environments with 'CANOCO for Windows' v4.02 (ter Braak and Smilauer, 1999). The rest of the statistical analyses were performed with IBM SPSS Statistics 24 and PRIMER v6 software packages. Data were transformed when they did not fulfil the assumptions of normal distribution (Shapiro-Wilk's test) and/or homogeneity of variances (Levene's test). One-way ANOVA followed by Tukey post-hoc test was used to check for differences among the study environments. Repeated measures

ANOVA (RM-ANOVA) was used to check for the evolution of AWCD over time. The factors included in the analysis were: 1) an inter-subject factor, the environment, with six levels (B, P, P+S, DP+S, FN, and FA); 2) an intra-subject factor, the time - repeated factor, with many levels as sampling times (9). Univariate F statistics using a corrector index of epsilon were applied when data did not fulfil the Mauchly's sphericity requirement (SPSS Inc., 2006). A significant effect of time indicates that AWCD evolves significantly over the incubation period. A significant effect of time x environment interaction indicates that the evolution of AWCD over the incubation period differed among the study environments. A significant effect of environment indicates that the average AWCD value differed among the study environments. When the environment was a significant factor, Bonferroni post-hoc test was used to identify differences. Pearson's correlations were performed to evaluate the relationships among all the assessed parameters.

A two-dimensional Principal Coordinate Analysis (PCoA) was used to examine the whole dataset. The PCoA was performed based on the Bray-Curtis similarity after the Biolog EcoPlate matrix was square root transformed (without restrictions – 0.06 and 0.25). A matrix gathering the carbon utilization by the microbial community (31 carbon substrates) per study environment (B, P, P+S, DP+S, FS, and FA) was used to calculate a Euclidean distance similarity matrix. This similarity matrix was simplified through the calculation of the distance among centroids matrix based on the carbon utilization per each environment. Briefly, the centroid is the arithmetic mean for a group of data points in an n-dimensional space. Thus, in this study, 4 replicates per each environment were used to construct these centroids. The soil parameters presenting good correlation with the Biolog EcoPlate matrix (Pearson's correlation coefficient ≥0.5) are indicated as vectors in the PCoA plot.

6.3. Results

6.3.1. Vegetation characteristics

In mine tailings bare soils (B) the only plant species found was the hemicryptophyte *Zygophyllum fabago* (Table 6.1). The individuals recorded (19 in total) were very small (<≈2.5 cm), with a low plant cover (<≈1%), and they died after few weeks. Therefore, these data were not considered for the evaluation of the vegetation. In the case of the vegetated environments (P, P+S, DP+S, FN, and FA) a total of 36 plant species belonging to 20 families were recorded (Table 6.1). Fewer species appeared in P (2; significantly lower) and FA (5) compared to the other vegetated environments (8); and no significant differences were observed for the number of individuals (≈12-61) (Table 6.2). In the P environment only two plant families were growing (Compositae and Pinaceae), while the rest of the vegetated environments counted with 7-11 families (Table 6.1). Therophytes (annual plants) were only present in P (17%) and P+S (3%), but the other life forms were found in the rest of the vegetated environments (Tables 6.1 and 6.2). The number of pioneer and nurse plants were higher in P+S and DP+S (Tables 6.1 and 6.2). Among the pioneer species appeared the therophyte *Sonchus tenerrimus* (annual plant), hemicryptophytes such as *Leontodon taraxacoides* (dwarf shrub), and chamaephytes such as *Brachypodium retusum* (perennial grass) and *Helianthemum syriacum* (dwarf shrub) (Table 6.1). Apart from the tree species *P. halepensis*, other nurse plants present in tailing environments included the hemicryptophytes *Lygeum spartum*, *Stipa tenacissima*, *Hyparrhenia sinaica* and *Piptatherum miliaceum* (perennial grasses), the nanophanerophyte *Chamaerops humilis* (a shrub-like clumping palm), and the chamaephytes *Teucrium carthaginense*, *Helichrysum decumbens* and *Thymus hyemalis* (dwarf shrubs) (Table 6.1). The Shannon-Weaver (H´, diversity) and Margalef (R, richness) indexes were different in P+S and DP+S from the forests outside the tailings (FN and FA) (H´ ≈1.8-2.6; R≈1.6-2.4), but Pielou evenness index was quite similar in all of the vegetated study environments (J´ ≈0.7-0.9) (Table 6.2).

Table 6.1. Plant species, family, life form, functional group, and total number of individuals of the plant species recorded in the study environments. See Section 6.2 for meaning of the environments (B, P, P+S, DP+S, FN and FA). Functional group: Ds: dwarf shurbs; S: shrubs; Pg: perennial grass; H: herb; T: tree. Functional role: P, pioneer species; N, nurse species; n.d., not defined.

Plant species	Abbr. for Fig.6.1	Family	Life form	Functional group[a]	Functional role[b]	Total number of individuals					
						B	P	P+S	DP+S	FN	FA
Arenaria montana L.	Am	Caryophyllaceae	Chameophyte	Ds	n.d.			123			
Asparagus acutifolius L.	Aa	Liliaceae	Hemicryptophyte	Ds	P				1	3	
Asparagus horridus L.	Ah	Liliaceae	Hemicryptophyte	Ds	P			1		2	
Brachypodium retusum (Pers.) Beauv.	Br	Gramineae	Chameophyte	Pg	P			1	3	4	
Calicotome intermedia (C. Presl) Guss.	Ci	Leguminosae	Nanophanerophyte	S	P						13
Chamaerops humilis L.	Ch	Arecaceae	Nanophanerophyte	S	N			2			4
Cheirolophus intybaceus (Lam.) Dostál	Cy	Compositae	Hemicryptophyte	Ds	n.d.					7	
Convolvulus althaeoides L.	Ca	Convolvulaceae	Hemicryptophyte	Ds	P				6		
Eryngium campestre L.	Ec	Umbeliferae	Geophyte	Ds	n.d.					1	
Helianthemum syriacum (Jacq.) Dum. Cours.	Hs	Cistaceae	Chameophyte	Ds	P			1	5		
Helichrysum decumbens (Lag.) Camb	Hd	Compositae	Chameophyte	Ds	N				3		
Hyparrhenia sinaica (Delile) Llauradó	Hy	Gramineae	Hemicryptophyte	Pg	N			55			
Lapiedra martinezii Lag.	Lm	Amaryllidaceae	Geophyte	Ds	n.d.						6
Leontodon taraxacoides (Willd.) Merat.	Lt	Compositae	Hemicryptophyte	H	P			4			
Limonium carthaginense (Rouy) Hubbard a Sandwith	Lct	Plumbaginaceae	Chameophyte	Ds	N					1	

Plant species	Abbr. for Fig.6.1	Family	Life form	Functional group[a]	Functional role[b]	Total number of individuals					
						B	P	P+S	DP+S	FN	FA
Limonium cossonianum Kunthze, Revis.	Lc	Plumbaginaceae	Chameophyte	Ds	P				23		
Lygeum spartum L.	Ls	Gramineae	Hemicryptophyte	Pg	N			9	5		2
Paronychia suffruticosa (L.) DC.	Pf	Caryophyllaceae	Chameophyte	Ds	N			2			
Phagnalon saxatile (L.) Cass.	Ps	Compositae	Chameophyte	Ds	P			1	5		
Phillyrea angustifolia L.	Pg	Oleaceae	Microphanerophyte	S	n.d.					10	
Phragmites australis (Cav.) Trin.	Pa	Gramineae	Geophyte	Pg	P			4	39		
Pinus halepensis Miller	--	Pinaceae	Macrophanerophyte	T	N		14	14	36	6	4
Piptatherum miliaceum (L.) Cosson	Pm	Gramineae	Hemicryptophyte	Pg	N			7	8		
Pistacia lentiscus L.	Pl	Anacardiaceae	Microphanerophyte	S	n.d.			11	8	1	
Rhamnus lycioides L.	Ry	Rhamnaceae	Microphanerophyte	S	n.d.				4	10	
Rosmarinus officinalis L.	Ro	Lamiaceae	Nanophanerophyte	Ds	n.d.						1
Ruta angustifolia Pers.	Ra	Rutaceae	Chameophyte	Ds	P			3			
Salsola genistoides Juss. ex Poir.	Sg	Amaranthaceae	Nanophanerophyte	S	P					12	
Satureja obovata Lag.	So	Lamiaceae	Chameophyte	Ds	n.d.						1
Sonchus tenerrimus L.	St	Compositae	Therophyte	H	P		11	3			
Stipa tenacissima L.	Ss	Gramineae	Hemicryptophyte	Pg	N					25	3
Tetraclinis articulata (Vahl) Mast.	Ta	Cupressaceae	Phanerophyte	T	n.d.						12
Teucrium capitatum L.	Tc	Lamiaceae	Chameophyte	Ds	P					1	
Teucrium carthaginense Lange	Tct	Lamiaceae	Chameophyte	Ds	n.d.					7	
Thymelaea hirsuta (L.) Endl.	Ty	Thymelaeaceae	Nanophanerophyte	Ds	P			4			
Thymus hyemalis Lange	Th	Lamiaceae	Chameophyte	Ds	N				1		
Zygophyllum fabago L.	--	Zygophyllaceae	Hemicryptophyte	Ds	P	19*					

* Small plants (<≈2.5 cm high), with low cover (<≈1%), and that died in a short time period.

a: Paula et al. (2009); Colin et al. (2019)

b: Navarro-Cano et al. (2018)

The different types of vegetated study environments (P, P+S, DP+S, FN, and FA) were segregated by the DCA ordination analysis based on species cover (27.2% of the total variance explained by the first two axes; Fig. 6.1A), and the results revealed the predominance of species belonging to different functional groups in each environment. FA plots were depicted in the positive side of Axis 1, characterized by shrubs and the tree species *Tetraclinis articulata*, while plots from the mine tailings (P+S and DP+S) and forest nearby (FN) were depicted in the negative side of Axis 1. Dwarf shrubs species tended to increase towards P+S plots, while perennial grasses and shrubs were more related with DP+S and FN plots. A second DCA was applied after excluding FA environment. The results (33.4% of the total variance explained by the first two axes; Fig. 1B) clearer reflected changes in predominant biological forms from P+S to FN environments.

Table 6.2. Vegetation characteristics in the study environments (average ± SE, n=4). See Section 6.2 for meaning of the environments (B, P, P+S, DP+S, FN and FA). Tot. fam: total families. H': Shannon-Weaver diversity index; R: Margalef richness index; J´: Pielou: evenness index. Different letters indicate significant differences among environments (one-way ANOVA followed by Tukey post-hoc test, p<0.05). n.c. (not calculated).

Parameter	Environments					
	B	**P**	**P+S**	**DP+S**	**FN**	**FA**
Total cover	0	100	100	91±4	57.7±7	97±2.5
Species number	0	2±1 a	8±1 b	8±1 b	8±1 b	5±1 a
Individual numbers	0	17±15 a	61±30 a	37±7 a	22±4 a	12±3 a
H'	n..c.	0.81±0.30 a	2.63±0.32 b	2.34±0.24 b	2.51±0.32 b	1.83±0.29 b
R	n.c.	0.48±0.34 a	1.95±0.27 b	2.04±0.11 b	2.45±0.11 b	1.58±0.13 b
J´	n.c.	0.94±0.06 b	0.69±0.08 a	0.80±0.05 ab	0.88±0.02 ab	0.89±0.01 ab
Total families	0	2	10	11	11	7
Total % of life forms						
Therophytes	0	17	3	0	0	0
Geophytes	0	17	16	15	3	16
Hemicryptophytes	0	0	23	15	26	5
Chamaephytes	0	0	19	40	26	10
Micro/Nano phanerophytes	0	0	23	18	32	37
Macrophanerophytes	0	67	16	12	12	32
Functional role: total species (total individuals)						
Pioneer	0	1 (11)	8 (19)	7 (38)	5 (22)	1 (13)
Nurse	0	1 (14)	5 (57)	5 (53)	5 (32)	4 (13)

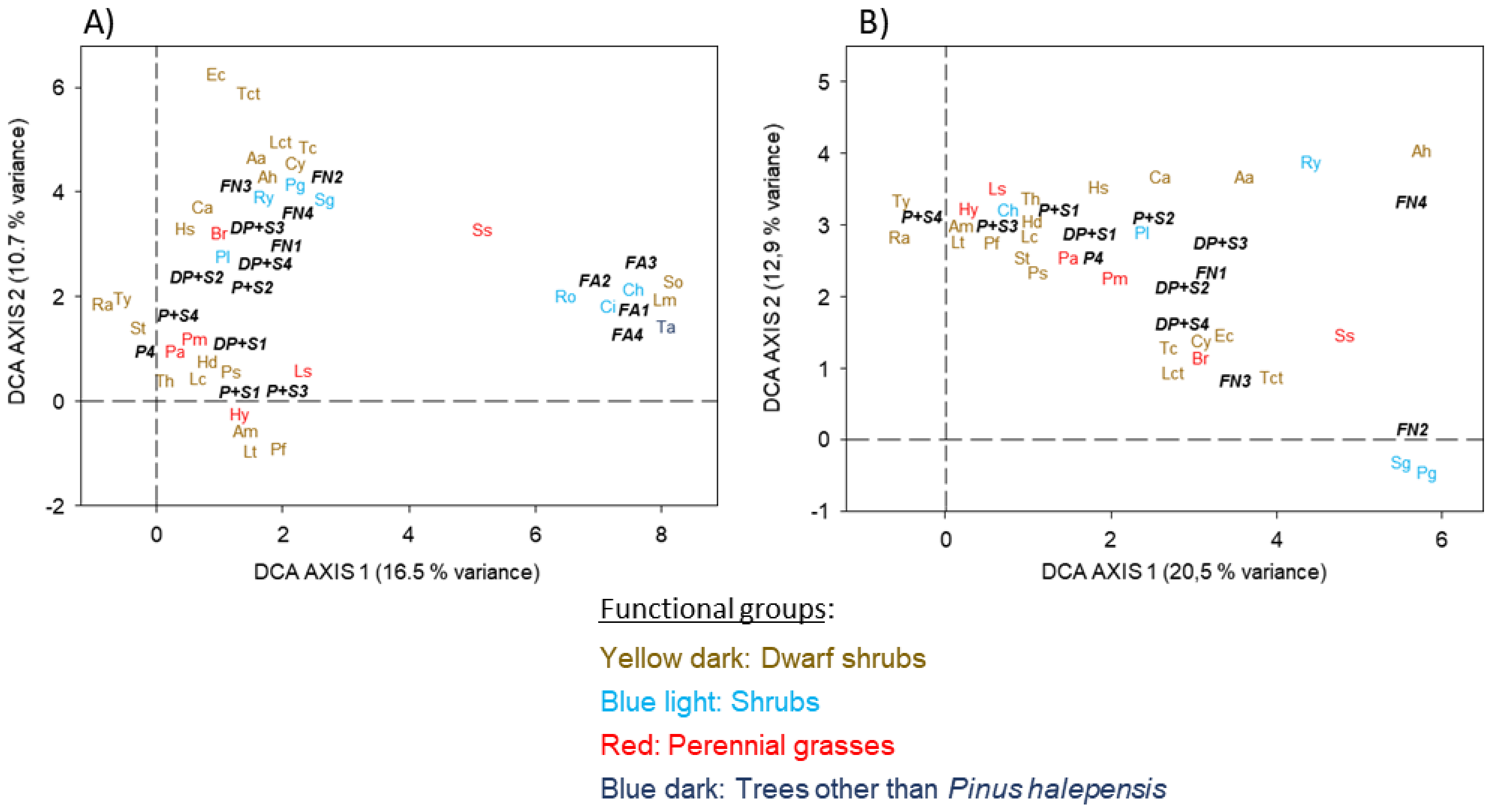

Figure 6.1. Results of Detrended Correspondence Analyses (DCA) carried out with data of plant cover: A) P, P+S, DP+S, FN and FA environments; B) P, P+S, DP+S and FN environments. See Table 6.1 for species abbreviations. See Section 6.2 for meaning of the environments (P, P+S, DP+S, FN and FA). Three P plots with the only presence of *Pinus halepensis* do not appear since this species (common to all environments) was excluded from the analysis.

