Departamento de Química Agrícola y Bromatología

Facultad de Ciencias

Universidad Autónoma de Madrid

**Tesis Doctoral**

# Enmiendas de hierro y materiales orgánicos para la recuperación de suelos contaminados con arsénico y cobre

## Iron and organic amendments for the remediation of arsenic- and copper-contaminated soils

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**Madrid, 2016**

UNIVERSIDAD AUTONOMA DE MADRID

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Que Dña. María Teresa Fresno García ha realizado bajo nuestra dirección y en este Departamento el trabajo que lleva por título “Enmiendas de hierro y materiales orgánicos para la recuperación de suelos contaminados con arsénico y cobre”, que constituye su Memoria de Tesis Doctoral. Dicho trabajo reúne todas las condiciones necesarias para su presentación y defensa.

Y para que conste a los efectos oportunos firmamos el presente certificado en Madrid, a 5 de Diciembre de 2016.

Fdo.: D. Jesús M. Peñalosa Olivares D. Eduardo Moreno Jiménez

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Memoria que presenta Dña. María Teresa Fresno García para obtener el título de Doctora con Mención Internacional por la Universidad Autónoma de Madrid.

**Fdo.: Mª Teresa Fresno García**

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# Abstract

Soil contamination is a problem at a global scale and is attracting increasing attention in the last decades. The remediation of contaminated soils is a challenge to protect ecosystems and human health but the remediation needs to be economically feasible. Conventional remediation technologies, such as excavation or vitrification, present multiple limitations, such as high cost and disturbance of soil properties and functioning. To overcome these limitations, gentle remediation strategies are attracting more attention from researchers.

Phytoremediation or phytotechnologies use green plants to remove or stabilise metals in soil and offer a low-cost and sustainable alternative. Selecting plants can optimise the remediation process and help to pursue economical sustainability, i.e. producing biomass for energy, safe food and plant products. In combination to plant-based technologies, the addition of soil amendments often decrease metal(loid)s availability and reduce their phytotoxicity, creating a suitable environment for the establishment of a plant cover. Inorganic amendments are in general effective at immobilising metals in soil; however, their application rarely improves soil physico-chemical properties. Iron amendments have been often studied for the remediation of As-contaminated soils, since iron oxides are known to be major scavengers of As in soils and to mainly control its mobility. Amongst different iron amendments in soils, reactive iron salts, such as iron sulfate, have demonstrated high efficiency to adsorb As in short- and long-term experiments. Organic amendments lead often to metals immobilisation but may occasionally provoke As mobilization. The main advantage of using organic materials as soil amendments is the improvement of soil functions.

In multi-contaminated soils, each amendment will have different, sometimes contrasting, effects on each contaminant. When negative effects appear (i.e. organic matter on As mobility), the combination of different amendments may be necessary to counteract the drawbacks with another amendment (iron amendments to prevent As mobilisation). However, so far little research has focused on the combination of reactive iron salts and organic materials as single amendment for multi-contaminated sites.

The main objective of this PhD Thesis is to evaluate a soil remediation strategy based on the combination of iron sulfate and organic materials as a single amendment for its application to As- and Cu-contaminated soils. To achieve this objective, two

investigation lines were established, which are developed in two different parts of this dissertation.

In the first part of the Thesis, the influence of several soil treatments combining iron sulfate and organic amendments on As and Cu fractionation and mobility in the soil was evaluated. The influence of the treatments on soil functioning was also studied, especially their effects on plant establishment and growth.

For that, three experiments were carried out using a slightly acidic soil contaminated mainly with As, but also with high content of Cu. The contaminated soil was treated with iron sulfate in combination with several materials differing in their content in organic matter and their physico-chemical characteristics: paper mill sludge (Fe+PS); two compost, produced with olive mill waste (Fe+OMWC) or municipal green waste (Fe+GWC), and biochar, produced from olive tree pruning or holm oak woodchip (Fe+BC). A treatment combining iron sulfate and calcium carbonate was included in all the experiments to isolate the effects of iron addition (Fe+lime) and a non-treated soil was used as a control.

The experiments were carried out at different size and time scales: two experiments were performed in pots, where the effect of soil treatments was investigated for 45 or 48 days (short-term) and a third experiment was carried out in macrocosm (240 $dm^3$-lysimeters), where the remediation strategy was implemented for almost two years (medium/long term).

The effect of the treatments on As and Cu distribution in different soil fractions, and their solubility and phytoavailability was investigated. Soil quality was evaluated in terms of nutrient availability, soil health parameters (enzymatic activities) and its ability to establish a *healthy* plant cover. The latter was studied with three plant species previously identified as interesting for phytostabilisation strategies: *Arrhenatherum elatius* L., *Lupinus albus* L. (white lupin) and *Secale cereale* L. (rye).

In the pot experiments, the combination of iron sulfate and the organic materials led to a decrease in As mobility, due to the formation of additional amorphous iron oxides, which mainly governed As dynamics in soil, while the addition of organic matter did not result in As mobilisation. Other factors, especially soil pH, also influenced As mobility. However, despite the treatments increased soil pH, when it was adjusted to a neutral range (5.5-7) there was no As mobilisation. The treatments were less effective at stabilising As in the macrocosm experiment, where organic matter addition showed greater influence than in the pot experiments. An increase in As mobility was found in all soils towards the end of the macrocosm experiment, but this cannot be attributed to amendment effect but rather to ageing, besides other factors. It is noteworthy that,

despite the intense addition of organic matter with treatments Fe+OMWC, Fe+OMWC and Fe+BC, significant mobilisation of As was not observed, pointing out the preventing effect of iron coapplication with the organic amendment. Cu mobility was efficiently reduced in all the experiments and was strongly influenced by soil pH and the addition of organic matter.

The combination of iron sulfate with olive mill waste compost (Fe+OMWC), green waste compost (Fe+GWC) and holm oak biochar (Fe+BC) generally resulted in an increase in nutrients availability (K, Mg, P), total organic carbon and total nitrogen contents in soil, both in short and medium/long term experiments. Plant growth was also increased by these treatments. *A. elatius* biomass was enhanced by Fe+OMWC and *L. albus* by Fe+OMWC and Fe+BC. In the macrocosm experiment, the treatments Fe+OMWC, Fe+GWC and Fe+BC improved *S. cereale* germination and biomass production, especially Fe+BC, which resulted in the greatest plant cover.

In the second part of the Thesis, two experiments were carried out to investigate the interaction of iron treatments with As in the rhizosphere and the effects on plant As uptake.

In a hydroponic experiment, it was investigated whether root iron plaque, formed under aerobic conditions, affected As uptake, metabolism and distribution in *Lupinus albus* plants. For that, iron plaque was induced on white lupin roots by supplying 30 mg $L^{-1}$ iron as $FeSO_4$ to the aerated nutrient solution. Then, three doses of As, supplied as sodium arsenate, were established: 0, 5 and 20 μM. Results on As uptake, speciation and distribution in the root and the whole plant were compared to a treatment consisting on supplying 3 mg $L^{-1}$ iron as Fe/EDDHA.

Similar total As concentration was found in roots from both iron treatments ($FeSO_4$ and Fe/EDDHA), but As translocation to shoots was greatly reduced when iron plaque was formed ($FeSO_4$ treatment). Results on As speciation showed that As was mainly accumulated as As(V) in $FeSO_4$-treated roots, whereas As(III) was the major species in Fe/EDDHA-treated roots. Laser ablation coupled to ICP-MS (LA-ICP-MS) accomplished in root sections showed surficial co-accumulation of As and Fe in $FeSO_4$-trated roots, but not in Fe-EDDHA-treated ones.

These results showed that iron plaque was formed on the surface of white lupin roots under aerobic conditions. This plaque sequestered As on roots surface, mitigating its uptake and translocation to the shoots.