6.3.2. Soil texture, pH, salinity, total $CaCO_3$ and metal(loid) concentrations

All the study environments had a sandy loam texture (≈59-72% sand, ≈19-23% silt, and ≈6-19% clay), except the P environment which showed a loamy fine sand texture (≈86% sand, ≈10% silt, and ≈5% clay) (data not shown). Soil pH was slightly acid in mine tailings bare soils (B) (≈6.4; significantly lower than in the other environments), while the rest of the study environments showed neutral or slightly basic pH values (≈7.5-7.9) (Table 6.3). Bare soils (B) had significantly higher salinity (EC ≈6.3 dS m^{-1}) than the vegetated environments inside the mine tailings (EC ≈1.3-2.3 m^{-1}) (Table 6.3). The P+S and DP+S environments were the ones showing the closest EC values to the forest soils outside the mine tailings (FN and FA, EC ≈0.5-1.0 dS m^{-1}; no significant differences between DP+S and FA). Total $CaCO_3$ content was significantly lower in B (≈0.6 g kg^{-1}) than in the vegetated environments within the mine tailings (P, P+S and DP+S, ≈28-56 g kg^{-1}) (Table 6.3). Of the latter, the DP+S environment was the one that showed the closest values to the forest soils outside the mine tailings (FN and FA, ≈90-149 g kg^{-1}; significantly higher in FA).

In general, total metal(loid) concentrations were higher in mine tailing soils than in forest soils (Table 6.3). The highest concentration of T-As (≈1251 mg kg^{-1}) and T-Pb (≈14570 mg kg^{-1}) were found in the P+S environment, of T-Cd (≈56 mg kg^{-1}), T-Cu (≈277 mg kg^{-1}) and T-Zn (≈17858 mg kg^{-1}) in the P environment, and of T-Mn (≈10830 mg kg^{-1}) in the DP+S environment. Forest away (FA) soils had significantly lower T-As (≈70 mg kg^{-1}), T-Cd (<10 mg kg^{-1}), T-Cu (≈49 mg kg^{-1}), T-Pb (≈1343 mg kg^{-1}) and T-Zn (≈758 mg kg^{-1}) than the rest of the study environments. Similar to total metal(loid)s, mine tailings soils generally showed higher concentrations of water-soluble metal(loid)s than forest soils (Table 6.3). Inside the mine tailings, bare soils (B) showed significantly higher concentrations of W-Cd (≈1878 µg kg^{-1}), W-Mn (≈11502 µg kg^{-1}), W-Pb (≈1891 µg kg^{-1}) and W-Zn (≈119161 µg kg^{-1}). The water-soluble concentrations of these elements tended to be lower in the DP+S environment that showed the closest values to the forest soils, especially for W-Cd (≈2.5 µg kg^{-1}), W-Mn (≈638 µg kg^{-1}) and W-Zn (≈371 µg kg^{-1}). However, the highest water-soluble concentrations of As and Cu were found in the forest soils outside the mine tailings (FN and FA, W-As ≈28-162 µg kg^{-1} and W-Cu ≈94-158 µg kg^{-1}).

Table 6.3. Soil characterization of the study environments (average ± SE, n=4). Values are expressed on a soil dry weight basis. See Section 6.2 for meaning of the environments (B, P, P+S, DP+S, FN and FA). EC (electrical conductivity). T-Metal(loid) (total metal(loid) concentration). W-Metal(loid) (water soluble metal(loid) concentration). Different letters indicate significant differences among environments (one-way ANOVA followed by Tukey post-hoc test, $p<0.05$).

Parameter	Environment					
	B	**P**	**P+S**	**DP+S**	**FN**	**FA**
pH	6.37 ± 0.35 a	7.55 ± 0.07 b	7.46 ± 0.26 b	7.93 ± 0.12 b	7.77 ± 0.15 b	7.47 ± 0.08 b
EC (dS m^{-1})	6.28 ± 1.29 e	2.31 ± 0.08 d	2.01 ± 0.28 cd	1.33 ± 0.35 bc	0.53 ± 0.03 a	1.0 ± 0.09 b
$CaCO_3$ (g kg^{-1})	0.64 ± 0.07 a	28.2 ± 1.4 b	35.9 ± 11.0 b	56.0 ± 3.8 bc	89.8 ± 10.0 cd	149 ± 35 d
T-As (mg kg^{-1})	223 ± 28 b	253 ± 21 b	1251 ± 165 d	794 ± 24 c	623 ± 27 c	70 ± 11 a
T-Cd (mg kg^{-1})	32.5 ± 8.3 a	56.0 ± 4.1 b	40.5 ± 6.6 ab	38.8 ± 10.2 a	27.8 ± 1.4 a	<10
T-Cu (mg kg^{-1})	233 ± 4 d	277 ± 9 e	198 ± 12 c	179 ± 8 c	142 ± 3 b	49 ± 3 a
T-Mn (mg kg^{-1})	1828 ± 159 a	3253 ± 59 b	9634 ± 1190 c	10830 ± 268 c	7435 ± 234 c	1497 ± 254 a
T-Pb (mg kg^{-1})	5345 ± 373 b	7961 ± 228 c	14570 ± 804 e	10196 ± 243 d	8649 ± 149 cd	1343 ± 243 a
T-Zn (mg kg^{-1})	8596 ± 2213 b	17858 ± 685 c	12210 ± 963 b	13235 ± 704 bc	9227 ± 362 b	758 ± 72 a
W-As (µg kg^{-1})	1.09 ± 0.14 a	4.22 ± 0.44 b	6.36 ± 0.82 bc	11.2 ± 2.1 c	27.6 ± 4.8 d	162 ± 17 e
W-Cd (µg kg^{-1})	1878 ± 685 c	126 ± 29 b	50.7 ± 30.1 ab	2.51 ± 0.69 a	2.51 ± 0.52 a	2.44 ± 0.21 a
W-Cu (µg kg^{-1})	13.2 ± 4.4 a	23.3 ± 4.3 ab	23.1 ± 4.6 ab	48.8 ± 3.8 bc	93.5 ± 23.9 cd	158 ± 9 d
W-Mn (µg kg^{-1})	11502 ± 4379 b	991 ± 211 ab	6442 ± 3461 ab	638 ± 280 a	245 ± 92 a	922 ± 96 ab
W-Pb (µg kg^{-1})	1891 ± 1090 d	19.6 ± 3.2 ab	23.7 ± 10.3 ab	14.7 ± 3.3 a	99.9 ± 21.6 bc	181 ± 11 c
W-Zn (µg kg^{-1})	119161 ± 41229 c	3312 ± 963 b	2529 ± 1456 ab	371 ± 121 a	331 ± 72 a	617 ± 56 ab

6.3.3. Soil organic C and N contents

Mine tailings soils had lower TOC (≈3-36 fold) and TN (≈3-14 fold) contents than forest soils (Figs. 6.2A and 6.2B). Inside the mine tailings, the B and P environments showed significantly lower TOC and TN contents (≈3-4 g kg^{-1} and ≈0.31-0.38 g kg^{-1}, respectively) than P+S and DP+S (TOC ≈11-13 g kg^{-1} and TN≈0.66-0.71 g kg^{-1}). Outside the mine tailings, forest away (FA) soils had the highest TOC (≈99 g kg^{-1}) and TN (≈5 g kg^{-1}) contents. TOC:TN ratios (Fig. 6.2C) significantly increased within the mine tailings from bare soils (B) (≈7) to the P+S and DP+S environments (≈12-17). The latter ones had similar ratios to that of the forest soils outside the mine tailings (FN and FA, ≈20-21).

Mine tailing soils showed lower WSOC (≈2-153 fold) and WSON (≈4-15 fold) concentrations than forest soils (Figs. 6.2D and 6.2E). Within the mine tailings, there was a progressive and significant increase of WSOC concentrations from B (≈8 mg kg^{-1}) to P (≈50 mg kg^{-1}), P+S (≈73 mg kg^{-1}) and DP+S (≈165 mg kg^{-1}) (Fig. 6.2D). The concentrations of WSON were very low in the B and P environments (<1.5 mg kg^{-1}), while increased in P+S and DP+S (≈1.2-5.2 mg kg^{-1}) (Fig. 6.2E). For both WSOC and WSON, the DP+S environment showed concentrations closest to the forest soils outside the mine tailings, mainly to FN (WSOC ≈371 mg kg^{-1} and WSON ≈18 mg kg^{-1}; no significant differences). Outside the tailings, forest away (FA) soils had the highest concentrations (WSOC ≈1223 g kg^{-1} and WSON ≈61 g kg^{-1}; significant). In relation to the WSOC:WSON ratios (Fig. 6.2F), the mine tailings soils showed higher ratios (≈89 in P+S and ≈38 in DP+S) than the forest soils (≈20-22).

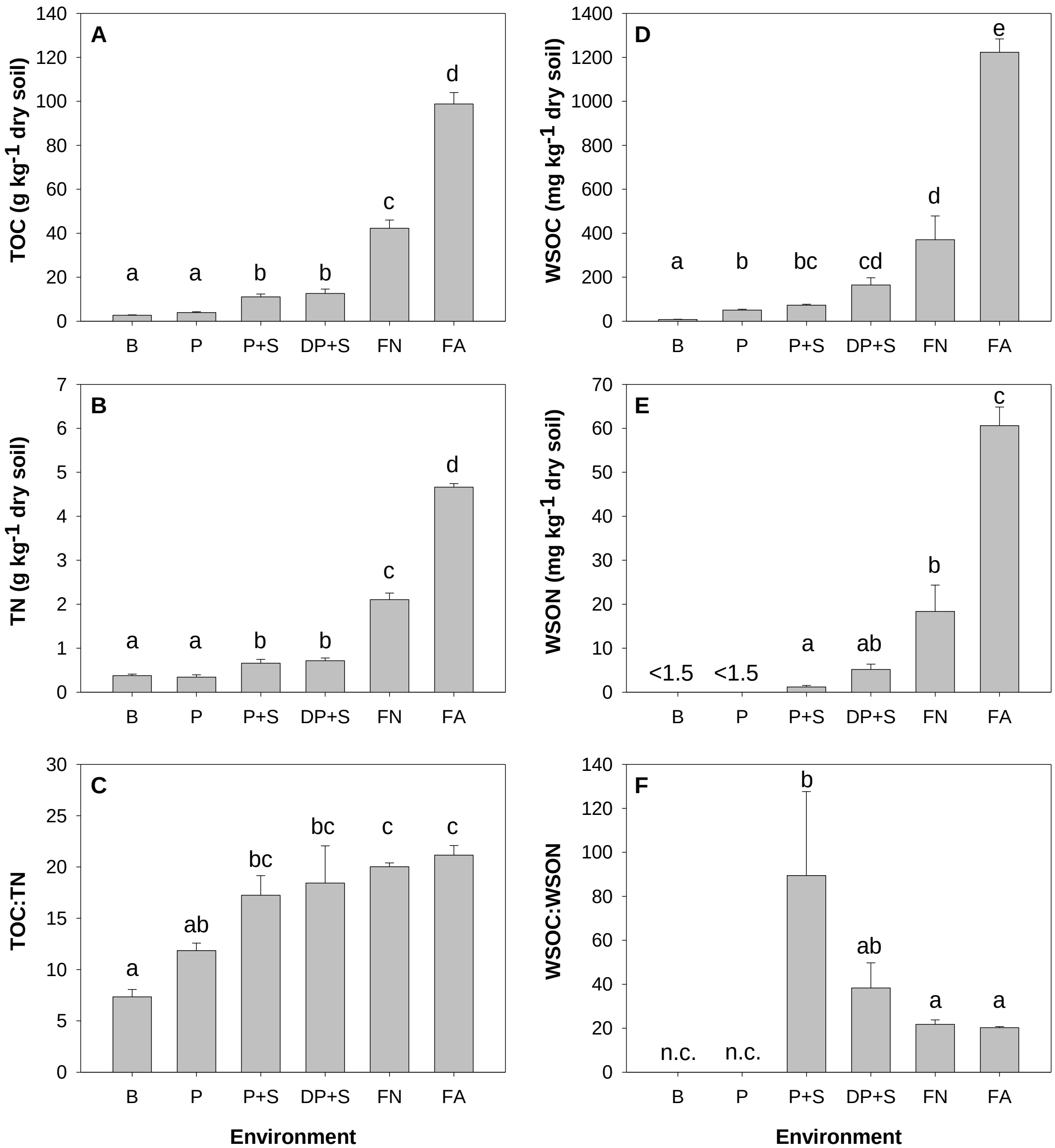

Figure 6.2. Carbon and nitrogen contents in the study environments. Columns represent average values and bars on columns standard error (n=4). Values are expressed on a soil dry weight basis. TOC (total organic carbon). TN (total nitrogen). WSOC (water soluble organic carbon). WSON (water soluble organic nitrogen). Different letters indicate significant differences among environments (one-way ANOVA followed by Tukey post-hoc test, $p<0.05$). n.c. (not calculated). See Section 6.2 for meaning of the environments (B, P, P+S, DP+S, FN and FA).

6.3.4. Soil microbial biomass C and β-glucosidase activity

Mine tailings soils generally showed lower MBC and β-glucosidase activity than forest soils (Fig. 6.3). Inside the mine tailings, a progressive and significant increase of MBC occurred from B (≈12 mg C kg^{-1}) to P (≈132 mg C kg^{-1}), P+S (≈263 mg C kg^{-1}) and DP+S (≈292 mg C kg^{-1}) (Fig. 6.3A). Outside the mine tailings, the FA environment showed the highest MBC content (≈1489 mg C kg^{-1}; significant). Similarly, bare soils (B) had much lower β-glucosidase activity than the vegetated environments inside the mine tailings (Fig. 6.3B). Among the latter ones, the β-glucosidase activity increased from P (≈0.38 µmol pNF g^{-1} dry soil h^{-1}) to P+S and DP+S (≈1.02-1.50 µmol pNF g^{-1} dry soil h^{-1}). In this case, the P+S environment showed the closest β-glucosidase activity values to the forest soils outside the mine tailings (FN and FA, ≈2.10-2.29 µmol pNF g^{-1} dry soil h^{-1}).

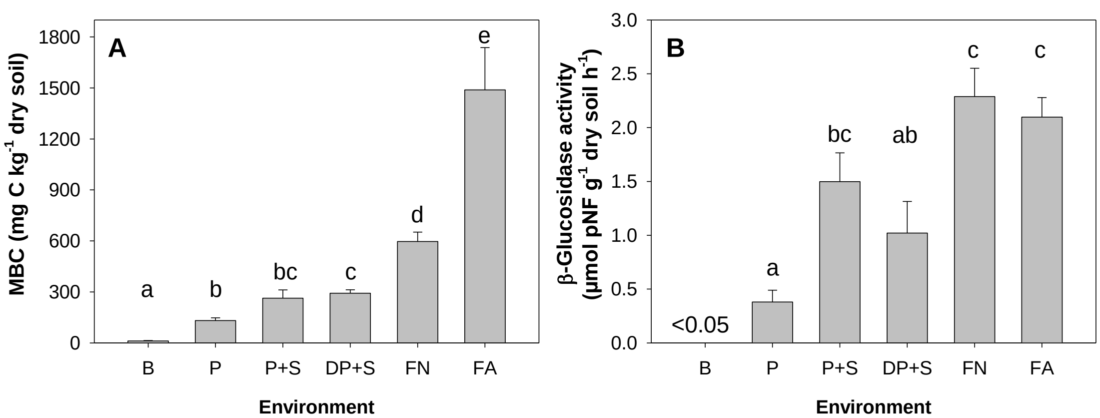

Figure 6.3. Microbial biomass carbon (MBC) and β-glucosidase activity in the study environments. Columns represent average values and bars on columns standard error (n=4). Values are expressed on a soil dry weight basis. See Section 6.2 for meaning of the environments (B, P, P+S, DP+S, FN and FA). Different letters indicate significant differences among environments (one-way ANOVA followed by Tukey post-hoc test, $p<0.05$).

6.3.5. Soil community-level physiological profile

Average well-color development (AWCD) showed a 24 h delay before metabolic reactions began to take place (Fig. 6.4). After that, AWCD tended to increase with time in most of the study environments (significant effect of time, RM-ANOVA) and reached the highest values upon 168-192 h of incubation. This was not the case of mine tailing bare soils (B) that showed a very low consumption of carbon substrates throughout the incubation period (absorbance values at 590 nm <0.06; significant effect of environment and time x environment interaction, RM-ANOVA). Within the vegetated mine tailing soils, AWCD progressively increased from P (from ≈0.06 at 48 h to ≈0.48 at 192 h) to P+S (from ≈0.09 at 48 h to ≈0.70 at 192 h) and DP+S (from ≈0.12 at 48 h to ≈0.93 at 192 h). The DP+S environment showed the closest AWCD values to the forest soils outside the mine tailings (FN and FA, ≈0.19-0.33 at 48 h to ≈0.88-1.14 at 192 h). From all the incubation times registered, the greatest differences observed among the study environments were found at 144 h (Fig. 6.4). Therefore, this incubation time was selected to show the remaining parameters derived from the CLPP analysis (Li et al., 2016).

No significant differences were found for SAWCD among the vegetated study environments (Fig. 6.5), which indicates that they showed similar patterns in terms of consumption of C source groups. In the mine tailings soils the most consumed substrates were polymers (≈26-30% in P and DP+S) and amino acids (≈23% in P+S), while in the forest soils polymers (≈23% in FN) and carbohydrates (≈23% in FA). In all the cases, amines/amides and phenolic acids were the least consumed substrates (≈6-13%). The SAWCD was not calculated for mine tailings bare soils (B) due to the low absorbance values registered. Despite the similar SAWCD values for the vegetated study environments, significant differences were observed for the consumption of some specific substrates (L-asparagine, L-phenylalanine, α-D-lactose, β-methyl-D-glucoside, D-mannitol, D,L-α-glycerol phosphate, N-acetyl-D-glucosamine, and glycogen) (Table 6.4). For these specific substrates, lower consumption was found in the mine tailings soils compared to the forest soils. Inside the mine tailings, the P+S and DP+S environments were those that showed the closest substrate consumption values to the forest soils.

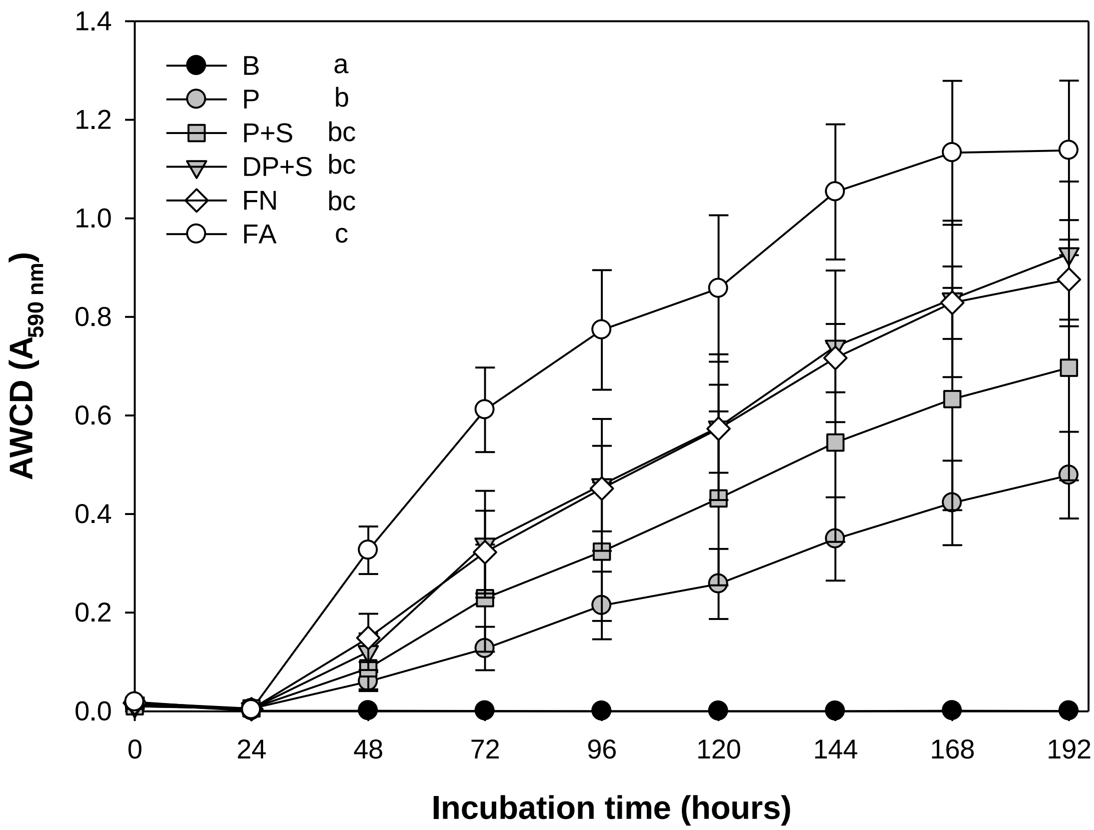

Figure 6.4. Average well-color development (AWCD) at 590 nm in the study environments at the different incubation times (average ± SE, n=4). See Section 6.2 for meaning of the environments (B, P, P+S, DP+S, FN and FA). Different letters indicate significant differences among environments (repeated measures ANOVA followed by Bonferroni post-hoc test, $p<0.05$).

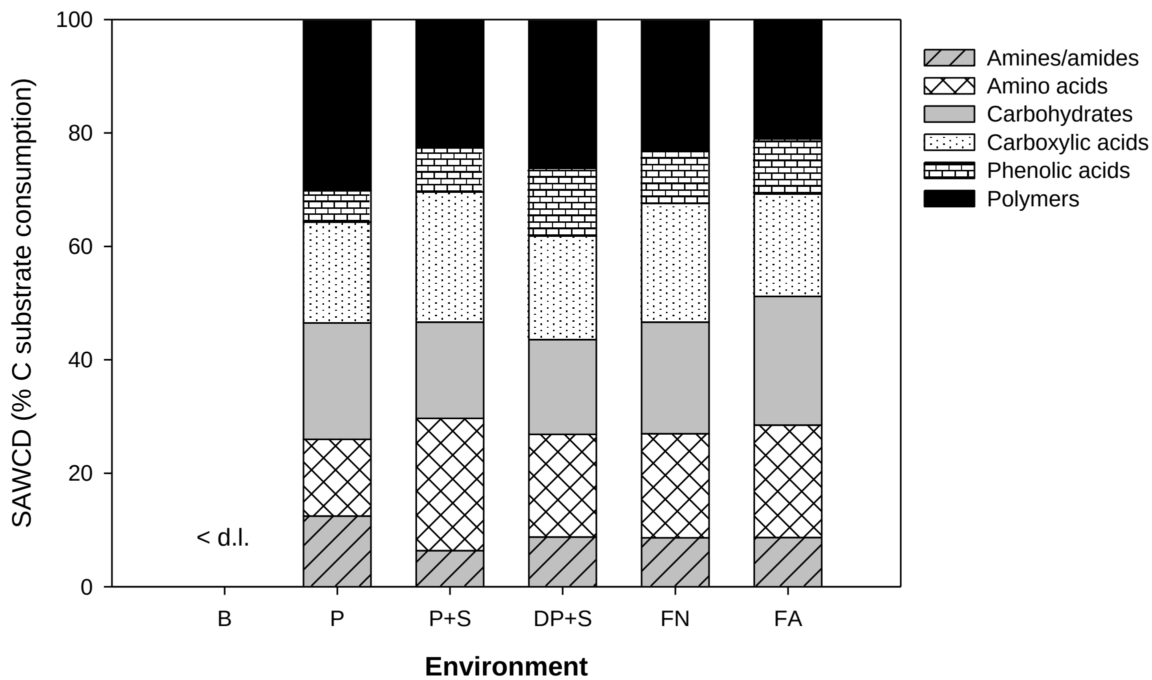

Figure 6.5. Substrate average well-color development (SAWCD) in the study environments at 144 h of incubation expressed as percentage of substrate consumption (n=4). See Section 6.2 for meaning of the environments (B, P, P+S, DP+S, FN and FA). d.l. (detection limit: absorbance values at 590 nm <0.06).

The ecological diversity indices calculated were generally lower in the mine tailings soils than in the forest soils (Table 6.5), although the differences were not so marked as for the other parameters derived from the CLPP analysis. Inside the mine tailings, all the indices increased from P to P+S and DP+S (S: from ≈19 to ≈21-24; H': from ≈2.6 to ≈2.8-3.0; J': from ≈0.91 to ≈0.93-0.94). No significant differences were observed among the P+S, DP+S, FN, and FA environments for S and J'. As for H', no significant differences were observed among all the study environments.

Table 6.4. Consumption of specific carbon substrates in the study environments. Data are absorbance values at 590 nm at 144 h of incubation (average ± SE, n=4). See Section 6.2 for meaning of the environments (B, P, P+S, DP+S, FN and FA). Different letters indicate significant differences among environments (one-way ANOVA followed by Tukey post-hoc test, p<0.05). d.l. (detection limit: absorbance values at 590 nm <0.06).

Carbon source group	Substrate	Environment					
		B	P	P+S	DP+S	FN	FA
Amino acids	L-asparagine	< d.l.	0.67 ± 0.22 a	1.53 ± 0.55 ab	1.90 ± 0.35 ab	1.32 ± 0.19 ab	2.22 ± 0.11 b
Amino acids	L-phenylalanine	< d.l.	0.04 ± 0.02 a	0.34 ± 0.08 ab	0.29 ± 0.15 ab	0.46 ± 0.17 ab	0.63 ± 0.13 b
Carbohydrates	α-D-lactose	< d.l.	0.19 ± 0.06 a	0.21 ± 0.09 a	0.18 ± 0.05 a	0.54 ± 0.14 ab	1.23 ± 0.24 b
Carbohydrates	β-methyl-D-glucoside	< d.l.	0.35 ± 0.14 a	0.25 ± 0.10 a	0.48 ± 0.21 a	0.62 ± 0.12 ab	1.43 ± 0.30 b
Carbohydrates	D-mannitol	< d.l.	0.58 ± 0.18 a	1.07 ± 0.44 ab	1.80 ± 0.44 ab	2.14 ± 0.21 b	1.95 ± 0.42 ab
Carbohydrates	D,L-α-glycerol phosphate	< d.l.	0.09 ± 0.03 a	0.14 ± 0.06 a	0.16 ± 0.04 ab	0.14 ± 0.05 a	0.42 ± 0.10 b
Carbohydrates	N-acetyl-D-glucosamine	< d.l.	0.55 ± 0.20 a	0.77 ± 0.27 ab	1.20 ± 0.34 ab	0.99 ± 0.24 ab	1.73 ± 0.22 b
Polymers	Glycogen	< d.l.	0.06 ± 0.02 a	0.27 ± 0.14 ab	0.59 ± 0.08 abc	1.03 ± 0.27 c	0.88 ± 0.20 bc

Table 6.5. Substrate richness (S), Shannon-weaver index (H´) and Pielou index (J´) in the study environments at 144 h of incubation (average ± SE, n=4). See Section 6.2 for meaning of the environments (B, P, P+S, DP+S, FN and FA). Different letters indicate significant differences among environments (one-way ANOVA followed by Tukey post-hoc test, p<0.05). d.l. (detection limit: absorbance values at 590 nm <0.06).

Index	Environment					
	B	P	P+S	DP+S	FN	FA
Substrate richness (S)	<d.l.	19.00 ± 3.44 a	20.50 ± 3.80 ab	23.75 ± 2.17 ab	26.00 ± 0.82 ab	28.75 ± 0.63 b
Shannon-Weaver (H´)	<d.l.	2.62 ± 0.22 a	2.76 ± 0.20 a	2.95 ± 0.09 a	3.05 ± 0.05 a	3.20 ± 0.04 a
Pielou (J´)	<d.l.	0.91 ± 0.01 a	0.93 ± 0.01 ab	0.94 ± 0.00 ab	0.94 ± 0.01 ab	0.95 ± 0.01 b

6.3.6. Principal coordinate analysis

The results of the PCoA analysis (79.7% of the total variance explained by the first two axes) showed the spatial distribution of each study environment in relation with the metabolic profiling of the soil bacterial communities (based on the combined C substrate utilization values) (Fig. 6.6A). The primary axis (PCoA 1, total variance explained 68.8%) depicted bare soils (B) at the positive side, as the most acidic and saline sites and with the highest concentrations of W-Cd, W-Mn, W-Pb and W-Zn. The secondary axis (PCoA 2, total variance explained 10.9%) affected vegetated environments. The P environment was depicted on the positive side while the forest away (FA) was highlighted on the negative side as the sites with the greatest values of total $CaCO_3$, TOC, TN, WSOC, WSON, MBC and β-glucosidase activity, but also with the highest W-As and W-Cu concentrations. To gain more insight about the differences among the vegetated study environments, a second PCoA was performed excluding mine tailings bare soils (B) (Fig. 6.6B). The results (72.2% of the total variance explained by the first two axes) separated FA environment on the negative side of the primary axis, again as the sites with better soil physicochemical and microbiological conditions, opposite to P and P+S environments, which were depicted on the positive side with higher total metal(loid)s and W-Cd concentrations (Fig. 6B). The DP+S and FN environments were clustered on the negative side of the primary axis, closer to FA than to P and P+S.

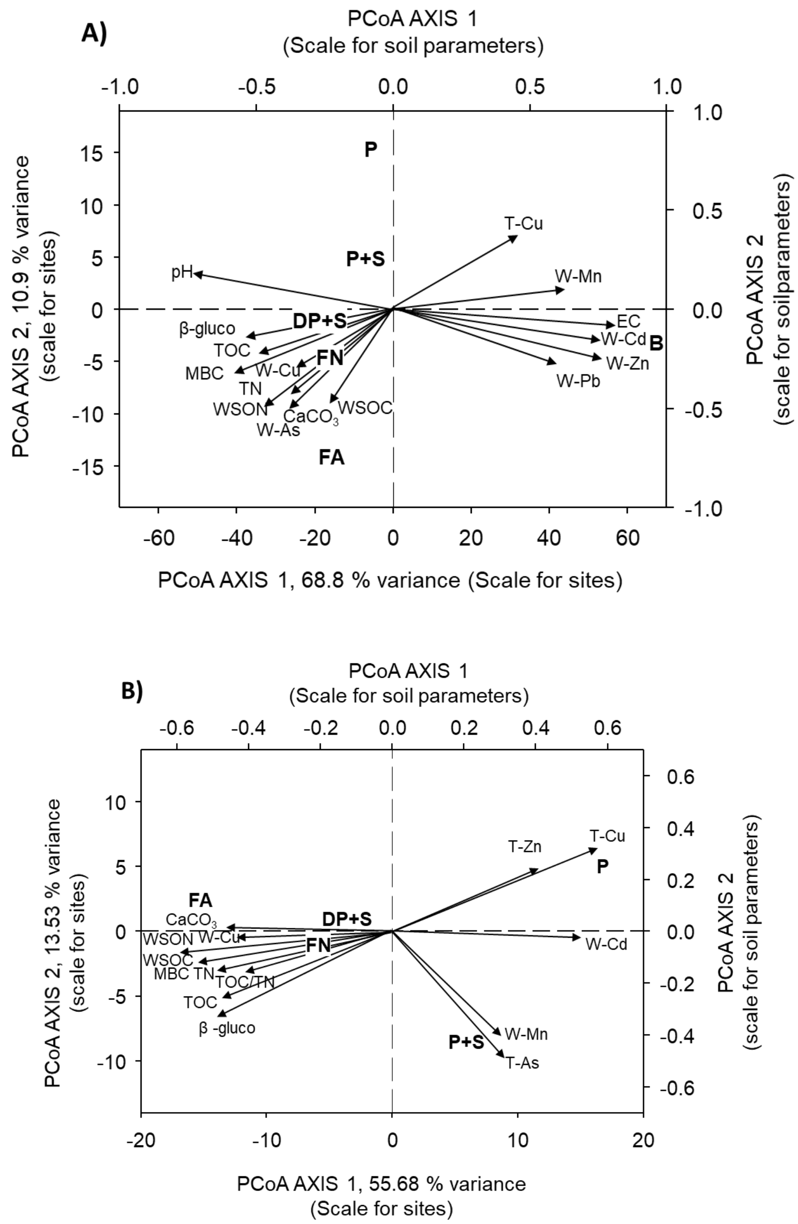

Figure 6.6. Results of the two-dimensional Principal Component Analyses (PCoA). Centroids for all the study environments (A) and vegetated environments (B) are represented. Vectors imposed for those soil parameters with Pearson's correlation coefficient ≥0.5. See Section 6.2 for meaning of the environments (B, P, P+S, DP+S, FN and FA). EC (electrical conductivity). T-Metal(loid) (total metal(loid) concentration). W-Metal(loid) (water soluble metal(loid) concentration). TOC (total organic carbon). TN (total nitrogen). WSOC (water soluble organic carbon). WSON (water soluble organic nitrogen). MBC (microbial biomass carbon). β-glu (β-glucosidase activity).