A pot experiment was conducted to investigate As and Cu mobility in the rhizosphere of white lupin grown in soil treated with iron sulfate and lime (Fe+lime) or

iron sulfate and biochar (Fe+BC). Non-treated soil was used as a control. A rhizobag system was used to differentiate between rhizosphere and bulk soil. Porewater chemistry was monitored throughout the experiment. Extractable As, Cu, Fe and P and As and Cu plant uptake were evaluated at the end of the experiment. The distribution of As, Cu, P and Fe in the lupin rhizosphere was evaluated by chemical mapping using diffusive gradients in thin films (DGT) sampling and LA-ICP-MS analysis of the gel.

The treatments reduced the soluble and extractable As and Cu fractions in the bulk soil, but slightly higher soluble As was found in the rhizosphere along the experiment in all cases. Soluble P was also higher in the rhizosphere after two weeks of plant growth. The increment in soluble As was greater in the Fe+lime-treated soil, where porewater pH and extractable As and Fe were also higher in the rhizosphere than in the bulk soil. Chemical mapping showed mobilisation of As and Fe in the rhizosphere of white lupin grown in the non-treated soil. In spite of the reduction of As and Cu mobility provoked by the treatments, plant uptake was similar in the treated and the control soils.

These results emphasise the importance of considering root activities and their effect on metal(loid)s biogeochemistry in the rhizosphere, when selecting plants and amendments for an aided phytostabilisation strategy.

As a main conclusion, the results obtained in this Thesis show the combination of iron sulfate and organic materials, such as compost and biochar, as a suitable amendment for the remediation of As- and Cu- contaminated soils. Besides, these results highlight the importance of performing long-term experiments and investigating rhizosphere processes before *in situ* implementation of a soil remediation strategy.

# Resumen

La contaminación del suelo es un problema a escala global y está recibiendo más tención en las últimas décadas. La recuperación o remediación de los suelos contaminados debe basarse en proteger los ecosistemas y la salud humana, pero también debe ser económicamente sostenible, lo que en ocasiones supone un reto. Las técnicas de remediación convencionales, tales como la vitrificación, presentan ciertas limitaciones, como el alto coste de su aplicación o la alteración de las propiedades y la funcionalidad del suelo. Por ello, en los últimos años la investigación se centra en impulsar la implementación de técnicas respetuosas con el medioambiente.

La fitorremediación se basa en el empleo de plantas vasculares para eliminar o estabilizar metales en el suelo y supone una alternativa económica y sostenible. La selección de plantas con valor añadido puede además optimizar el proceso de remediación y su sostenibilidad económica, por ejemplo, mediante la producción de biomasa para generar energía y mediante la obtención de productos vegetales seguros para la salud. La aplicación de enmiendas del suelo se puede utilizar para disminuir la biodisponibilidad de los contaminantes y para crear un ambiente adecuado para el establecimiento de una cubierta vegetal, acelerando así el proceso de la fitorremediación. Las enmiendas inorgánicas son por lo general efectivas en la inmovilización de metales en el suelo; sin embargo, su aplicación rara vez mejora las propiedades físico-químicas del suelo. Las enmiendas de hierro han sido ampliamente estudiadas para la recuperación de suelos contaminados con As, ya que los óxidos de hierro controlan en gran parte la movilidad de este metaloide en el suelo. De entre las posibles formas de aplicar hierro al suelo, la utilización de sales reactivas, como sulfato de hierro, ha demostrado una elevada eficacia en la adosrción de As tanto a corto como a largo plazo. Las enmiendas orgánicas por lo general dan lugar a la inmovilización de metales, pero en ocasiones han provocado la movilización de As. La mayor ventaja del empleo de materiales orgánicos como enmiendas es la mejora de las funciones del suelo.

En aquellos suelos en los que se da multicontaminación, cada enmienda puede tener diferentes efectos sobre cada tipo de contaminante, en ocasiones efectos opuestos. Por eso en estos casos a veces se hace necesario recurrir a la combinación de diferentes enmiendas para evitar efectos negativos (como la movilización de As al aplicar materia orgánica). Sin embargo, hasta el momento existen pocos estudios

centrados en la combinación de sales de hierro y materiales orgánicos como única enmienda para la recuperación de suelos con multicontaminación.

El principal objetivo de esta Tesis Doctoral es la evaluación de una estrategia de remediación de suelos contaminados con As y Cu basada en la combinación de sulfato de hierro y materiales orgánicos como única enmienda. Para alcanzar este objetivo se establecieron dos líneas de investigación, que se desarrollan en dos partes diferenciadas de esta memoria.

En la primera parte de esta Tesis, se estudió la influencia de varios tratamientos consistentes en la combinación de sulfato de hierro y enmiendas orgánicas sobre el fraccionamiento y la movilidad de As y Cu en un suelo. Además, se estudió el efecto de los tratamientos sobre la funcionalidad del suelo, prestando especial atención al establecimiento de una cubierta vegetal.

Para ello se llevaron a cabo tres experimentos en los que se utilizó un suelo ligeramente ácido y principalmente contaminado con As, que presentaba además una elevada concentración de Cu. El suelo contaminado se trató con sulfato de hierro en combinación con varios materiales que diferían en su cantidad de materia orgánica y en sus propiedades físico-químicas: un residuo de la industria papelera (Fe+PS); dos compost, producidos a partir de *alperujo* (Fe+OMWC) o de restos de poda urbanos (Fe+GWC) y biochar, producido a partir restos de poda de olivo o de encina (Fe+BC). Además, se incluyó un tratamiento en el que se combinó sulfato de hierro con carbonato cálcico para aislar los efectos de la aplicación de hierro (Fe+lime) y se utilizó como control el suelo no tratado.

Los experimentos se llevaron a cabo a diferentes escalas de tamaño y tiempo: dos experimentos se realizaron en tiestos y el efecto se los tratamientos se investigó durante 45 o 48 días (corto plazo), mientras que un tercer experimento se realizó en macrocosmos (contenedores o lisímetros de 240 $dm^3$ de capacidad) y la estrategia de remediación se implementó durante casi dos años (medio-largo plazo).

Se estudió el efecto de los tratamientos sobre la distribución de As y Cu en diferentes fracciones del suelo y sobre su solubilidad y fitodisponibilidad. Se evaluó la calidad del suelo en términos de disponibilidad de nutrientes, parámetros indicadores de la salud del suelo (actividades enzimáticas) y la capacidad para establecer una cubierta vegetal “sana”. Esto último se evaluó con tres especies previamente identificadas como adecuadas para estrategias de fitoestabilización: *Arrhenatherum elatius* L., *Lupinus albus* L. (altramuz blanco) y *Secale cereale* L. (centeno).

En los experimentos en tiestos, la combinación de sulfato de hierro y materiales orgánicos dio lugar a una reducción de la movilidad de As, debido a la formación de óxidos de hierro que controlaron mayoritariamente la dinámica del As en el suelo, mientras que la adición de materia orgánica no provocó su movilización. Otros factores, en especial el pH del suelo, influyeron en cierta medida en la movilidad del As. Sin embargo, a pesar de que en general los tratamientos aumentaron el pH del suelo, cuando este se ajustó a un rango de neutralidad (5,5-7), este efecto no provocó la movilización del As. Los tratamientos fueron menos efectivos en la estabilización de As en el experimento en macrocosmos, donde la adición de materia orgánica pareció tener una mayor influencia que en los experimentos en tiestos. Hacia el final del experimento se observó un aumento de la movilidad de As en todos los casos, aunque no parece que se pueda atribuir a un efecto de los tratamientos, sino al efecto del paso del tiempo sobre la estructura de los óxidos de hierro formados, entre otros factores. En este experimento es destacable el hecho de que, a pesar de que se añadió materia orgánica con los tratamientos Fe+OMWC, Fe+OMWC y Fe+BC, no se observó una movilización significativa de As, lo que sugiere que la aplicación de hierro junto con materiales orgánicos puede prevenir la movilización de As. La movilidad de Cu se redujo eficazmente en todos los experimentos y fueron el pH del suelo y la adición de materia orgánica los factores que más influencia tuvieron sobre este metal.