6.4. Discussion

We evaluated the relationships between physicochemical and functional soil parameters and plants in vegetation patches of metal(loid) mine tailings abandoned ≈40 years ago, relative to forest areas outside the tailings. It is assumed that microclimatic/microtopographic conditions in all the tailing study sites, and vegetation cover close to each tailing, could be non-identical, and this could influence biota colonization (plants and soil microorganisms). However, it is reasonable to think that the current situation, after ≈40 years, represents a realistic picture of the effects of spontaneous plant colonization and the concomitant changes in soil functionality. The results indicated that metal(loid) mine tailings soils may reach a functional state comparable to that of the surrounding forests along with spontaneous plant colonization. This shows the value of preserving spontaneously formed vegetation patches when implementing restoration programs in areas degraded by mining activities, as valuable nucleation spots not only for vegetation but also for soil microbiota. Even more, the creation of new vegetation patches by favoring the establishment of nurse species could be a suitable strategy to trigger soil functional restoration. This could be implemented through the placement of local woody and organic debris, to ameliorate the extreme conditions of these environments, as suggested by other authors (Oreja et al., 2020).

6.4.1. Soil physicochemical conditions and vegetation in the metal(loid) mine tailings

High levels of total metal(loid)s were found throughout mine tailings in the study area, without a consistent pattern of decreasing concentrations from barren to vegetation patches or among these patches (Table 6.3). Compared to bare soils (B), vegetation patches (P, P+S, and DP+S) showed better conditions in terms of higher pH and $CaCO_3$ and lower salinity (EC) and water soluble metals (Table 6.3). Higher soil pH in vegetation patches led to decreasing W-Cd, W-Mn, W-Pb and W-Zn concentrations (negative correlation; $r \leq -0.608$, $p \leq 0.002$) and, consequently, to lower potential toxic effects on soil microorganisms (Giller et al., 2009; Abdu et al., 2017). Plants could also contribute to reductions in the concentrations of water soluble metals in the underlying soils by taking up and/or immobilizing them in their rhizospheres (Antoniadis et al., 2017). Particularly, in the case of the P environment, the coarser texture (attributable to the accumulation of sand particles blown up by wind around pine trunks) (Navarro-Cano et al., 2018), could contribute to lower salinity and water soluble metals concentrations, but also lower water availability for plants. Reduced water soluble metals concentrations were, in general, more evident in the DP+S environment, which showed similar concentrations of W-Cd, W-Mn and W-Zn to the forest soils outside the mine tailings (Table 6.3). Nevertheless, despite the low total metal(loid) levels, the forest soils outside the mine tailings showed the highest concentrations of W-As and W-Cu from all the study environments (Table 6.3). The latter could have been related to the greater solubility of these elements with increasing labile organic C, as shown by the positive correlation with WSOC ($r \leq 0.929$, $p<0.001$), and, in the case of W-As, to the limited soil fixation with high $CaCO_3$ content (positive correlation between W-As and $CaCO_3$; $r=0.780$, $p<0.001$) (Simón et al., 2010, 2015; Pardo et al., 2017; Parraga-Aguado et al., 2017).

Vegetation presence on mine tailings was mainly reflected in higher levels of soil TOC, TN, WSOC and WSON (Figs. 6.2a, 6.2b, 6.2d and 6.2e). Moreover, among the vegetation patches under study, these parameters tended to increase in P+S and DP+S, which were the environments with the greatest plant diversity and richness values (Shannon-Weaver index – H´ and Margalef index – R) and the highest number of total plant families and different life forms (Tables 6.1 and 6.2). The tendency to higher values for vegetation indexes in P+S, DP+S and even in FN than in FA can be explained because ecosystem perturbations facilitate the development of opportunistic species, so increasing diversity, while invasive plants tend to disappear in mature stages. These findings are of interest for restoration approaches that consider assemblages of species with distinct characteristics particularly suitable for the establishment of native perennial vegetation (Brown et al., 2008). In fact, significant positive correlations between H´ and R with TOC, TN and WSON ($r \geq 0.596$, $p \leq 0.05$), and marginally significant between H´ and WSOC ($r=0.574$, $p=0.083$), were found when only considering the data from the vegetation patches. In particular, the DP+S environment showed the closest conditions to the forest soils outside the mine tailings, especially for WSOC and WSON concentrations (Figs. 6.2d and 6.2e).

Similar to TOC, TN, WSOC and WSON, the C to N ratios (TOC:TN and WSOC:WSON) also showed differences between vegetation patches and barren areas (Figs. 6.2c and 6.2f). The tendency of higher TOC:TN ratios in P+S and DP+S than in B and P suggests a greater incorporation and stabilization of soil organic matter in vegetation patches with higher plant diversity and richness (i.e., better balance between organic matter mineralization and immobilization) (Brust, 2019), reaching similar values to those of the forest soils. This could be favored by the abundance of nurse plants in these environments (Tables 6.1 and 6.2). Plant species with this functional role often generate leaf litter with a low C:N ratio and, therefore, greatly contribute to increase soil fertility and bacterial abundance (Colin et al. 2019).

WSOC and WSON composition in forest soils is a complex issue that is controlled by several factors (Kooch et al., 2018). The WSOC:WSON ratio is often related to the decomposition of soil organic matter and soluble organic matter leached from senescent leaves, fine roots, and living fine root exudates (Uselman et al., 2012; Vestgarden et al., 2012; Kooch and Bayranvand, 2017). Uselman et al. (2012) reported lower WSOC:WSON ratios from root-derived soluble organic matter than from leaf litter. In our study, the occurrence of dense pine stands with continuous understory shrubs in the DP+S environment could have led to higher amounts of root-derived soil organic matter and lower WSOC:WSON rations than in the P+S environment (vegetation patches formed by isolated pines trees). Lower WSOC:WSON ratios could imply more easily accessible soil organic matter for microorganisms and, hence, favor C and nutrients cycling (Uselman et al., 2012). Moreover, an increase in the amount and diversity of root exudates has been related with the presence of nurse plants (Eisenhauer, 2017; Colin et al., 2019).

6.4.2. Soil functional conditions and vegetation in metal(loid) mine tailings

Vegetation not only provides organic matter and nutrients to the soil through plant debris and root exudates, but also offers active root surfaces for microorganisms to adhere to and function (Caffery and Kemp, 1990; Hinsinger et al., 2009). The latter can trigger and/or accelerate organic matter turnover and biogeochemical cycling in these degraded environments, which, in turn, helps providing a more suitable habitat to other soil-dwelling (micro)organisms (Wall et al., 2012; Morgado et al., 2018). Relief from soil abiotic stress conditions of barren metal(loid) mine tailings with spontaneous plant colonization, along with increased C and N levels, could be simultaneously accompanied by an improvement in the functional status of the soil microbial communities. Most of the soil microbiological parameters determined were positively correlated with pH, $CaCO_3$, TOC, TN, TOC:TN, WSOC and WSON ($r \geq 0.424$, $p \leq 0.039$), while negatively with EC, W-Cd, W-Mn and W-Zn

($r \leq -0.465$, $p \leq 0.022$). Compared to bare soils (B), higher MBC, β-glucosidase activity, overall bacterial metabolic activity (AWCD) and functional diversity (ecological diversity indices) were found in vegetation patches (Figs. 6.3 and 6.4; Table 6.5). Our results agree with other studies showing increasing functionality of soil microorganisms in the rhizosphere of plants spontaneously growing on metal mine tailings (Li et al., 2011) and in areas affected by metal mine wastes after the introduction of vegetation as a phytomanagement strategy (Zhang et al., 2007; Zhou et al., 2020). Higher resource availability coming from plants could have provided energy and nutrients for microbial colonizers, stimulating the transition from the autotrophic structure typically found in barren tailings to more heterotrophic communities beneath vegetated spots (Huang et al., 2012; Colin et al., 2019; Risueño et al., 2020). This could have led to more abundant microbial communities, with greater capability to consume more diverse organic substrates and, hence, functionally more diverse.

The degree of functional improvement in tailing soils seemed to be related not only to the mere presence of plants but also to the characteristics of the species growing in vegetation patches. The different environments considered showed similar plant cover, but the vegetation of P+S and DP+S had greater diversity and richness and more pioneer and nurse species than that of P (Tables 6.1 and 6.2). The establishment of a variety of plants with different life forms triggers a cascade of benefits on ecosystem functioning, increases resilience and stimulates positive plant-soil feedbacks and synergistic interactions between soil microbial communities and spontaneous vegetation colonizing metal(loid) mine tailings, hence promoting microbial functionality and providing longer stability to these vegetated spots (Parraga-Aguado et al., 2014; Navarro- Cano et al., 2018). Moreover, previous studies have shown concomitant successional trajectories of vegetation and soil bacterial communities in spontaneously colonized mine tailings, with trees, shrubs, and perennial grasses significantly increasing bacterial diversity, but not dwarf shrubs (Colin et al., 2019). Our work was not specifically designed to study the relationships between the functional role of plants and soil function, but the results showed that shrubs and perennial grasses were more relevant in DP+S than in P+S (Fig. 6.1), coinciding with a tendency of higher bacterial metabolic activity (AWCD) in the first environment (Fig. 6.4). In fact, DP+S, and in less extent P+S, showed a microbial functional status closer to that of the forest soils outside the mine tailings (Fig. 6.6). In this sense, the P+S and DP+S environments, or something in between (i.e., patches with intermediate vegetation composition), could represent a soil microbial functional threshold or tipping point within mine tailings. In a previous work focused on successional trajectories of soil bacterial communities in mine tailings, Colin et al. (2019) identified root architecture as a plant functional key trait for soil bacterial community structure during primary succession. Although it is well known that rhizosphere environment provides a better structure and resources for microorganisms (Hinsinger et al., 2009), competition with plants for resources in dense rhizospheres can negatively affect bacterial communities (Moreau et al., 2015; Colin et al., 2019). Hence, not very dense root systems could favor species coexistence in vegetated patches, which would lead to higher diversity of roots exudates and, consequently, higher microbial diversity. The greater vegetation diversity and richness in P+S and DP+S environments could facilitate microbial functionality to move towards that of the forest soils outside the mine tailings. Furthermore, the presence of more species with more favorable functional traits, such as shrubs and perennial grasses, could provide more suitable organic compounds to the soil medium, which, in turn, could favor more diverse heterotrophic microbial communities (Hartmann et al., 2009; El Moujahid et al., 2017; Sun et al., 2018; Risueño et al., 2020).

Regardless of the increasing soil microbial functionality with vegetation composition, no differences were found for the consumption pattern of the different C source groups (SAWCD) among the vegetation patches (Fig. 6.5). Moreover, the forest soils outside the mine tailings had similar metabolic fingerprints to the tailing vegetation patches (Fig. 6.5). In all the cases (P, P+S, DP+S, FN, and FA), polymers, carbohydrates and amino acids were the most widely used C groups while amines/amides and phenolic acids the least used. This overlap in the C use potential could indicate a certain degree of functional redundancy of the soil bacterial communities present in the vegetated study environments (i.e., communities capable of performing similar metabolic functions even if they differ in composition) (Louca et al., 2018; Waymouth et al., 2020), regardless of their location and/or their soil contamination status. Nevertheless, given that the methodology used in this study is culture-based and so only a small fraction of the soil microbial community is evaluated, specific studies must be done to prove this functional redundancy.

6.5. Conclusions

Different types of vegetation patches formed by spontaneous plant colonization in metal(loid) mine tailings under Mediterranean semiarid climate showed only small differences in physicochemical parameters related to soil abiotic stress conditions (pH, salinity, and total and water soluble metals). However, vegetation patches with greater vegetation diversity and richness, and presence of plants with contrasted life forms and, particularly, abundance of shrubs and perennial grasses, showed an improvement in terms of soil microbial functional-related parameters (higher MBC, β-glucosidase activity, bacterial metabolic activity, and functional diversity). Furthermore, these vegetation patches showed scores of functional-related parameters comparable to that of the forest soils outside the mine tailings.

Our findings contribute to support the value of spontaneously colonized patches as valuable components of phytomanagement strategies for abandoned metal(loid) mine tailings. Vegetation patches should be preserved during restoration programs since they can act as nucleation spots not only for plant recruitment but also for biological propagules that may help to accelerate the functional recovery of these degraded environments. Management practices should consider the establishment of species with diverse life forms and functional roles, to create vegetation patches with the capacity to trigger plant and soil microbial succession, which would contribute to the functional restoration of these environments.

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CHAPTER 7

Biochar and urban solid refuse ameliorate the inhospitality of acidic mine tailings and foster effective spontaneous plant colonization under semiarid climate

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Abstract

Phytomanagement is considered a suitable option in line with nature-based solutions to reduce environmental risks associated to metal(loid) mine tailings. We aimed at assessing the effectiveness of biochar from pruning trees combined with compost from urban solid refuse (USR) to ameliorate the conditions of barren acidic (pH ≈5.5) metal(loid) mine tailing soils (total concentrations in mg kg^{-1}: As ≈220, Cd ≈40, Mn ≈1800, Pb ≈5300 and Zn ≈8600) from Mediterranean semiarid areas and promote spontaneous plant colonization. Two months after amendment addition were enough to observe improvements in chemical and physico-chemical tailing soil properties (reduced acidity, salinity and water-soluble metals and increased organic carbon and nutrients content), which resulted in lowered ecotoxicity for the soil invertebrate *Enchytraeus crypticus*. Recalcitrant organic carbon provided by biochar remained in soil whereas labile organic compounds provided by USR were consumed over time. These improvements were consistent for at least one year and led to lower bulk density, higher water retention capacity and higher scores for microbial/functional-related parameters in the amended tailing soil. Spontaneous growth of native vegetation was favored with amendment addition, but adult plants of remarkable size were only found after three years. This highlights the existence of a time-lag between the positive effects of the amendment on tailing soil properties being observed and these improvements being translated into effective spontaneous plant colonization.

Keywords: mine wastes; phytomanagement; organic amendments; ecotoxicity; community-level physiological profile; soil functionality

7.1. Introduction

As explained in Chapter 1 (see Section 1.5), due to the difficulties for the establishment and survival of plants in mine tailings (Huang et al., 2012), the use of soil amendments to improve soil conditions and favoring the natural recruitment of native species might help to create nucleation spots and fertility islands to trigger vegetation expansion within mine tailings (Oreja et al., 2020). Particularly, biochar has been proposed as an effective amendment to improve tailing soils by immobilizing metals, reducing their mobility and availability (Beesley et al., 2011; Wu et al., 2017; Nie et al., 2018; Palansooriya et al., 2020). However, because biochar contains high levels of recalcitrant organic matter, it is poor in labile organic carbon and readily available nutrients (Rodríguez-Vila et al., 2014; Ghosh et al., 2015; Wu et al., 2016, 2017). These limitations can be solved by mixing biochar with composted raw organic materials that are richer in more labile organic matter such as urban solid refuse (USR) (Karami et al., 2011; Wu et al., 2017).

This chapter includes the work planned to respond to the third specific objective of the PhD Thesis (see Section 3.1): To assess the effectiveness of an organic amendment composed of biochar from pruning trees and compost from USR to ameliorate the conditions of barren metal(loid) mine tailings soils from Mediterranean semiarid areas. In particular, the work sought to: 1) assess the effects of the organic amendment on tailings soil conditions and if these effects persist seasonally over a year; 2) assess whether the organic amendment favors spontaneous plant colonization. The initial hypotheses were: 1) the organic amendment would improve tailings soil conditions and these effects would be modulated by the time lasted from the application and the season of the year; 2) the organic amendment would favor the spontaneous colonization of tailing soils by pioneer native plants from surrounding areas.