La combinación de sulfato de hierro con compost de alperujo (Fe+OMWC), compost de restos de poda (Fe+GWC) y con biochar de encina (Fe+BC) resultó en general en un aumento en la disponibilidad de nutrientes (K, Mg, P) y en el contenido de carbono orgánico y nitrógeno total en los experimentos a tanto corto como a medio-largo plazo. Además, estos tratamientos mejoraron el crecimiento de las plantas. La biomasa de *A. elatius* fue mayor con Fe+OMWC y la de *L. albus* aumentó con la aplicación de Fe+OMWC y Fe+BC. En el experimento en macrocosmos, los tratamientos Fe+OMWC, Fe+GWC y Fe+BC mejoraron la germinación y producción de biomasa de centeno, especialmente Fe+BC, que dio lugar a una mayor cobertura vegetal.

En la segunda parte de esta Tesis se llevaron a cabo dos experimentos en los que se investigó la interacción de tratamientos de hierro con el As en la rizosfera y el efecto en su absorción por la planta.

Se realizó un experimento hidropónico para investigar si la presencia de una placa de hierro formada en condiciones aeróbicas podría afectar a la absorción, metabolismo

y distribución de As en plantas de *L. albus*. Para ello se indujo la formación de placa en las raíces de altramuz añadiendo 30 mg $L^{-1}$ de Fe a la disolución nutritiva como $FeSO_4$, manteniendo siempre la aireación. Después se establecieron tres dosis de As, añadido como arseniato sódico: 0, 5 y 20 μM. Los resultados obtenidos referentes a la absorción, especiación y distribución de As en la raíz y la planta se compararon con un tratamiento en el que el Fe se añadió como el quelato Fe/EDDHA (3 mg $L^{-1}$ de Fe).

Se encontró una concentración de As total en raíz similar en ambos tratamientos de hierro ($FeSO_4$ y Fe/EDDHA), pero la translocación a la parte aérea fue mucho menor cuando se formó la placa de hierro (tratamiento $FeSO_4$). Los resultados de especiación de As mostraron que la mayor parte del As presente en las raíces tratadas con $FeSO_4$ era As(V), mientras que en las raíces tratadas con Fe/EDDHA el As(III) era la especie mayoritaria. El análisis de cortes transversales de raíces mediante ablación láser acoplada a ICP-MS (LA-ICP-MS) mostró una co-acumulación superficial de Fe y As en las raíces tratadas con $FeSO_4$, pero no se observó lo mismo en las tratadas con Fe/EDDHA).

Estos resultados confirmaron la formación de una placa de hierro sobre las raíces de altramuz en condiciones aerobias. Esta placa retuvo el As en la superficie de la raíz, reduciendo así su absorción y por tanto su translocación a la parte aérea.

Se llevó a cabo un experimento en tiestos para investigar la movilidad de As y Cu en la rizosfera de plantas de altramuz cultivadas en un suelo tratado con sulfato de hierro y carbonato cálcico (Fe+lime) o sulfato de hierro y biochar (Fe+BC). Además, se incluyó un suelo no tratado como control. Se utilizó un sistema de *rhizobags* (rizobolsas) para diferenciar el suelo rizosférico del resto del suelo (*bulk*). Se analizó a lo largo del experimento el agua de poro y la fracción extraíble de As, Cu, Fe y P al final, así como la concentración de As y Cu en planta. Además se evaluó la distribución de As, Cu, P y Fe en la rizosfera de altramuz utilizando la técnica de muestreo *diffusive gradients in thin films* (DGT) y el análisis del gel mediante LA-ICP-MS.

Los tratamientos redujeron las fracciones soluble y disponible de As y Cu en el suelo no afectado directamente por la raíz (suelo *bulk*), pero se encontró un ligero aumento de la concentración de As soluble en todos los casos a lo largo del experimento, además de un aumento del P soluble en la rizosfera tras dos semanas de cultivo del altramuz. El aumento en la solubilidad de As fue mayor en los suelos tratados con Fe+lime, en los que el pH del agua de poro y el As y el Fe extraíbles también fueron mayores en la rizosfera que en el suelo *bulk*. El mapero químico de los geles demostró una cierta movilización de As y Fe en la rizosfera de altramuz en el

suelo no tratado. A pesar de la reducción en la movilidad de As y Cu provocada por los tratamientos, su acumulación en la planta fue similar en los suelos tratados y en el control.

Estos resultados enfatizan la importancia de considerar la actividad de las raíces y su efecto sobre la biogeoquímica de los metales y metaloides en la rizosfera cuando se seleccionen plantas y enmiendas para llevar a cabo una estrategia de fitoestabilización.

Como conclusión principal, los resultados obtenidos en esta Tesis muestran que la combinación de sulfato de hierro y materiales orgánicos, tales como compost y biochar, es una enmienda adecuada para la recuperación de suelos contaminados con As y Cu. Además, estos resultados resaltan la importancia de realizar ensayos a largo plazo y de investigar los procesos rizosféricos antes de implementar un proceso de remediación *in situ*.

# ÍNDICE

# Capítulo 1

# Introducción

## 1.1. Contaminación del suelo y legislación

### *El suelo, un recurso natural limitado*

Aunque generalmente se define el suelo como la capa superior de la corteza terrestre, una definición más completa y que aporta más matices puede ser la establecida en el Real Decreto 9/2005, que define como suelo "la capa superior de la corteza terrestre, situada entre el lecho rocoso y la superficie, compuesto por partículas minerales, materia orgánica, agua, aire y organismos vivos y que constituye la interfaz entre la tierra, el aire y el agua, lo que le confiere capacidad de desempeñar tanto funciones naturales como de uso".

La Carta de los Suelos del Consejo de Europa, redactada en Estrasburgo en 1972, reconoció la importancia del suelo como uno de los bienes más preciados de la humanidad, ya que "permite la vida de los vegetales, de los animales y del hombre sobre la superficie de la Tierra". Este documento enfatizó la vulnerabilidad del suelo y la necesidad de su conservación y protección contra la erosión y la contaminación. Más tarde, en el año 1981, la FAO redactó la primera Carta Mundial de los Suelos con el objetivo de promover e institucionalizar la gestión sostenible de este recurso limitado. Este documento fue revisado en el año 2015, coincidiendo con el Año Mundial del Suelo, por parte de la Alianza Mundial de los Suelos (AMS). En esta Carta se reconoce la suprema importancia del suelo para la supervivencia y el bienestar de los pueblos y destaca la importancia de una utilización racional de los recursos edafológicos mundiales, así como la necesidad de su protección contra la degradación irreversible.

### *Contaminación del suelo*

La contaminación del suelo es uno de los principales procesos que dan lugar a su degradación. Según la Ley 22/2011 de residuos y suelos contaminados, se considera suelo contaminado "aquel cuyas características han sido alteradas negativamente por la presencia de componentes químicos de carácter peligroso procedentes de la actividad humana, en concentración tal que comporte un riesgo inaceptable para la salud humana o el medio ambiente, de acuerdo con los criterios y estándares que se determinen por el Gobierno, y así se haya declarado mediante resolución expresa". Según la Agencia Europea del Medio Ambiente (EEA), una zona contaminada se

considera como un área bien definida donde la contaminación del suelo se ha confirmado y presenta un riesgo potencial para el ecosistema, para los seres humanos o para otros receptores.

La relación entre la contaminación del aire y del agua y sus efectos sobre la salud está hoy en día relativamente clara. Sin embargo, históricamente no se ha prestado tanta atención a la contaminación del suelo y a los efectos que ésta puede tener sobre nuestra salud. En un informe realizado recientemente por la Comunidad Europea (European Comission, 2013a) se abordó este tema, reconociendo la necesidad de seguir investigando sobre esta relación. Como se indica en este informe, y de acuerdo a lo declarado por la EEA en el año 2007, se estima que más de 3 millones de terrenos en la Unión Europea han sido expuestos a actividades que puedan ser fuente de contaminación del suelo. De ellos, alrededor de 250.000 necesitan medidas urgentes de remediación o descontaminación. En el mismo año, Panagos et al. (2013) realizaron una revisión en la que integraron datos concernientes a la contaminación del suelo aportados por 27 países miembros de la Unión Europea, junto con otros países del territorio europeo, con el objetivo de tener una visión global de cuántas zonas hay afectadas en Europa por este problema y cuáles son las principales causas. Como indican los autores, hasta esa fecha se han identificado en Europa hasta 1.170.000 zonas potencialmente contaminadas, de las cuales alrededor de 127.000 se han confirmado, aunque se estima que esta cifra puede ascender a 342.000. De entre los principales sectores que contribuyen a la contaminación del suelo, las actividades industriales y comerciales lo hacen en un 33.3%, de las cuales un 7% corresponde a actividades mineras. Ambos informes coinciden en que, de entre los contaminantes más comunes, los aceites minerales y en mayor medida los metales pesados son los más frecuentes tanto en suelos como en aguas subterráneas (Fig. 1.1). Estos últimos representan más del 30% de los contaminantes en las zonas declaradas como contaminadas hasta el 2011 en la UE.

Aunque algunos metales pesados (incluyendo semimetales como el As o el Sb), también denominados elementos traza, son esenciales para los seres vivos, se consideran contaminantes cuando existen en una zona indeseada o cuando la forma y concentración en las que están presentes pueden causar algún perjuicio sobre la salud humana o el medio ambiente (Panagos et al., 2013). Debido al desarrollo industrial y económico, la contaminación del suelo por metales pesados es cada vez más frecuente en todo el mundo, pero presenta el problema de que es difícil de identificar debido a su “invisibilidad” (Su et al., 2014). La presencia de metales pesados en el suelo tiene diversas fuentes, que se pueden clasificar en geológicas y antropogénicas.

Como la mayoría de ellos forman parte del material parental del suelo, su meteorización da lugar a la liberación de los metales y su enriquecimiento en la capa más superficial del suelo.

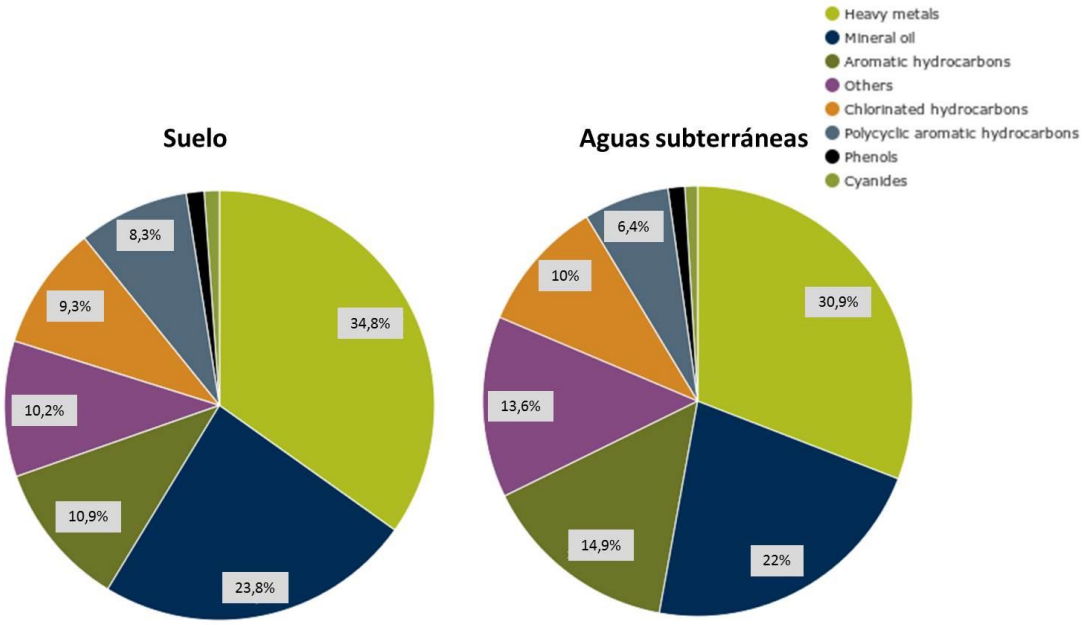


**Fig. 1.1**. Distribución de algunos contaminantes en suelos y aguas subterráneas de Europa (Fuente: EEA, 2011).

Las fuentes antropogénicas pueden ser muy variadas, ya que están presentes en muchas aspectos de nuestra vida diaria; de entre las principales se encuentran los procesos industriales, el sector de la producción y los desechos, tanto domésticos como industriales (Bolan et al., 2014). En la Tabla 1.1 se recogen las principales fuentes de los metales más comunes. Los metales pesados pueden llegar al suelo por diferentes vías, como son la deposición atmosférica, las aguas residuales, el suministro de productos agrícolas y los residuos sólidos, de entre los cuales los que provienen de la minería y los procesos industriales representan un problema importante (Su et al., 2014), como se ha recalcado anteriormente.

En la Figura 1.2 se muestran varios mapas de la distribución de metales pesados en suelos del continente europeo elaborados por Tóth et al. (2016). Los autores indican que aproximadamente el 28% del total de la superficie de la UE debería someterse a una mayor evaluación de la contaminación de suelos por metales, especialmente teniendo en cuenta zonas en las que actividades como la industria y la minería han dado lugar a elevadas concentraciones de algunos elementos traza, especialmente de As, Cd, Pb y Hg.

La contaminación del suelo con metales o elementos traza, ya se haya producido de manera puntual o continuada, da lugar a un detrimento en la calidad del suelo afectado. La calidad del suelo se puede definir como su capacidad para llevar a cabo de forma sostenible sus procesos ecológicos, sus funciones y su servicio al ecosistema a un nivel similar al de un suelo de referencia, sin causar un impacto negativo en los ecosistemas adyacentes o la salud humana (Garbisu et al., 2011; Burges et al., 2015). Debido a que el suelo es la base de la cadena trófica, uno de los principales riesgos asociados a la presencia en exceso de metales pesados es la seguridad alimentaria, especialmente cuando los suelos afectados se utilizan para la producción agrícola y ganadera. Aunque la gran mayoría de los suelos en la Unión Europea se pueden considerar seguros para la producción alimentaria, recientemente se ha sugerido que aproximadamente un 6% del terreno agrícola debería someterse a controles más estrictos y procesos de descontaminación (Tóth et al., 2016).

**Tabla 1.1.** Emisión de metales pesados hacia suelos alrededor del mundo generados a partir de diferentes fuentes (1000 t · año$^{-1}$). Modificado de Su et al. (2014) [Fuente: Nriagu y Pacyna (1988)].

| Fuente | As | Cd | Cr | Cu | Hg | Ni | Pb | Zn |
|---|---|---|---|---|---|---|---|---|
| Residuos agrícolas y alimentarios | 0-0,6 | 0-0,3 | 4,5-90 | 3-38 | 0-1,5 | 6-45 | 1,5-27 | 12-150 |
| Estiércoles | 1,2-4,4 | 0,2-1,2 | 10-60 | 14-80 | 0-0,2 | 3-36 | 3,2-20 | 150-320 |
| Residuos de la tala y la industria maderera | 0-3,3 | 0-2,2 | 2,2-18 | 3,3-52 | 0-2,2 | 2,2-23 | 6,6-8,2 | 13-65 |
| Residuos municipales | 0,09-0,7 | 0,88-7,5 | 6,6-33 | 13-40 | 0-0,26 | 2,2-10 | 18-62 | 22-97 |
| Aguas residuales municipales | 0,01-0,24 | 0,02-0,34 | 1,4-11 | 4,9-21 | 0,01-0,8 | 5,0-22 | 2,8-9,7 | 18-57 |
| Residuos orgánicos | 0-0,25 | 0-0,01 | 0,1-0,48 | 0,04-061 | - | 0,17-3,2 | 0,02-1,6 | 0,13-2,1 |
| Metalurgia | 0,01-0,21 | 0-0,08 | 0,65-2,4 | 0,95-7,6 | 0-0,08 | 0,84-2,5 | 4,1-11 | 2,7-19 |
| Cenizas de carbón | 6,7-37 | 1,5-13 | 149-446 | 95-335 | 0,37-4,8 | 56-279 | 45-242 | 112-484 |
| Fertilizantes | 0-0,02 | 0,03-0,25 | 0,03-0,38 | 0,05-0,58 | - | 0,20-3,5 | 0,42-2,3 | 0,25-1,1 |
| Impurezas de materias primas | 36-41 | 0,78-1,6 | 305-610 | 395-790 | 0,55-0,82 | 6,5-32 | 195-390 | 310-620 |
| Deposición atmosférica | 8,4-18 | 2,2-8,4 | 5,1-38 | 14-36 | 0,63-4,3 | 11-37 | 202-263 | 49-134 |