7.2. Material and Methods

This chapter contains the data corresponding to the organic amendment addition experiment carried out in barren mine tailings soils: bare soil treatment (B); amended bare soil treatment (AB). In particular, it includes the initial characterization of the treatments (two months after amendment addition) and the seasonal monitoring for one year (summer

2017, autumn 2017, winter 2018, and spring 2018). The following parameters are displayed (for a complete description of the methodologies used see Chapter 4):

- Vegetation parameters. Plant cover; number of species; number of individuals per species. Additionally, vegetation data were newly recorded two years after finishing the seasonal sampling program (spring 2020).
- Soil parameters. Bulk density; water retention capacity; particle size distribution; cation exchange capacity (CEC); total $CaCO_3$; total organic carbon (TOC); total nitrogen (TN); total metal(loid)s concentration (T-metal(loid)s); pH; electrical conductivity (EC); water soluble salts (Cl^-, SO_4^{2-}, Na^+, K^+, Ca^{2+}, and Mg^{2+}); water soluble metal(loid)s (W-metal(loid)s); water soluble organic carbon (WSOC); microbial biomass carbon (MBC); dehydrogenase activity (DH); community-level physiological profile (average well-color development – AWCD, substrate average well-color development – SAWCD, substrate richness index – S, Shannon-Weaver heterogeneity index – H', and Pielou evenness index – J′); organic matter decomposition (TBI); feeding activity of soil dwelling organisms; soil temperature; soil moisture; soil respiration (CO_2 emission); ecotoxicity bioassays.

Related to the statistical analyses, differences were considered significant at $p < 0.05$. Data were transformed when they failed to pass the Shapiro-Wilk's test (normal distribution) and/or Levene's test (homogeneity of variance). Student's *t*-test was used to check for differences between treatments (B vs. AB). Repeated-measures ANOVA followed by Bonferroni post-hoc test was used to compare how parameters evolved over seasons and between treatments. The factors included in the analysis were: an intra-subject factor, the season - repeated factor, with four levels (summer 2017, autumn 2017, winter 2018 and spring 2018); an inter-subject factor, the treatment, with two levels (B and AB). A significant effect of season indicates that the parameter evolves significantly over the study period. A significant effect of treatment indicates that the average parameter value differed among treatments. A significant effect of season x treatment interaction indicates that the evolution of the parameter over the study period differed among treatments. Person's rank correlations were performed to analyze the relationships between parameters.

7.3. Results

7.3.1. Particle size distribution, total $CaCO_3$, CEC, TN and total metal(loid)s

Soils were sandy loam in both treatments with ≈71-75% sand, ≈20-22% silt and scarce clay content (≈5-7%) (Table 7.1). Two months after the application of the amendment the contents of total $CaCO_3$ (≈14-fold higher, significant) and TN (≈1.8-fold higher) had increased, but no differences were found for CEC values between treatments (≈4-5 $cmol_c$ kg^{-1}) (Table 7.1). Amendment application slightly decreased total metal(loid) concentrations (≈1.2-1.7-fold lower), probably due to a dilution effect, but differences between treatments were only significant for Pb (Table 7.1).

Table 7.1. Initial effects of organic amendment addition on soil characteristics (average ± SE, n=4). Treatments: B (bare soil) and AB (amended bare soil). CEC (cation exchange capacity). TN (total nitrogen). $Metal(loid)_{tot}$ (total metal(loid) concentration). Asterisks (*) indicate significant differences between treatments (Student's *t*-test, p<0.05).

Parameter	B		AB
Sand (%)	71 ± 5		75 ± 4
Silt (%)	22 ± 4		20 ± 4
Clay (%)	7 ± 1		5 ± 1
Total $CaCO_3$ (g kg^{-1})	0.64 ± 0.07	*	8.99 ± 4.14
CEC ($cmol_c$ kg^{-1})	5.0 ± 0.5		4.1 ± 0.7
TN (g kg^{-1})	0.31 ± 0.08		0.55 ± 0.09
T-As (mg kg^{-1})	223 ± 28		173 ± 13
T-Cd (mg kg^{-1})	37.7 ± 8.5		21.9 ± 8.0
T-Fe (mg kg^{-1})	153350 ± 3384		123375 ± 8007
T-Mn (mg kg^{-1})	1828 ± 159		1508 ± 107
T-Pb (mg kg^{-1})	5345 ± 373	*	4099 ± 299
T-Zn (mg kg^{-1})	8596 ± 2213		6182 ± 1168

7.3.2. Evolution of pH, EC, water soluble salts and water soluble metal(loid)s

Seasonal monitoring showed that the amendment significantly affected soil pH (season, treatment, and season x treatment interaction) and EC (season and treatment) (Figs. 7.1a and 7.1b). Soil pH had increased two months after the application of the amendment (from ≈5.5 in B to ≈7.6 in AB in summer 2017) and differences between treatments were maintained over the study period (Fig. 7.1a). In treatment B soil pH increased over time, with significant higher values in spring 2018 (≈6.4). On the contrary, lower pH variations were observed in treatment AB (≈7.6-8.0). Soil EC decreased with the application of the amendment (from ≈6.6 dS m^{-1} in B to ≈3.9 dS m^{-1} in AB in summer 2017), although differences between treatments were only significant in spring 2018 (Fig. 7.1b). Treatment B showed marked seasonal changes in EC, which was significantly lower in autumn 2017 (≈3.8 dS m^{-1}) compared to summer 2017 (≈6.5 dS m^{-1}), and tended to increase again in winter (≈4.6 dS m^{-1}) and spring 2018 (≈6.3 dS m^{-1}). However, treatment AB showed no significant variations in EC over the study period (≈2.7-3.9 dS m^{-1}). The Cl^-, SO_4^{2-}, Na^+ and Mg^{2+} were more abundant in B, while Ca^{2+} and K^+ reached higher concentrations in treatment AB (Fig. 7.2).

Water soluble Cd, Mn, Pb and Zn concentrations were significantly affected by the amendment (season and treatment). Concentrations were extremely high in treatment B and significantly decreased two months after amendment application (W-Cd, W-Pb and W-Zn ≈100-fold lower and W-Mn ≈10-fold lower in summer 2017) (Figs. 7.1c to 7.1f). These differences remained of similar magnitude over the study period. In treatment B no significant seasonal variations were found for these four metals. On the contrary, in treatment AB, a general tendency to decrease from summer 2017 to spring 2018 was observed. Water soluble Fe concentrations were not affected by the amendment and similar seasonal variations were observed for both treatments, with higher concentrations in autumn 2017 and winter 2018 and lower in summer 2017 and spring 2018 (Fig. 7.1g). Amendment application led to a progressive increase of W-As in treatment AB, with significant higher concentrations than treatment B in autumn 2017, winter 2018 and spring 2018 (≈2.6-3.5-fold) (Fig. 7.1h).

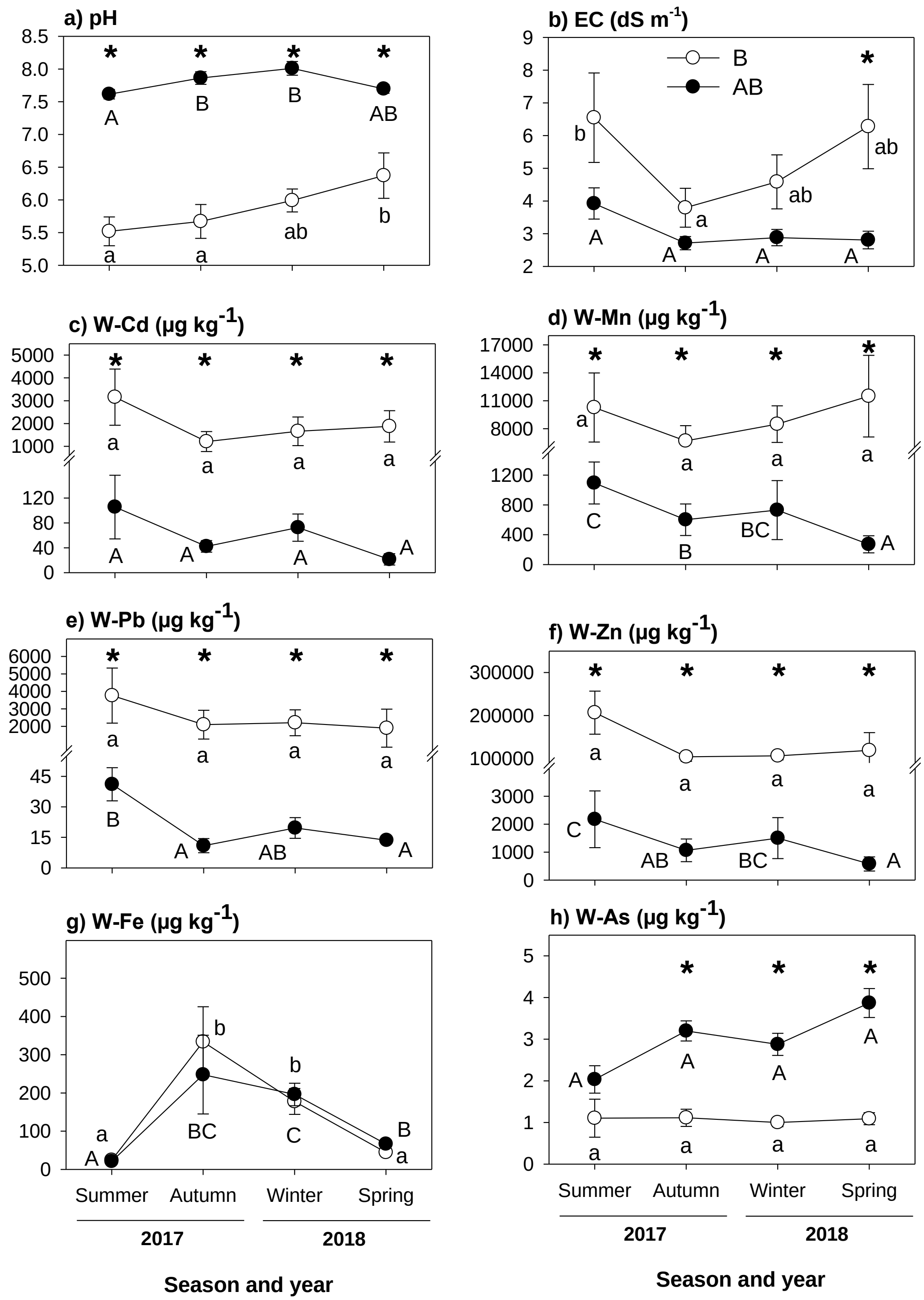


Figure 7.1. Seasonal evolution of pH, electrical conductivity (EC) and water soluble metal(loid) concentrations (W- metal(loid)) during the study period in both soil treatments: B (bare soil) and AB (amended bare soil). Dots represent average values and bars SE (n=4). Asterisks (*) indicate significant differences between treatments per season (Student's *t*-test, $p<0.05$). Different letters (lowercase for B, uppercase for AB) indicate significant differences among seasons (repeated-measures ANOVA followed by Bonferroni post-hoc test, $p<0.05$).

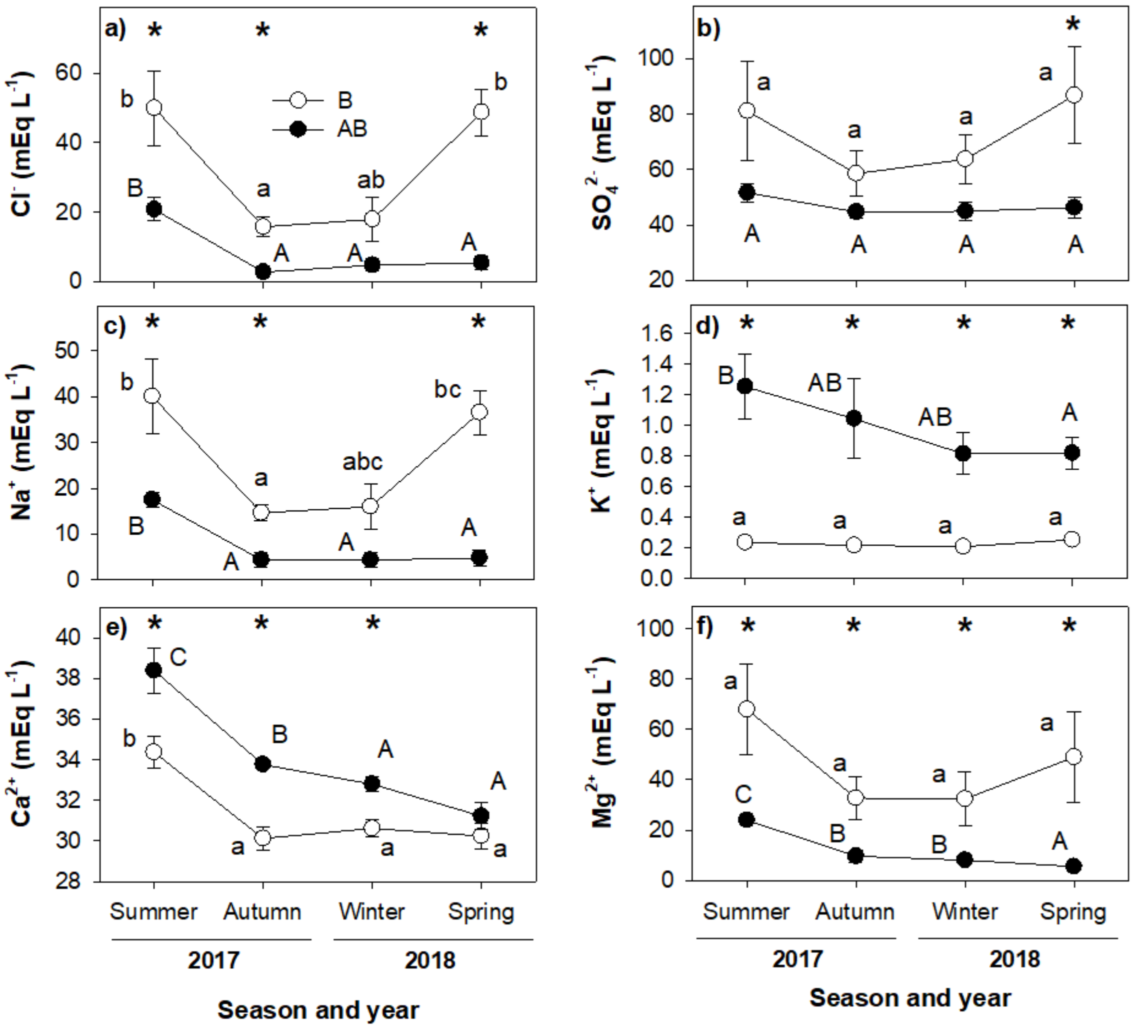


Figure 7.2. Seasonal evolution of water soluble salts during the study period in both soil treatments: B (bare soil) and AB (amended bare soil). Dots represent average values and bars SE (n=4). Asterisks (*) indicate significant differences between treatments per season (Student's *t*-test, $p<0.05$). Different letters (lowercase for B, uppercase for AB) indicate significant differences among seasons (repeated-measures ANOVA followed by Bonferroni post-hoc test, $p<0.05$).

7.3.3. Evolution of TOC, WSOC, MBC, DH, CLPP, TBI, temperature, moisture and feeding activity

Two months after the application of the amendment (summer 2017) TOC, WSOC, MBC and DH had increased significantly (≈16-, ≈1.7, ≈12- and ≈4.7-fold higher, respectively)

(Figs. 7.3a to 7.3d). For TOC, the differences between treatments were maintained during the study period and no seasonal variations were found (significant effect of treatment) (Fig. 7.3a). For WSOC and MBC, both parameters decreased in autumn 2017, without differences between treatments (WSOC ≈12-17 mg kg^{-1}; MBC ≈13-28 mg C kg^{-1}). WSOC continued to decrease throughout the study period and reached the lowest concentrations in spring 2018 (significant effect of season) (Fig. 7.3b). However, MBC remained stable from autumn 2017 onwards and it was significantly higher in treatment AB in spring 2018 (significant effect of season x treatment interaction) (Fig. 7.3c). For DH, it tended to increase in both treatments throughout the study period and was significantly higher in treatment AB in spring 2018 (Fig. 7.3d).

Amendment application favored higher soil microbial catabolic activity throughout the study period (Fig. 7.4a). In both treatments, AWCD values were almost negligible during the first 96 h of incubation. From this time on and for all the study seasons, AWCD tended to increase in treatment AB and reached the highest values upon 192 h of incubation (≈0.09 in summer 2017, ≈0.06 in autumn 2017 and ≈0.04 in spring 2018). This was not the case of treatment B that showed a very low consumption of carbon substrates in all the study seasons. Regarding substrates consumption pattern (SAWCD) (Fig. 7.4b), the consumption of amino acids (from ≈18% to ≈10%), carbohydrates (from ≈30% to ≈26%) and phenolic acids (from ≈7% to ≈2%) tended to decrease from summer 2017 to spring 2018, while that of amines/amides (from ≈5% to ≈8%) and carboxylic acids (from ≈15% to ≈24%) to increase. Polymer’s consumption tended to increase from summer 2017 (≈25%) to autumn 2017 (≈35%) and decreased again in spring 2018 (≈30%). SAWCD was not calculated for B treatment due to the low absorbance values registered. Regarding the ecological diversity indices derived from CLPP analysis in treatment AB, S and H´ showed higher values in summer 2017 and tended to decrease towards spring 2018 (S from ≈8.5 to ≈4.8; H´ from ≈1.8 to ≈1.5) (Table 7.2). On the contrary, J´ tended to increase throughout the study period in treatment AB (from ≈0.9 in summer 2017 to ≈0.9 in spring 2018) (Table 7.2). Diversity indices could not be calculated for treatment B.

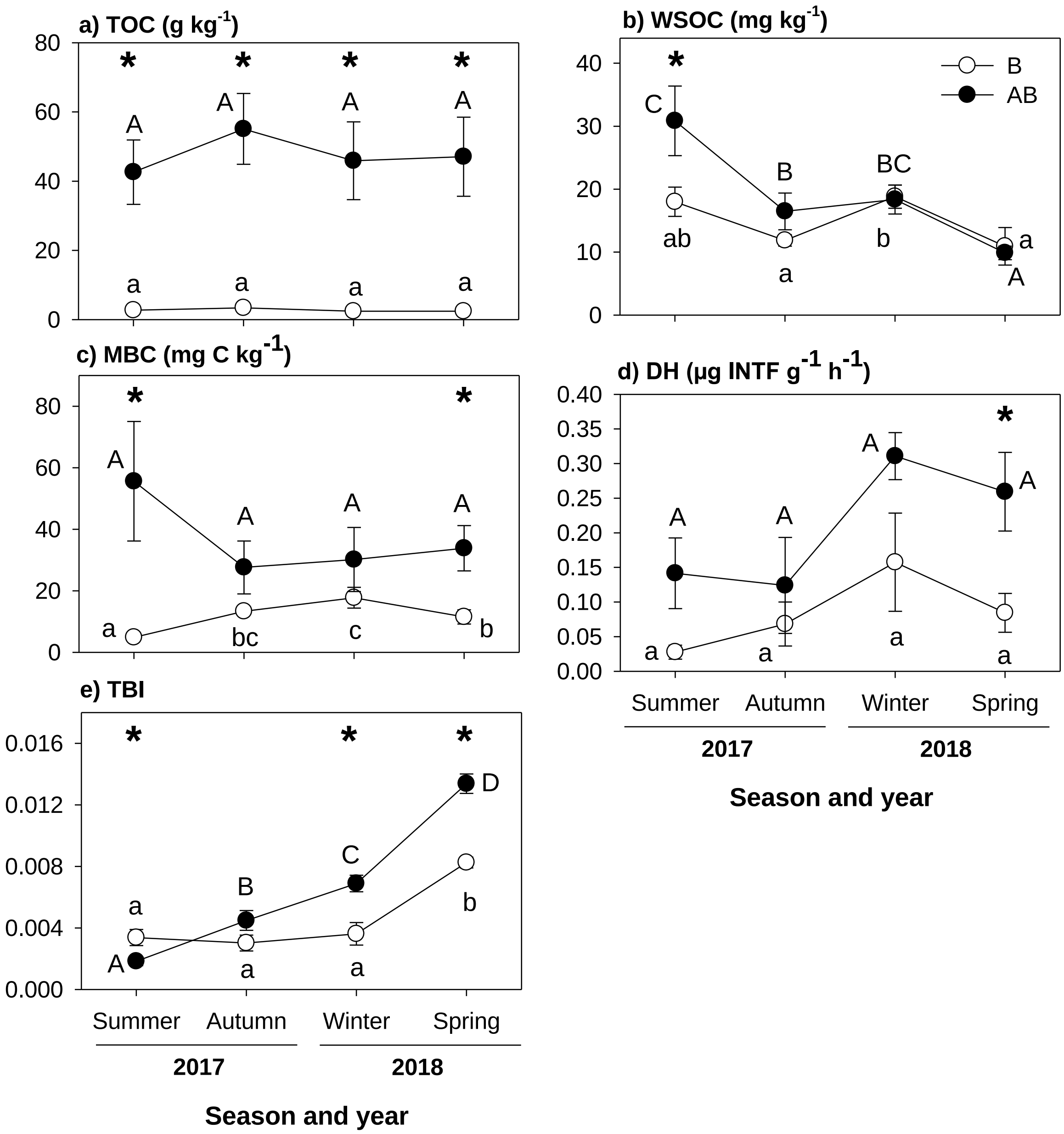


Figure 7.3. Seasonal evolution of total organic carbon (TOC), water soluble organic carbon (WSOC), microbial biomass carbon (MBC), dehydrogenase activity (DH) and tea bag index (TBI) during the study period in both soil treatments: B (bare soil) and AB (amended bare soil). Dots represent average values and bars SE (n=4). Asterisks (*) indicate significant differences between treatments per season (Student's *t*-test, $p<0.05$). Different letters (lowercase for B, uppercase for AB) indicate significant differences among seasons (repeated-measures ANOVA followed by Bonferroni post-hoc test, $p<0.05$).

Table 7.2. Substrate richness (S), Shannon-weaver index (H´) and Pielou index (J´) during the study period in both soil treatments: B (bare soil) and AB (amended bare soil). Values are average ± SE (n=4). Different letters indicate significant differences among seasons (repeated-measures ANOVA followed by Bonferroni post-hoc test, $p<0.05$). d.l. (detection limit: absorbance values at 590 nm <0.06).

Index	**Summer 2017**		**Autumn 2017**		**Spring 2018**	
	B	**AB**	**B**	**AB**	**B**	**AB**
Substrate richness (S)	<d.l.	8.50 ± 1.94 a	<d.l.	6.75 ± 2.29 a	<d.l.	4.75 ± 0.48 a
Shannon-Weaver (H´)	<d.l.	1.81 ± 0.30 a	<d.l.	1.61 ± 0.37 a	<d.l.	1.45 ± 0.08 a
Pielou (J´)	<d.l.	0.89 ± 0.02 a	<d.l.	0.93 ± 0.02 a	<d.l.	0.94 ± 0.01 a

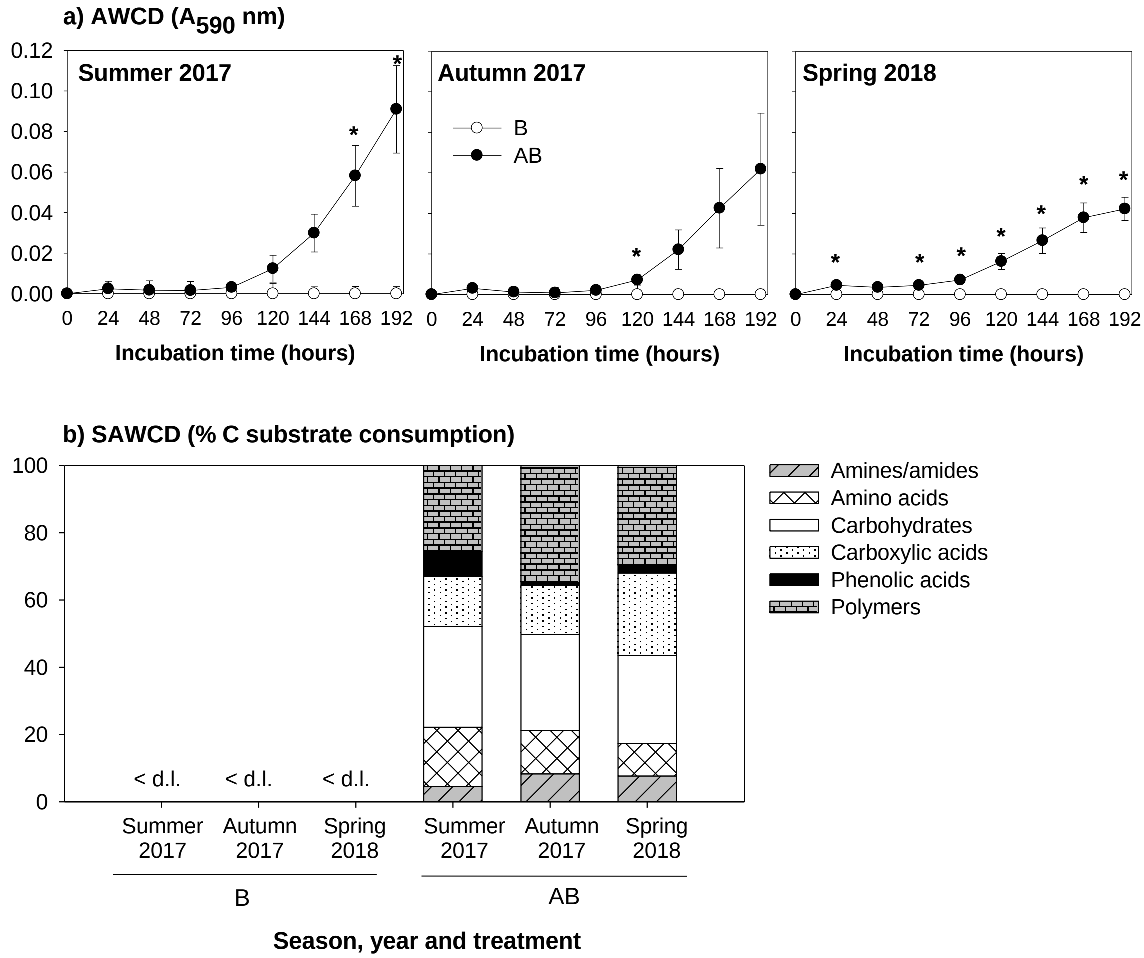


Figure 7.4. a) Average well color development (AWCD) at 590 nm during the study period. Dots represent average values and bars SE (n=4). Asterisks (*) indicate significant differences between treatments per incubation time (Student's *t*-test, $p<0.05$). b) Substrate average well color development (SAWCD) during the study period expressed as percentage of substrate consumption (n=4). d.l. (detection limit: absorbance values at 590 nm <0.06). Soil treatments: B (bare soil) and AB (amended bare soil).

Opposite to the previous parameters described, TBI was negatively affected by the amendment two months after its application (≈1.9-fold lower) (Fig. 7.3e). However, in treatment AB, it increased significantly throughout the study period and reached significant higher values than treatment B in winter and spring 2018 (≈1.6-1.9-fold higher) (significant effect of season and treatment).

Soil temperature and moisture significantly varied during the study period (effect of season), but without effects of the treatment and treatment x season interaction (Fig. 7.5). The highest soil temperatures were registered in summer 2017 (≈24 to ≈31 °C) and the lowest in winter 2018 (≈6 to ≈11 °C). Soil moisture was always <10% and slightly higher in treatment B than in AB. Soils were drier in summer 2017 and wetter in winter 2018.

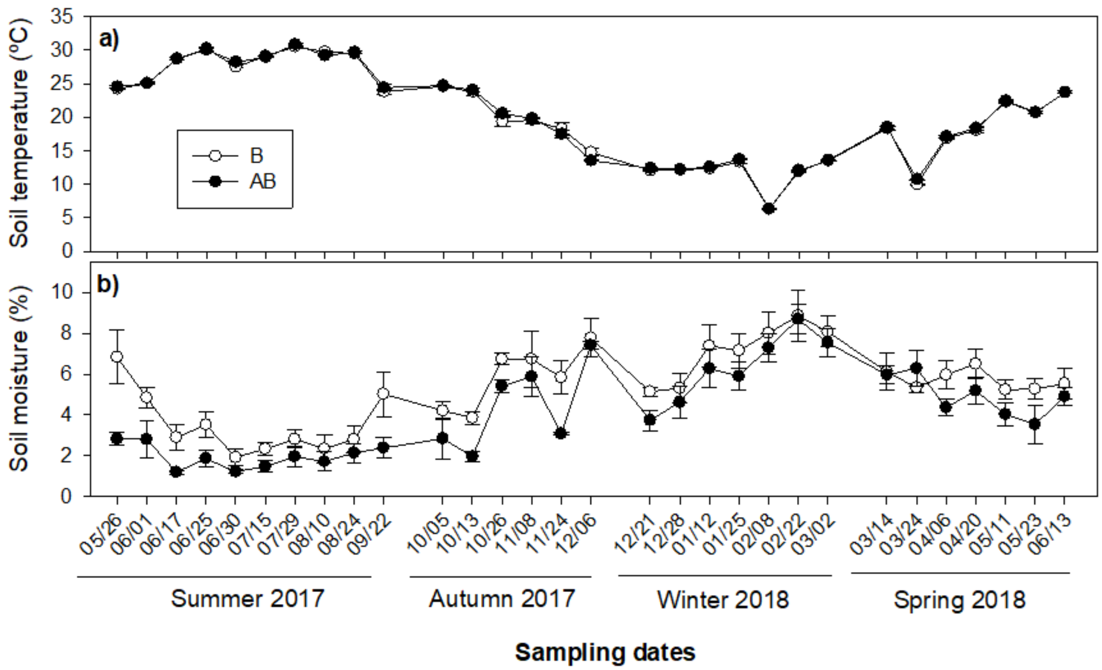


Figure 7.5. Evolution of soil temperature and moisture content (upper ≈10 cm) during the study period in both soil treatments: B (bare soil) and AB (amended bare soil). Dots represent average values and bars SE (n=4).

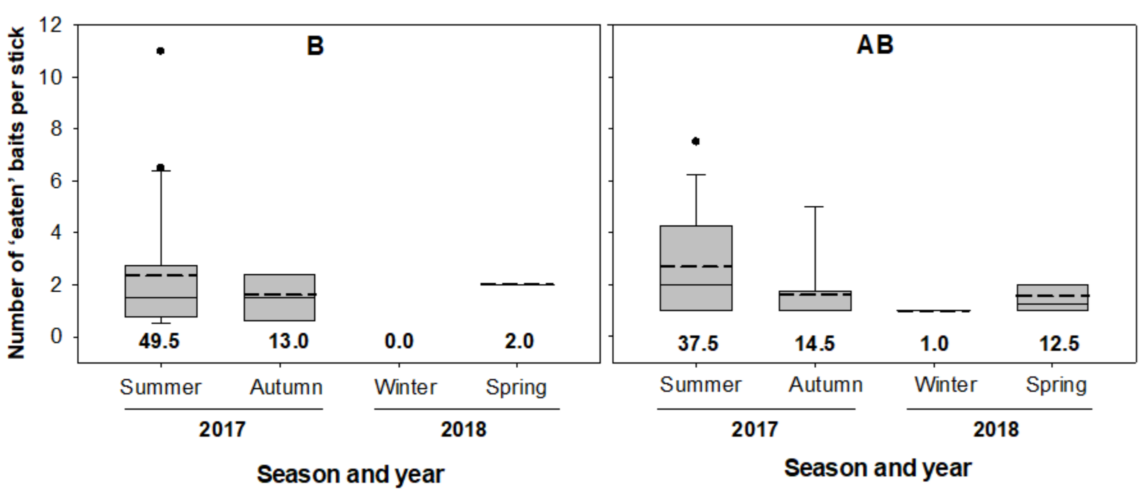


Figure 7.6. Box plots for the feeding activity of soil dwelling organisms during the study period in both soil treatments: B (bare soil) and AB (amended bare soil). Solid lines within boxes indicate the median value and dotted lines the mean. Boxes include data within the 25th and 75th percentiles; whisker lines refer to the 5th and 95th percentiles. Numbers below boxes indicate the total number of holes fed upon in each environment.

Feeding activity of soil dwelling organisms was scarce and widely variable (Fig. 7.6). No clear tendencies were found in relation to the application of the amendment, except in spring 2018 when the feeding activity seemed to be favored in treatment AB (≈6.3-fold higher).

7.3.4. Bulk density, water retention capacity and CO_2 emission

One year after starting the experiment (spring 2018), the amendment had significantly decreased soil bulk density (≈1.1-fold lower) and increased water retention capacity (≈1.4-fold higher) (Figs. 7.7a and 7.7b). Soil CO_2 emissions had also increased significantly in treatment AB in spring 2018 (≈4.5-fold higher) (Fig. 7.7c).