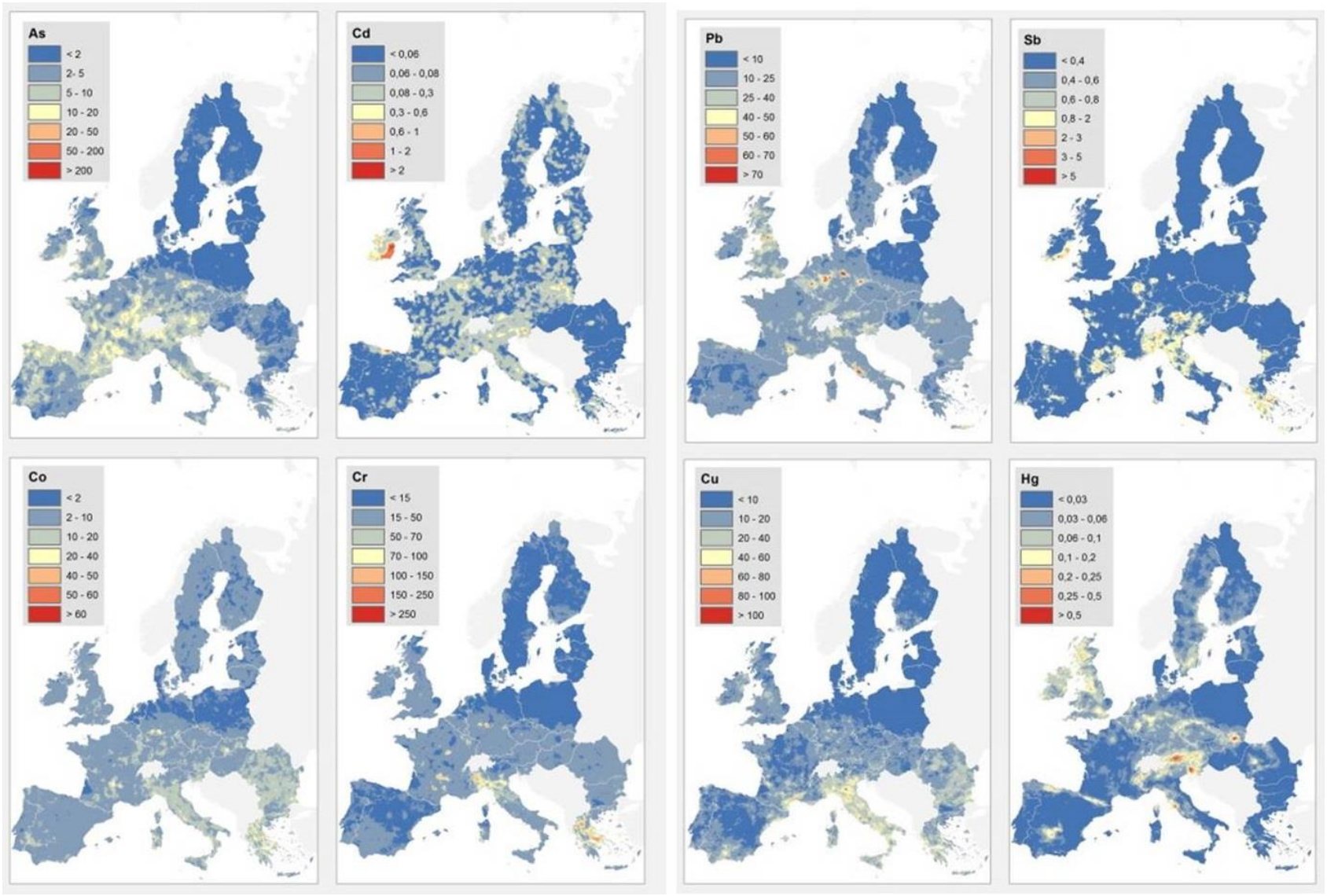


**Figura 1.2.** Mapas de distribución de algunos metales en la capa superficial de los suelos de la Unión Europea. La concentración viene dada en mg $kg^{-1}$. Modificado de Tóth et al. (2016).

Contaminación por As

El arsénico ha sido identificado por la organización mundial de la salud (OMS, o en inglés WHO) como una de las diez sustancias químicas de mayor preocupación pública. La principal fuente del As en el suelo es de origen geológico, ya que se encuentra normalmente asociado a minerales de azufre como la arsenopirita (FeAsS), el rejalgar ($As_4S_4$) o el oropimente ($As_2S_3$), además de que sustituye fácilmente a Si, Al o Fe en los silicatos (Fitz y Wenzel, 2002; Zhao et al., 2010). La variación de la concentración de As en los suelos depende en mayor medida del material parental que de las características geomorfológicas de la capa más superficial del suelo (Smith et al., 1998; Tóth et al., 2016). En suelos no contaminados, la concentración de As suele estar por debajo de 10 mg $kg^{-1}$, variando habitualmente entre 1,5 y 2 mg $kg^{-1}$ (Adriano, 2001; Fitz y Wenzel, 2002). Entre las principales fuentes antropogénicas de As se encuentran la industria, la minería, la siderurgia, la aplicación de productos agroquímicos y los conservantes de la madera (Adriano, 2001; Mandal y Suzuki, 2002; Fitz y Wenzel, 2002).

La principal vía de exposición de As para el ser humano es su presencia en altas concentraciones en el agua, como ocurre en países del sudeste asiático como la India o Bangladesh, donde por causas naturales la concentración de As en el agua de más de 50 distritos supera el límite de 10 μg $L^{-1}$ establecido por la OMS (European Comission, 2013a; Rasheed et al., 2016). Para las poblaciones no expuestas directamente a aguas potables contaminadas, la principal fuente de ingesta de As es la alimentación, ya sea a través de cereales eficientes en la absorción de As como el arroz (la base de la alimentación en muchos países asiáticos), o a través de la carne si el ganado ha sido alimentado con piensos con altas concentraciones de As (Zhao et al., 2010; Rasheed et al., 2016). Un ejemplo es el caso de la provincia china de Hunan, donde años de un tratamiento inadecuado de los residuos de sus múltiples explotaciones mineras han dado lugar a altas concentraciones de As en suelos agrícolas y en consecuencia en el arroz cultivado (Lei et al., 2013; Wang et al., 2015).

Contaminación por Cu

El Cu es un elemento esencial para las plantas y los animales, pero un exceso de este en suelos y aguas subterráneas puede dar lugar a efectos nocivos para la salud. La acumulación de Cu en los suelos tiene su origen principalmente en actividades antropogénicas como la minería o los procesos industriales, que han derivado en focos

localizados con altas concentraciones de Cu en la capa más superficial del suelo. El uso de productos agroquímicos que contienen Cu en su composición, tales como el sulfato de Cu o el oxicloruro de Cu, ha generado la contaminación de suelos agrícolas, especialmente viñedos y huertos (Chaignon et al., 2003; Wuana y Okieimen, 2011; Mackie et al., 2014). Esto puede explicar por qué la zona mediterránea, en la que este uso del suelo es más común, presenta áreas con concentraciones más elevadas de Cu (Fig. 1.2) (Tóth et al., 2016).