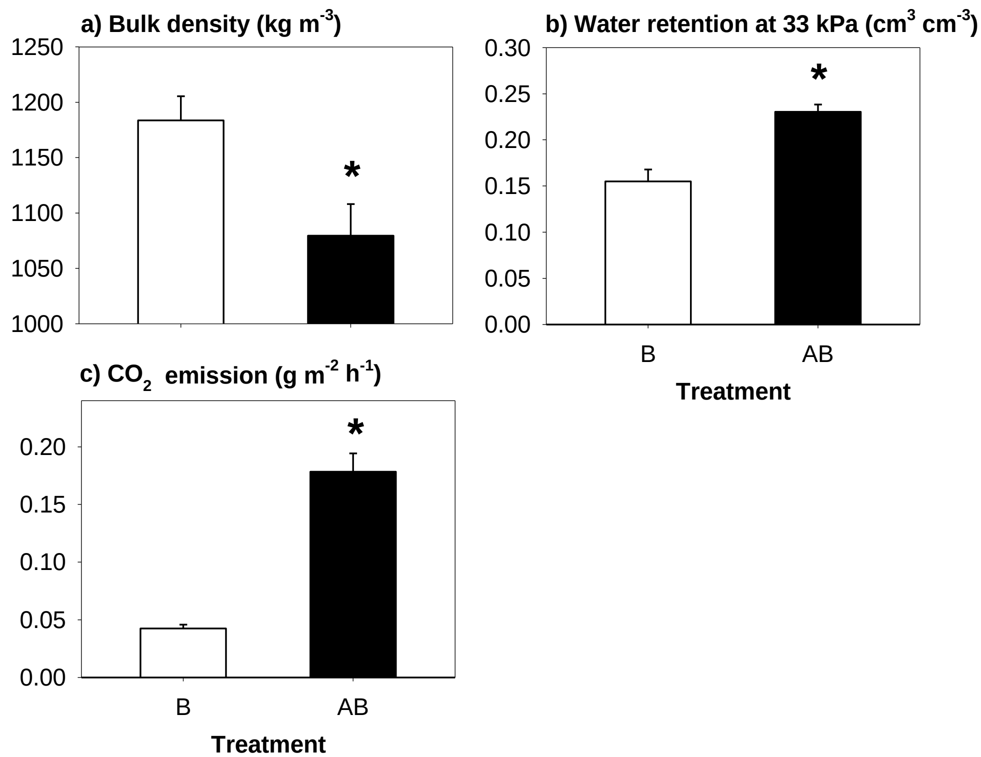


Figure 7.7. Bulk density, water retention capacity at 33 kPa and CO_2 emission in spring 2018 in both soil treatments: B (bare soil) and AB (amended bare soil). Columns represent average values and bars on columns standard error (n=4). Asterisks (*) indicate significant differences between treatments (Student's *t*-test, $p<0.05$).

7.3.5. Soil ecotoxicity

The survival of *E. crypticus* was not affected by the organic amendment, both in summer 2017 (≈60-80%) and spring 2018 (≈66-74%) (Fig. 7.8a). However, *E. crypticus* reproduction was significantly stimulated two months after the application of the amendment (≈4 juveniles in B vs. ≈32 juveniles in AB in summer 2017) and this effect was maintained one year later (≈1 vs. ≈40 juveniles in spring 2018) (Fig. 7.8b).

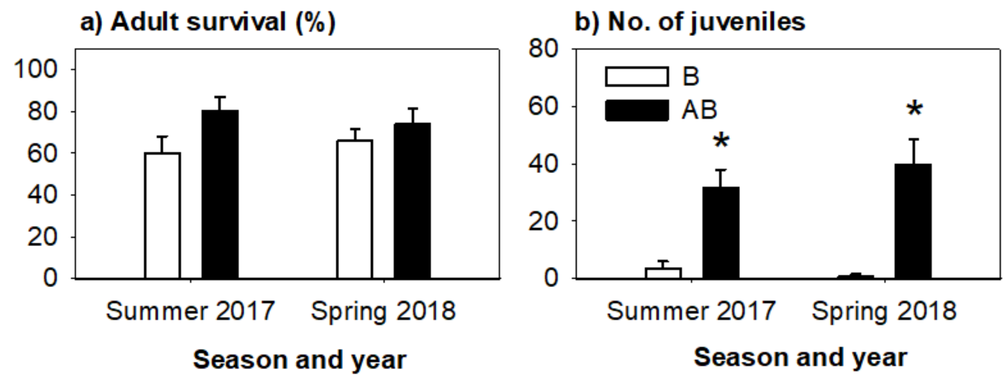


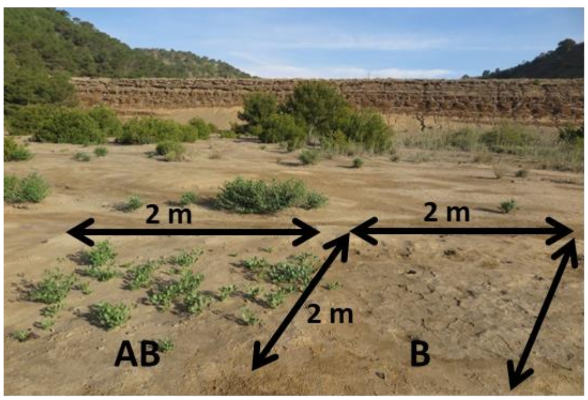
Figure 7.8. Adult survival and reproduction (number of juveniles produced) of the soil invertebrate species *Enchytraeus crypticus* in summer 2017 and spring 2018 in both soil treatments: B (bare soil) and AB (amended bare soil). Columns represent average values and bars on columns standard error (n=4). Asterisks (*) indicate significant differences between treatments per season (Student's *t*-test, $p<0.05$).

7.3.6. Plant colonization

The application of the amendment significantly influenced the spontaneous plant colonization of the study plots (season, treatment, and season x treatment interaction). *Zygophyllum fabago* L. (Zygophylaceae) was the only plant species that appeared in the study plots between summer 2017 and spring 2018 (Fig. 7.9a). The plots of treatment B remained bare or very low covered throughout the study period (≈1 individual in autumn 2017 and ≈5 individuals in spring 2018) (Fig. 7.10a). However, the plots of treatment AB had significantly higher number of individuals from autumn 2017 onwards, reaching a maximum average value in spring 2018 with ≈41 individuals (Fig. 7.10a). Between summer 2017 and spring 2018, the *Zygophyllum* seedlings that colonized the plots were very small (height <≈2.5 cm) and, therefore, plant cover was very low (<1%), even in treatment AB (Fig. 7.10b). However, in spring 2020, in the plots of treatment AB, *Z. fabago* had reached ≈25% average cover with plants up to ≈50-60 cm high. Furthermore, it was also observed the presence of the perennial herb *Piptatherum miliaceum* (L.) Cosson (Gramineae) with an average cover ≈2% (Fig. 7.9d).

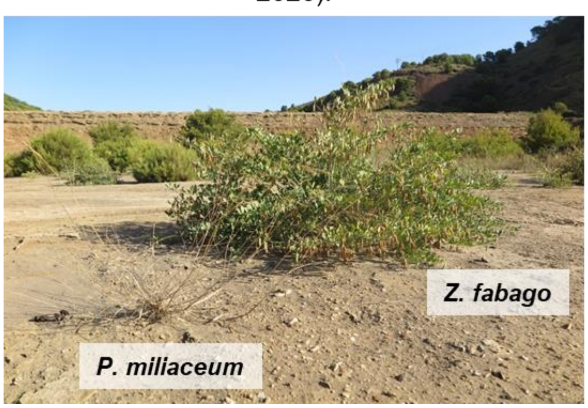
a) *Zygophyllum fabago seedlings* in a plot with amendment (spring 2018).

b) Paired plots (summer 2020). B: bare soil. AB: amended bare soil.



c) *Zygophyllum fabago* growing in treatment AB (summer 2020).

d) *Piptatherum miliaceum* and *Zygophyllum fabago* growing in treatment AB (summer 2020).



e) Wind-blow sandy material accumulated around *Zygophyllum fabago* plants in treatment AB (summer 2020).

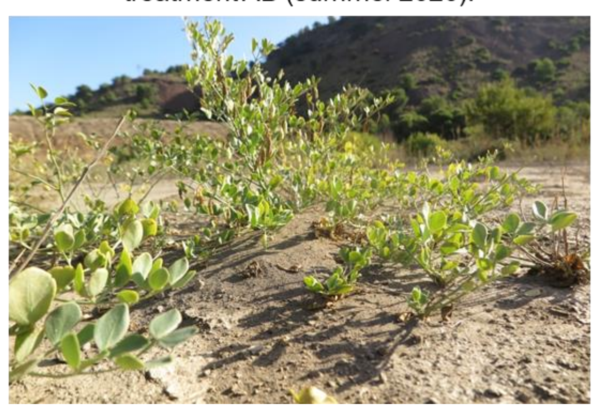


Figure 7.9. Different views of the treatment with the application of the organic amendment in spring 2018 (a) and spring 2020 (b to e).

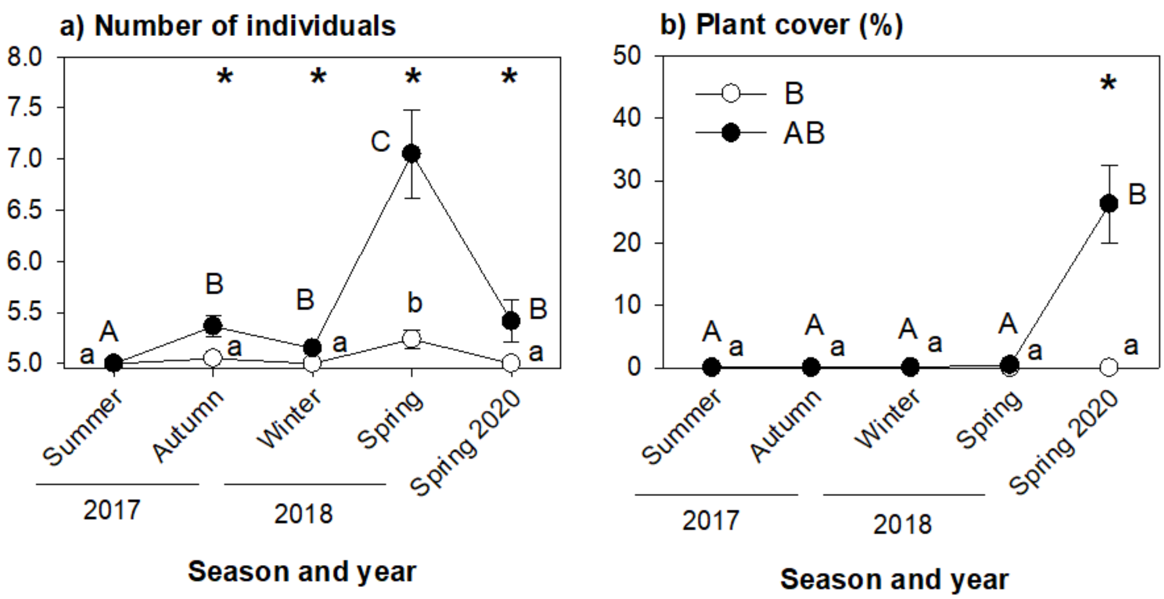


Figure 7.10. Evolution of the number of individuals and plant cover during the study period in both soil treatments: B (bare soil) and AB (amended bare soil). Dots represent average values and bars SE (n=4). Asterisks (*) indicate significant differences between treatments per season (Student's *t*-test, $p<0.05$). Different letters (lowercase for B, uppercase for AB) indicate significant differences among seasons (repeated-measures ANOVA followed by Bonferroni post-hoc test, $p<0.05$).

7.4. Discussion

The organic amendment applied (mixture of biochar from pruning trees and composted USR) improved the unfavorable chemical and physico-chemical conditions of the acidic mine tailing soil from the beginning of its application. This improvement, which, for some parameters, was maintained over time, resulted in a decrease in the ecotoxicity of the tailing soil and, consequently, in an increase in the scores of parameters related to the microbiology and functionality of the edaphic system. Besides, relief from the stressful tailing soil conditions allowed some plants to germinate and grow, covering a significant part of the amended plots three years later. All this highlighted the suitability of the organic amendment used for the transition of a barren mine tailing towards a self-sustainable soil-plant system.

7.4.1. Effects of the organic amendment on soil pH, salinity, water soluble metal(loid)s and ecotoxicity

The alkalinity of the biochar and the high content of $CaCO_3$ of the USR contributed to rising the pH of the mine tailing soil, and this effect was sustained over the study seasons (Figs. 7.1a; Table 4.3). Soil ploughing at the beginning of the experiment could boost the oxidation of sulfides such as pyrite (a common geochemical process in these types of mine wastes when they are aerated) (Huang et al., 2012; Pellegrini et al., 2016), which was reflected by the lower pH in summer 2017. Re-settling of soil material with time must have slowed down this process and the pH tended to rise. This was not observed in treatment AB due to the alkalizing and buffer effect of the amendment applied. Despite the high salinity of the USR, its combination with biochar led to a decrease in soil salinity in treatment AB (Fig. 7.1b; Table 4.3). This could be related to the adsorption capacity of biochar (Ahmad et al., 2014), but also to other inherent properties of this type of organic amendments such as the high pH values. In fact, a significant negative correlation between pH and EC was found ($r=-0.540$, $p<0.001$), which indicated that the increased pH led to a decrease of some dissolved elements like metals by precipitation or co-precipitation mechanisms (as discussed later). In addition, the calcium added with the organic amendment directly as dissolved Ca^{2+} or in the form of $CaCO_3$ could react with SO_4^{2-} by forming gypsum ($CaSO_4·2H_2O$) and, therefore, lowered the concentration of soluble SO_4^{2-} (Fig. 7.2b) (Zornoza et al., 2015). It should be also noted that the concentration of K^+ in soil was 6-fold higher in treatment AB (Fig. 7.2), which could favor plants establishment due to its role as macronutrient (Marschner, 1995).

Soil salinity strongly varied over the study period. It increased in summer 2017 and spring 2018, the warmer and drier seasons, while decreased in autumn 2017 and winter 2018, the wetter seasons (Figs. 7.1b and 7.5). Seasonal salinity changes in the upper soil centimeters have been previously described in saline soils (González-Alcaraz et al., 2014). They are mainly caused by the upward capillary movement of soluble salts during drier and warmer periods and their leaching to deeper layers during wetter seasons. This phenomenon affected to a greater extent to the more mobile salts, such as Cl^- and Na^+, and was more evident in treatment AB (Fig. 7.2). Most likely the latter was related to the higher porosity induced by the organic amendment, as shown by the lower soil bulk density in treatment AB (Fig. 7.7a).

Interestingly, the increase in K^+ concentration in treatment AB was maintained throughout the study period and hardly changed over seasons (Fig. 7.2d).

The higher soil pH in treatment A was accompanied by a decrease in the concentration of water soluble Cd, Mn, Pb and Zn (significant negative correlation between pH and W-Cd, W-Mn, W-Pb and W-Zn; $r \leq -0.615$, $p \leq 0.002$) (Fig. 7.1). Precipitation or co-precipitation is considered the major mechanism involved in reducing metal solubility when pH increases after liming acidic soils (Simón et al., 2018). Nevertheless, biochar addition implies that other mechanisms can play relevant roles such as electrostatic interactions between metal cations and negatively charged biochar surfaces, cation exchange and/or ligand complexation with biochar surface functional groups (Kołodyńska et al., 2017; Wu et al., 2017). Houben and Sonnet (2015) found that the alkalinizing effect of biochar lowered the concentration of soluble Cd, Pb and Zn in an acidic contaminated soil, and that these metals were mainly sequestered in carbonates. Zornoza et al. (2016) found that Cd was mainly immobilized in carbonates and Fe/Mn oxides in acidic mine soils amended with biochar. Simón et al. (2018) stated that the fate of Cu, Pb and Zn on biochar implies the formation of metal complexes with functional groups of this carbonaceous material. Even though numerous studies have reported the effectiveness of organic amendments, mainly biochar, to reduce metal solubility, this matter is still under debate since the effects may change with aging (Chen et al., 2018; Manzano et al., 2020). For example, in a soil incubation experiment, He et al. (2019) stated that different biochar types had contrasting impacts on metal speciation and lability upon 2-years aging. The use of USR in mine tailing soils has also been discussed (Párraga-Aguado et al., 2017). The addition of organic amendments with labile fractions can effectively reduce metals mobility at the short-term, but metals could be released at the long-term due to organic matter mineralization (Schwab et al., 2007). According to Bian et al. (2014), in our study, the effect of the organic amendment was consistent over the study period and its effectiveness did not decrease by the time elapsed since the application was done. Nevertheless, if the presence of USR favored the release of some metals with aging the presence of biochar probably counteracted that effect.