***Legislación para la protección del suelo***

Tomando como referencia la Cumbre de la Tierra, celebrada en Río en el año 1992 y donde se reconoció la importancia de la protección de los suelos, en el año 2002 la Comisión Europea elaboró la Comunicación "Hacia una estrategia temática para la protección del suelo" [COM (2002)], la primera que abordaba la protección del suelo a nivel europeo, con el objeto de servir como base para una política comunitaria respecto a la prevención de la contaminación. Esta Comunicación señalaba la contaminación del suelo como una de las ocho amenazas que afectan a los suelos de la Unión Europea, además de la erosión, la pérdida de materia orgánica, la salinización, la compactación, la pérdida de la biodiversidad del suelo, el sellado, los deslizamientos de tierras y las inundaciones.

Tomando como punto de partida aquella primera Comunicación, en el año 2006 la Comisión Europea aprobó la Estrategia Temática para la Protección del Suelo en la Unión Europea [COM (2006), 231], sustentada en cuatro grandes pilares: sensibilización, investigación, integración en otras políticas y legislación. Además, se realizó la propuesta de la Directiva Europea de Protección de Suelos [COM (2006) 232], una Directiva Marco que establecía los principios comunes para la protección de los suelos en toda la Unión Europea. Sin embargo esta propuesta fue retirada en el 2014 como respuesta a la oposición de una minoría de Estados Miembros, por lo que actualmente no existe un marco legal a nivel europeo dirigido a la protección del suelo. Ahora la vista está puesta en el año 2020, cuando la UE espera que el suelo esté debidamente protegido y las zonas contaminadas se encuentren bajo procesos de recuperación (European Comission, 2013b).

En el territorio español, hasta que en el año 1998 se promulgó la Ley 10/1998 de Residuos, no existía ninguna ley que promoviera la protección del suelo. Esta ley permitió por primera vez la protección de este recurso contra la contaminación y la

identificación de los suelos ya contaminados, y quedó derogada por la entrada en vigor de la Ley 22/2011 de residuos y suelos contaminados, que regula el régimen jurídico en esta temática.

Actualmente, la Ley 22/2011 y el Real Decreto 9/2005 establecen en el territorio español la relación de actividades potencialmente contaminantes y los criterios y estándares para la declaración de suelos contaminados (MAPAMA). De acuerdo a estas leyes, se delega en las Comunidades Autónomas la declaración y delimitación de suelos contaminados en base a los criterios establecidos en el RD 9/2005.

En base a lo establecido en el RD 9/2005, la Comunidad de Madrid estableció en el año 2006 los niveles genéricos de referencia de metales pesados y otros elementos traza en suelos de la Comunidad dependiendo de los diferentes usos del suelo, que se recogen en la Orden 2770/2006 y se muestran en la Tabla 1.2.

**Tabla 1.2.** Niveles genéricos de referencia de metales pesados y otros elementos traza en suelos de la Comunidad de Madrid (Orden 2770/2006).

| | **Industrial (mg $kg^{-1}$)** | **Urbano (mg $kg^{-1}$)** | **Otros usos del suelo (mg $kg^{-1}$)** | **VR90 (mg $kg^{-1}$)** |
|---|---|---|---|---|
| Antimonio | 80 | 8 | 0,8 | 0,48 |
| Arsénico | 40 | 24 | 24 | 24 |
| Bario | 100000 | 15200 | 4200 | 138 |
| Berilio | 13 | 2 | 2 | 2,1 |
| Cadmio | 300 | 30 | 3 | 0,22 |
| Cobalto | 1500 | 150 | 15 | 12 |
| Cobre | 8000 | 800 | 80 | 20 |
| Cromo total | 2300 | 230 | 90 | 32 |
| Estaño | 100000 | 46730 | 46730 | 4,45 |
| Manganeso | 33900 | 3390 | 690 | 690 |
| Mercurio | 15 | 7 | 5 | 0,065 |
| Molibdeno | 1500 | 150 | 15 | 0,7 |
| Níquel | 15600 | 1560 | 405 | 21 |
| Plata | 500 | 50 | 5 | 0,12 |
| Plomo | 2700 | 270 | 75 | 30 |
| Selenio | 3900 | 390 | 85 | 0,24 |
| Talio | 30 | 3 | 2 | 0,39 |
| Vanadio | 3700 | 370 | 37 | 37 |
| Zinc | 100000 | 11700 | 1170 | 73 |

## 1.2. Arsénico y cobre en el sistema suelo-planta

La biodisponibilidad de un elemento, que podría definirse como la fracción susceptible de ser absorbida o modificada por un organismo vivo, es el resultado de la interacción de factores geoquímicos, climáticos y biológicos que controlan su movilidad en el suelo. El origen de los elementos traza (litogénico, pedogénico o antropogénico) influye en cierto modo en su comportamiento en el suelo y su biodisponibilidad. Aunque algunos factores como la capacidad de intercambio catiónico y la materia orgánica soluble tienen una elevada influencia, los óxidos de Fe, Al y Mn, debido a su ubicuidad y su sensibilidad a cambios en el potencial redox, son los componentes del suelo que principalmente gobiernan la movilidad y biodisponibilidad de metales pesados y otros elementos traza, especialmente aquellos que se presentan en forma aniónica (Kabata-Pendias, 2004). La distribución de los elementos traza entre las fracciones sólida y líquida (fracción disuelta) y su concentración en la disolución del suelo, así como los factores que más influyen en ella, proporcionan una importante información sobre su movilidad y biodisponibilidad (Sauvé et al., 2000; Kabata-Pendias, 2004).

### *Dinámica del As en el suelo*

En el suelo, el As puede formar parte de una variedad de compuestos orgánicos e inorgánicos, pero se presenta mayoritariamente en forma de oxoaniones inorgánicos de As(III) y As(V). Atendiendo a cálculos termodinámicos, en condiciones aerobias (pe+pH>10) la forma predominante de As es el As(V), mientras que en condiciones anaerobias (pe+pH<6) el As estaría mayoritariamente presente en su estado de oxidación +3, es decir como especies de As(III) (Sadiq, 1997).

La dinámica del As en el suelo es compleja y está determinada por la interacción de varios factores. La solubilidad del As está en parte determinada por su especiación, en la que influyen principalmente el pH y el potencial redox (Eh) del suelo. En el rango de pH común de los suelos (4-9), las especies de As predominantes son, en condiciones reductoras o anaerobias $H_3AsO_3$ (As(III)), y en condiciones aerobias $H_2AsO_4^-$, $HAsO_4^{2-}$ (As(V)) (Fig. 1.3) (Masscheleyn et al., 1991; Smith et al., 1998).

Diversos estudios han mostrado la elevada influencia del pH del suelo sobre la movilidad del As, aunque en general su efecto varía dependiendo de su especiación, de su concentración y de la composición del suelo. Smith et al. (1999) observaron que la influencia del pH es mayor en suelos con un alto contenido en óxidos y aumenta con la concentración de As en la disolución del suelo. En condiciones oxidantes o aerobias, cuando predomina el As(V), un aumento del pH del suelo da lugar por lo general a su movilización. Al aumentar el pH, la conversión de $H_2AsO_4^-$ a $HAsO_4^{2-}$ ($pK_2$ = 6,97) y el aumento de cargas negativas en la superficie de los óxidos y oxihidróxidos de hierro incrementan la repulsión electrostática y facilitan la desorción de As. En condiciones anaerobias el As se encuentra mayoritariamente como la especie neutra $H_3AsO_3$, cuyo $pK_1$ es 9,22; por tanto, su adsorción sobre la superficie del suelo es menor y su solubilidad es generalmente elevada y está principalmente controlada por la disolución de los óxidos de Fe (Masscheleyn et al., 1991; Fitz y Wenzel, 2002).

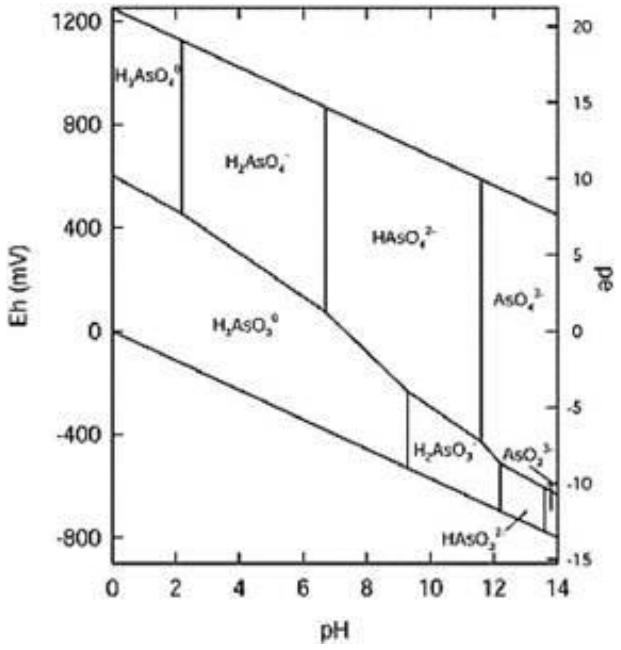


**Figura 1.3.** Diagrama de especiación del arsénico en función del pH-Eh en el sistema As-$O_2$-$H_2O$ a 25 ºC y 1 bar (Smedley y Kinniburgh, 2002).

Dada la elevada afinidad del As por las superficie de óxidos e oxihidróxidos metálicos, especialmente de Fe y Al, su movilidad y solubilidad en suelos está íntimamente relacionada con estos componentes. La capacidad de adsorción de los óxidos de hierro está condicionada por la especiación de As, por el pH del suelo y por la morfología y el grado de cristalización de los óxidos. En este sentido, Bowell (1994) observó que la proporción de As asociada a oxihidróxidos amorfos era mucho mayor

que la asociada a oxihidróxidos cristalinos. Al estudiar cómo afectaba la reducción del As(V) a (III) en su adsorción sobre diferentes tipos de óxidos de hierro (óxidos amorfos, goetita y magnetita), Dixit y Hering (2003) encontraron que la afinidad relativa del As(V) y el As(III) por los óxidos de hierro depende de las características de éstos y de la composición de la disolución, especialmente del pH. A pesar de la idea generalizada de que el As(III) es más móvil que el As(V), los autores mostraron que en un rango de pH de 6 a 9, la capacidad de adsorción del As(III) sobre óxidos de hierro amorfos y sobre goetita era similar o incluso mayor que la del As(V).

La presencia de otros aniones puede interferir en la capacidad del suelo, y en especial de los óxidos, para adsorber As. Dada la similitud entre arseniato y fosfato, la competencia entre estos dos aniones ha sido quizás la más estudiada. El fosfato forma complejos de esfera interna sobre la superficie de óxidos y muestra un comportamiento similar al del arseniato. Manning y Goldberg (1996) demostraron la competencia entre fosfato y arseniato por los mismos sitios específicos de adsorción en un amplio rango de pH (2-11). De forma similar, Kanematsu et al. (2013) encontraron que la presencia del anión fosfato reduce significativamente la adsorción de As(V) en un amplio rango de pH, mientras que la competencia con As(III) es más evidente en rangos de pH menores. La competencia arseniato-fosfato no es simple y depende de muchos factores, tales como la naturaleza de los componentes del suelo, la concentración inicial de arseniato y fosfato y el pH (Violante y Pigna, 1991). Por ejemplo, en un estudio de adsorción/desorción con diferentes suelos, Arco-Lázaro et al. (2016) observaron que la competencia arseniato-fosfato fue más evidente con una baja concentración de As en el equilibrio y en un suelo ligeramente ácido que en uno con un pH neutro/alcalino. La presencia de carbonato en la solución del suelo también puede dar lugar a cierta reducción en la adsorción de As dependiendo de las condiciones; se ha observado que este anión tiene un gran efecto en la adsorción de As(III) a bajo pH, mientras que reduce sólo ligeramente la adsorción de As(V) (Kanematsu et al., 2011, 2013; Stachowicz et al., 2008). Cationes como $Ca^{2+}$ y $Mg^{2+}$ pueden incrementar la adsorción de As(V) sobre la superficie de óxidos (Stachowicz et al., 2008).

El efecto de la materia orgánica sobre la movilidad del As es complejo y existe cierta controversia sobre ello. Bauer y Blodau (2006) sugirieron que la movilización del As provocada por la materia orgánica se debe principalmente a la competencia entre arseniato y aniones orgánicos, mientras que la reducción de As(V) a (III) sería menos relevante. Dobran y Zagury (2006) resaltaron la importancia del contenido de materia orgánica del suelo en la disponibilidad de As(V) y As(III), mostrando que el carbono

orgánico soluble es un factor crítico en la movilización de ambas especies de As. De forma similar, otros autores han observado que la adición de materia orgánica al suelo da lugar a una movilización de As (Tao et al., 2006; Moreno-Jiménez et al., 2013; Beesley et al., 2014). Sin embargo, la influencia de la materia orgánica sobre el As depende de las características del suelo, como demostraron Arco-Lázaro et al. (2016); estos autores observaron que la adición de materia orgánica procedente de un compost disminuía la capacidad de adsorción de As de un suelo rico en óxidos de hierro (con una elevada capacidad de adsorción) pero tenía el efecto contrario en un suelo agrícola con una capacidad de adsorción de As inicialmente baja. Además, algunos autores han mostrado la capacidad de la materia orgánica disuelta para reducir la biodisponibilidad de As (Williams et al., 2011). Esto último podría explicarse asumiendo la formación de complejos ternarios entre la materia orgánica y el As con Fe(III) como catión puente, planteada por varios autores, aunque aún existen pocos trabajos que evidencien la formación de estos complejos en el suelo y bajo diferentes condiciones (Mikutta y Kretzschmar, 2011; Sharma et al., 2010; Sundman et al., 2014).

### *Absorción, metabolismo y transporte de As en la planta*

Como se ha mencionado anteriormente, el arseniato es la especie predominante en suelos oxigenados, por lo que la mayor parte de las plantas terrestres absorben As en esta forma. Puesto que el arseniato y el fosfato se pueden considerar químicamente análogos, su absorción a través de la raíz está íntimamente relacionada. Ambos aniones se absorben a través de los mismos transportadores, que en general presentan una mayor afinidad hacia el fosfato (Meharg y Macnair, 1992). El mecanismo de absorción implica el cotransporte de fosfato o arseniato y protones, con una estequiometria de al menos $2H^+$ por cada molécula de $H_2AsO_4^-$ o $H_2PO_4^-$ (Ullrich-Eberius et al., 1989). Por su parte, la absorción de arsenito es de especial importancia en plantas como el arroz, que crece en suelos anaerobios donde esta es la especie de As predominante. Se ha descrito que la absorción del arsenito en arroz se hace principalmente a través de acuaporinas, que son canales de absorción de agua que permiten el paso de moléculas neutras y por tanto son permeables al arsenito (que en un amplio rango de pH se encuentra totalmente protonado) pero no al arseniato (Meharg y Jardine, 2003). Según estudios recientes, el arsenito y el Si comparten el mismo sistema de transporte en arroz, las proteínas intrínsecas de membrana (NIP) del tipo “nodulin26”; concretamente el transporte tanto de ácido arsenioso como de ácido silícico desde el medio externo al interior de la célula parece darse a través del

transportador OsLsi1 (Ma et al., 2008; Wang et al., 2015). Además de As inorgánico, se han encontrado especies metiladas en arroz, predominantemente ácido dimetilarsínico (DMA). La presencia de estas especies en la planta se debe a su formación en el suelo; su absorción, en la que el pH del medio tiene una elevada influencia, parece ser a través de los mismos transportadores de Si y arsenito (Zhao et al., 2013).