Unlike the aforementioned metals, the organic amendment did not affect the concentration of W-Fe and the seasonal variation of this element was similar in both treatments (Fig. 7.1g). Wetter soil conditions in autumn 2017 and winter 2018 (Fig. 7.5b) could have led to lower redox potential values that provoked the dissolution of Fe oxy-hydroxides (González-Alcaraz and Álvarez-Rogel, 2013) and, therefore, higher W-Fe concentrations during these seasons. In the case of As, the amendment applied was not only ineffective for reducing its solubility, but also progressively favored the increase of W-As concentration (Fig. 7.1h). The higher content of labile organic matter in treatment AB, as shown by the greater concentration of WSOC and MBC (Figs. 7.3b and 7.3c), could facilitate the mobilization of As (Simón et al., 2014). In addition, a significant positive correlation between pH and W-As was found ($r=0.732$, $p<0.001$). This agreed with Simón et al. (2010, 2014) who showed that As fixation is hindered under high pH and $CaCO_3$ conditions due to the predominance of $HAsO_4^{2-}$, which shows low adsorption capacity on solid surfaces (González et al., 2012; Simón et al., 2014). These authors stated that Fe-oxides are highly efficient in reducing the mobility and availability of As in the absence of $CaCO_3$. Hence, in treatment B, lower W-As concentration could be attributable to the immobilization of this metalloid by binding onto amorphous and crystalline Fe-oxides that are abundant in this type of mine wastes (Parraga-Aguado et al., 2015).

Amelioration of mine tailing soil acidity, salinity, and solubility of some metals (Cd, Mn, Pb and Zn), following amendment application, was translated into decreasing toxic effects to the soil invertebrate *E. crypticus* (Fig. 7.8). The possible ecotoxicological effect of biochar, recently reported by Godlewska et al. (2021), was clearly overweighted by its beneficial effects in the mine tailing soil. Ecotoxicity bioassays showed that invertebrates' survival was not affected neither by the organic amendment nor by the time elapsed since its application (adult survival ≈60-80% in summer 2017 and spring 2018 in both treatments) (Fig. 7.8a). However, reproduction, a parameter more sensitive to changes occurring in soil conditions (Castro-Ferreira et al., 2012; González-Alcaraz and van Gestel, 2015; Zhang and Van Gestel, 2019), showed a clear improvement with the amendment. The number of juveniles produced in treatment AB was 8-fold higher two months after the application of the amendment (summer 2017) and 40-fold higher one year later (spring 2018) (Fig. 7.8b), which evidenced the reduced ecotoxicity of the amended tailing soil. These results are in accordance with Beesley et al. (2014) who found decreasing ecotoxicity risks of a heavily contaminated metal(loid) mine soil after being amended with a mixture of olive mill waste compost and biochar from orchard pruning residues.

7.4.2. Effects of the organic amendment on soil organic carbon and microbial, functional-related and physical parameters

As expected, amendment addition was accompanied by an increase in tailing soil organic carbon. The contrasting composition of biochar and USR determined the way in which both materials contributed to the organic carbon pool in treatment AB (Wu et al., 2016). Biochar provided stable organic compounds (TOC:TN ratio ≈118) and USR more labile organic matter (TOC:TN ratio ≈9) (Table 4.3). Hence, the consisted higher TOC content in treatment AB along the study period was mainly attributable to biochar while USR was responsible for the initial boost of WSOC concentration, which tended to decrease to similar levels to treatment B from autumn 2017 onwards (Figs. 7.3a and 7.3b). The sharp increase in WSOC concentration followed by a fast decline after the addition of an amendment enriched in labile organic matter has been previously described, whereas organic carbon from biochar may persist in soils for longer periods (Moreno-Barriga et al., 2017a).

Since biochar provided a limited amount of easily available organic carbon for microorganisms, the initial increase in MBC observed in summer 2017 in treatment AB was mainly attributable to the extra energy source provided by the WSOC contained in USR (significant positive correlation; $r= 0.533$; $p< 0.005$) (Figs. 7.3b and 7.3c). Furthermore, increasing pH together with decreasing salinity and water soluble concentrations of Cd, Mn, Pb and Zn had to facilitate the growth of tailing soil microorganisms. In fact, MBC was significantly positively correlated with pH ($r=0.537$, $p=0.002$) and negatively with W-Cd, W-Mn, W-Pb and W-Zn ($r\geq-0.386$, $p\leq0.029$). Microorganisms colonizing the USR could also have contributed to the initial increase in MBC of treatment AB (Huang et al., 2012). This was probably not the case for biochar which probably had a very low microbial load (if any) as it was a commercially manufactured product. However, biochar could provide shelter for microorganisms and so facilitate their resistance against metal(loid) pollution as suggested by Tu et al. (2020). All this was reflected in a greater rate of substrate utilization (AWCD, i.e., catabolic potential of the microbial community) and a greater functional diversity (ecological diversity indices derived from CLPP analysis) in treatment AB, especially in summer 2017 (Fig. 7.4a; Table 7.3).

The concomitant decline in MBC and WSOC from summer to autumn 2017 in treatment AB (Figs. 7.3b and 7.3c) pointed out that the initial boost of the microbial population tended to disappear after the consumption of the initial load of labile organic matter. However, even though WSOC continued to decline, MBC stabilized or even increased slightly from autumn 2017 onwards. This could be related to the progressive disappearance of the exogenous microorganisms introduced with the organic amendment in favor of the proliferation of communities better adapted to local conditions (Huang et al., 2012). Zornoza et al. (2015, 2016) found changes in microbial communities' structure of amended acidic mine tailing soils and related these results to the increase in pH and organic matter content and reduced metal availability with amendment addition. Furthermore, these authors found that the microbial communities of the amended tailings were closer to those of the surrounding native forest soils than to the original mine waste microbial communities. In our case, the increasing soil functionality of the amended plots over time, reflected by the better scores for parameters such as DH, TBI and CO_2 emission (Figs. 7.3d, 7.3e and 7.7c), supported the possible shift of microbial populations. Moreover, the changes observed in the consumption pattern of the different carbon sources groups (SAWCD, i.e., metabolic fingerprint) towards spring 2018 (Fig. 7.4b) could be a consequence of the possible transition from exogenous to native microorganism species over time.

The higher content of soil organic carbon along with the improved microbial activity in the amended plots contributed to the decrease in bulk density and the increase in water retention capacity of the mine tailing soil (Figs. 7.7a and 7.7b), both parameters associated with higher soil porosity. The latter indicated soil structure development in treatment AB, which is of utmost importance for improving soil functioning and deliver ecosystem services since pores and channels harbor life underground and allow the growth of plant roots (Morgado et al., 2017; Rabot et al., 2018). Moreover, soil structure development is also paramount for air and water storage and solute movement including salts and metal(loid) lixiviation (Pellegrini et al., 2016).

7.4.3. Plant colonization

The hostile conditions that plants must cope with in metal(loid) mine tailings hinder the colonization of these environments, even after the application of soil treatments (Oreja et al., 2020). Our findings highlight that while amendments can be effective to modify certain chemical and physico-chemical soil conditions in the short time, a more prolonged time-lag might be necessary to reach an effective plant colonization. In this sense, although one year (until spring 2018) was enough to improve tailing soil conditions and a significant higher number of seedlings were observed in treatment AB, adult plants did not proliferate (Figs. 7.9 and 7.10a). It was in spring 2020, about three years after soil ploughing and application of the organic amendment, when a consistent plant cover with the presence of two pioneer species (*Z. fabago* and *P. miliaceum*) was observed in the amended plots (Figs. 7.9 and 7.10b). Moreno-Barriga et al. (2017b) also observed a delay in the response of *P. miliaceum* to the addition of biochar at a dose of 4.8% to an acidic mine tailing soil of similar characteristics in a pot experiment that lasted ten months. The authors found that biochar was ineffective in stimulating plant growth for the first five months after amendment addition probably due to the immobilization of nutrients by biochar, but that this effect did not persist after ten months. *Z. fabago* (Syrian bean-caper) is a perennial xerohalophyte shrub widely spread worldwide and that tolerates drought conditions (Menzel and Lieth, 2003). This species is one of the main primo colonizers of barren metal(loid) mine tailings growing in some of the most unfavorable edaphic niches (Párraga-Aguado et al., 2013). It has been shown that *Z. fabago* enhances soil microbial activity within the rhizospheric environment and that it can accumulate a wide range of metal(loid)s in the leaves but at concentrations lower than phytotoxic levels (Párraga-Aguado et al., 2016). Since belowground organs of this species are persistent, we cannot be sure whether some of these plants were survivors from previous years or new individuals. Anyway, it was only in spring 2020 that adult plants with remarkable size were observed in the amended plots, and hence three years were necessary to reach a consistent plant cover.

P. miliaceum (smilograss) is a perennial grass worldwide distributed in nitrified lands, roadsides, slopes, and other types of altered environments (Párraga-Aguado et al., 2013). It is characterized by presenting a dense well-developed root system, including rhizomes, that favors microbial activity (Moreno-Barriga et al., 2017a) and protects soil against erosion (De Baets et al., 2007). Results from field (Conesa et al., 2006; Parraga-Aguado et al., 2015) and pot (Párraga et al., 2015; Moreno-Barriga et al., 2017a) experiments revealed that this species is suitable for phytoremediation as it shows limited translocation of metals from roots to shoots. *P. miliaceum* is also considered a pioneer colonizer of metal(loid) mine tailings. However, its role may be controversial. Navarro-Cano et al. (2018), based on field works, classified this species as a nurse plant (plants with high capacity to cope with multi-stressed conditions that facilitate the further colonization by other less stress-tolerant species). However, Martinez-Oró et al. (2017), based on a pot experiment, stated that *P. miliaceum* can behave as an opportunistic competitor when growing together with the pine tree *P. halepensis*. The fact that *P. miliaceum* was only observed in the experimental field plots in spring 2020 further supports that three years after organic amendment application were necessary to reach a step forward in system sustainability.

7.5. Conclusions

The results obtained highlight the suitability of combining biochar from pruning trees and composted USR to ameliorate the hostile conditions of barren acidic metal(loid) mine tailings soils in Mediterranean semiarid areas and effectively favor spontaneous plant colonization. Improvements in chemical and physico-chemical soil properties were observed just two months after amendment addition (e.g., increase in pH and TOC and decrease in salinity, water soluble metals and ecotoxicity). The recalcitrant organic carbon provided by biochar remained in the soil system for at least one year. The more labile organic compounds provided by USR triggered an initial boost of microbial growth, although they were consumed over time. These improvements were consistent for at least one year and led to lower bulk density, higher water retention capacity and better functionality of the tailing soil as shown by the higher scores for microbial/functional-related parameters (e.g., MBC, microbial catabolic activity and CO_2 emission). Despite the overall improvement described, increased pH following amendment application led to higher water soluble As concentration. The stabilization (or slightly increase) of MBC from about seven months after amendment addition, despite the WSOC drop, and the changes occurring in the metabolic fingerprint, suggested a progressive shift from a microbial population dominated by microorganisms introduced with the amendment to one dominated by native species better adapted to tailing soil conditions. Although vegetation was able to spontaneously colonize the amended tailing soil, one year was not enough to reach a consistent plant cover with adult plants, which was only observed three years after soil treatment. This highlights the slowness of the recovery processes in these extreme environments and the existence of a time-lag between the positive effects of the amendment on soil properties being observed and these improvements being translated into effective spontaneous plant colonization.

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PART III

CONCLUSIONS

CHAPTER 8

General conclusions and management recommendations

8.1. General conclusions

The general conclusions of the PhD Thesis are three:

1) Spontaneous vegetation colonization of abandoned metal(loid) mine tailings from Mediterranean semiarid areas induces improvement of physical, physicochemical, functional and ecotoxicological soil conditions regardless of total metal(loid) levels.

2) Plant species richness and diversity with contrasting life forms and functional traits seem to be key factors for achieving effective functional soil improvement in spontaneously vegetated mine tailings patches.

3) The combination of biochar from pruning trees and composted urban solid refuse (USR) is, in general, a suitable amendment to improve barren acidic mine tailings, by promoting their functional recovery and triggering spontaneous vegetation colonization.

These general statements are supported by the following specific findings:

i) In vegetated mine tailings patches, when the presence and diversity of pioneer and nurse shrubs and grasses reaches a certain status, the improvement of soil conditions allows to achieve scores for soil microbial functional-related parameters (e.g., microbial biomass carbon, β-glucosidase activity, bacterial metabolic activity, functional diversity) comparable to that of the forest soils outside the tailings, regardless of the total metal(loid) levels.

ii) During the early stages of the soil-vegetation system development in mine tailings, the influence of individual abiotic stress factors (mainly physical and physicochemical soil parameters) seems to play a key role. However, when vegetated patches have reached a certain functional status, the influence of each physical and physicochemical soil parameter seems to be not as relevant.

iii) The presence of high levels of available As and Cu in the forest soil away from the mine tailings warns about ecotoxicity risks, even at low total metal(loid) levels. This emphasizes the importance of valuable ecosystem functions provided by vegetated tailings patches that include not only aspects such as metal(loid) and nutrient retention and habitat provision, but also the reduction of pollutants dispersion by promoting soil aggregation beneath plants and acting as phytobarriers against wind and water erosion.

iv) The addition of biochar and composted USR to barren acidic mine tailings ameliorated their hostile conditions and improved their physicochemical soil properties (e.g., increase in pH and total organic carbon and decrease in salinity, water soluble metals, and ecotoxicity) just two months after amendment addition, and the effects were maintained for at least one year. The improvement was also observed in soil structural and microbial/functional-related parameters (e.g., bulk density, water retention capacity, microbial biomass carbon, microbial catabolic activity, CO_2 emission).

- Despite the overall improvement observed, a possible drawback is that the increased pH following amendment application led to higher water soluble As levels. This aspect should be monitored for a longer period to evaluate the consequences at long term.

- The high levels of microbial biomass carbon two months after amendment addition indicated an initial boost of microbial growth, attributable to the labile organic compounds introduced with the USR (rich in water soluble organic carbon), which were consumed over time. The stabilization (or slightly increase) of microbial biomass carbon from about seven months after amendment addition, despite the drop in water soluble organic carbon, together with the changes observed in the microbial metabolic fingerprint, suggests a progressive shift from a microbial population dominated by microorganisms introduced with the amendment to one dominated by native species better adapted to tailing soils conditions.

- Although vegetation was able to spontaneously colonize the amended tailing soil, one year was not enough to reach a consistent plant cover with adult plants, which was only observed three years after soil treatment. The existence of this time lag highlights that the recovery of the soil-plant system in these extreme environments is slow even with management practices for improving soils.

8.2. Management recommendations

The findings of the PhD Thesis support the value of passive restoration as a suitable option for the regeneration of abandoned metal(loid) mine tailings from Mediterranean semiarid areas. This does not imply that spontaneous vegetation colonization has the capacity, by itself, to fully restore ecosystem functions and eliminate environmental hazards of abandoned mine tailings. Rather than, the results show that spontaneous colonization by native vegetation should be seriously considered as a valuable option to complement others. Moreover, mine tailings management, either by conventional techniques and/or phytomanagement options, should be preceded by a detailed knowledge of the already existing spontaneously colonized sites (i.e., fertility islands), which should be preserved to take advantage of their high potentiality. These vegetated patches could act as nucleation spots not only for plant recruitment but also for biological propagules that may help to accelerate the functional recovery of these degraded environments.

Spontaneous vegetation colonization could be favored by techniques of aided phytomanagement. Among them, the use of organic amendments, such as biochar and composted USR, to ameliorate the hostile conditions of tailing soils could be useful to stimulate spontaneous colonization. Moreover, since the latter process is slow under semiarid conditions, sowing and afforestation combined with amendment additions would accelerate the restoration. These practices should be managed with caution to promote functional diversity instead of the uncontrolled spread of ruderal opportunistic species that produce high biomass but low diversity, which does not favor succession processes. Hence, afforestation should include plant species with diverse life forms and functional roles, instead of monocultures.