El hecho de que la especie predominante en tejidos vegetales sea generalmente As(III), independientemente de que las plantas se hayan expuesto a As(V) o (III), indica que una vez absorbido el arseniato se reduce rápidamente a arsenito en las células de la raíz; este proceso está catalizado por las enzimas arseniato reductasas (Zhao et al., 2009a). El transporte de As al resto de la planta se hace principalmente a través del xilema y no es por lo general muy efectivo, excepto en plantas hiperacumuladoras como *Pteris vittata*, que presentan una eficiente carga y transporte de arsenito en el xilema (Su et al., 2008). Mientras que la carga de arseniato en el xilema se hace a través del sistema transportador de fosfato, se ha observado que en arroz el transportador de Si OsLSi2 está implicado en el transporte de arsenito hacia el xilema (Ma et al., 2007, 2008). El arsenito tiene una gran afinidad por los grupos tiol (-SH) de péptidos como el glutatión (GSH) y las fitoquelatinas (PC) (Zhao et al., 2009a). Uno de los principales mecanismos de detoxificación y tolerancia que utilizan las plantas frente al As es la complejación de arsenito con fitoquelatinas y su secuestro en las vacuolas (Schmöger et al., 2000; Hartley-Whitaker et al., 2001). Por ejemplo, en *Arabidopsis thaliana* esta complejación parece estar relacionada con un menor transporte de As de las raíces a la parte aérea de la planta (Liu et al., 2010). Se ha encontrado que algunas especies exudan As desde la raíz hacia el medio externo (lo que se conoce como *efflux*) rápidamente después de su absorción mediante lo que parece un mecanismo activo similar al del fosfato (Xu et al., 2007; Zhao et al., 2009). Este mecanismo parece tener un cierto peso en la detoxificación de As(III) en plantas hiperacumuladoras (Hatayama, 2012). Sin embargo, su implicación en la tolerancia que presentan algunas especies al As no está clara; por ejemplo, Logoteta et al. (2009) no observaron diferencias en cuanto a la proporción de As exudado entre dos fenotipos de *Holcus lanatus*, uno tolerante y otro no.

El As no es considerado un elemento esencial ni beneficioso para las plantas, aunque se han observado algunos efectos beneficiosos, como un mayor desarrollo y crecimiento de las raíces de maíz en presencia de baja concentración de arseniato

(Evans et al., 2005). El modo en que el As provoca toxicidad en las plantas depende de su especiación. El arseniato, por su analogía con el fosfato, interfiere en el metabolismo de éste y en la síntesis de ATP, mientras que el arsenito, la especie más tóxica, principalmente se une a los grupos tiol de las proteínas, modificando su estructura y funcionalidad (Zhao et al., 2010). Entre los principales síntomas visuales de toxicidad por As se encuentran la inhibición del crecimiento, una pobre germinación, débil desarrollo de la raíz, el marchitamiento y senescencia de las hojas, clorosis y necrosis, pudiendo provocar la muerte de la planta cuando se expone a altas concentraciones (Garg y Singla, 2011; Manzano et al., 2015). Además, la exposición a As puede inducir la formación de especies reactivas de oxígeno (ROS), probablemente como consecuencia del proceso de reducción de arseniato a arsenito en la planta (Meharg y Hartley-Whitaker, 2002).

***El Cu en el suelo y la planta***

La concentración media de Cu en los suelos es de 20 mg $kg^{-1}$ (He et al., 2005). Como la mayoría de elementos traza, el Cu se puede encontrar en el suelo en forma soluble, intercambiable, unido a óxidos, a carbonatos, a la materia orgánica y en la fracción residual del suelo, formando parte de minerales (Alloway, 1995; Kabata-Pendias, 2004). De entre los numerosos minerales de los que puede formar parte el Cu, la ferrita cúprica ($CuFe_2O_4$) es relativamente soluble, por lo que en parte controlaría la solubilidad del Cu en los suelos. Sin embargo este mineral es sensible a las condiciones redox del suelo y bajo condiciones reductoras se reduce, liberando Cu que puede reaccionar con sulfuros para dar lugar sulfuro de Cu, mucho menos soluble (He et al., 2005).

Los principales procesos físico-químicos que determinan la biodisponibilidad del Cu incluyen precipitación/disolución, adsorción/desorción y la complejación o quelación. Por lo general el Cu se encuentra en la disolución de suelos no contaminados en un rango de 0,5 a 135 μg $L^{-1}$ (Kabata-Pendias, 2004). La solubilidad del Cu está especialmente influida por el pH del suelo y, como la mayoría de los metales, un aumento en el pH del suelo por lo general disminuye su solubilidad, ya que precipita como hidróxidos y se une preferentemente a los grupos funcionales ionizados de los componentes del suelo (Sposito, 2008; Soler-Rovira et al., 2010).

El Cu forma complejos estables con la materia orgánica, por lo que su interacción con la materia orgánica soluble del suelo también va a determinar su movilidad y biodisponibilidad (Zhou and Wong, 2001; Uchimiya et al., 2011).

El Cu es un elemento esencial para las plantas. La mayoría de sus funciones en la planta se basan en que forma parte de enzimas que catalizan procesos redox y están relacionadas con la fotosíntesis, la respiración, el metabolismo de C y N y la protección contra el estrés oxidativo (Marschner, 2012).

Sin embargo, un exceso de Cu en el suelo puede tener un efecto citotóxico, induciendo estrés y causando daño a la plantas. Por ejemplo, la exposición a un exceso de Cu genera estrés oxidativo y la formación de ROS, lo que disturba algunos procesos metabólicos (Nagajyoti et al., 2010). Además, un exceso de Cu puede afectar a la ultrestructura de los cloroplastos y la capacidad fotosintética de algunas plantas (Sánchez-Pardo et al., 2014). Entre los principales síntomas de toxicidad de Cu se encuentran la reducción de la biomasa y la clorosis (Yruela, 2005). En la mayoría de los cultivos, el rango de concentración de Cu en parte aérea a partir del cual se pueden observar efectos tóxicos es de 20-30 mg $kg^{-1}$, aunque algunas especies tolerantes, como las metalofitas, pueden llegar a acumular concentraciones de hasta 1000 mg $kg^{-1}$ sin mostrar signos de toxicidad (Marschner, 2012).

## 1.3. Recuperación de suelos contaminados por metales

### *Técnicas de recuperación de suelos*

La mayor parte de los países europeos tienen una legislación nacional, o incluso regional, que regula la gestión de los suelos contaminados, sin embargo, como se ha indicado anteriormente, todavía no existe un marco legal europeo que permita homogeneizar los avances en este sentido.

De los 342.000 sitios que se estiman como contaminados en el territorio europeo (incluyendo países de la UE y otros adyacentes), alrededor de un 15% han sido ya sometidos a algún proceso de recuperación o remediación (European Comission, 2014).

En la Figura 1.4 se muestran algunas de las técnicas más frecuentemente utilizadas en algunos países de Europa (EEA, 2015). Las técnicas están divididas en tratamientos biológicos, físico-químicos y térmicos realizados *in situ*; tratamientos biológicos, físico-químicos y térmicos realizados *ex situ*, que requieren excavación y otras técnicas como la excavación y posterior emplazamiento en vertederos. Como se puede observar, las técnicas tradicionales de remediación, que incluyen la excavación y disposición en vertederos, aún prevalecen. Sin embargo, la ampliación del conocimiento de los riesgos ambientales asociados a las técnicas convencionales hace que este perfil esté cambiando, especialmente en países como Holanda, Bélgica y Malta, donde la remediación biológica y físico-química *in situ* va en aumento.

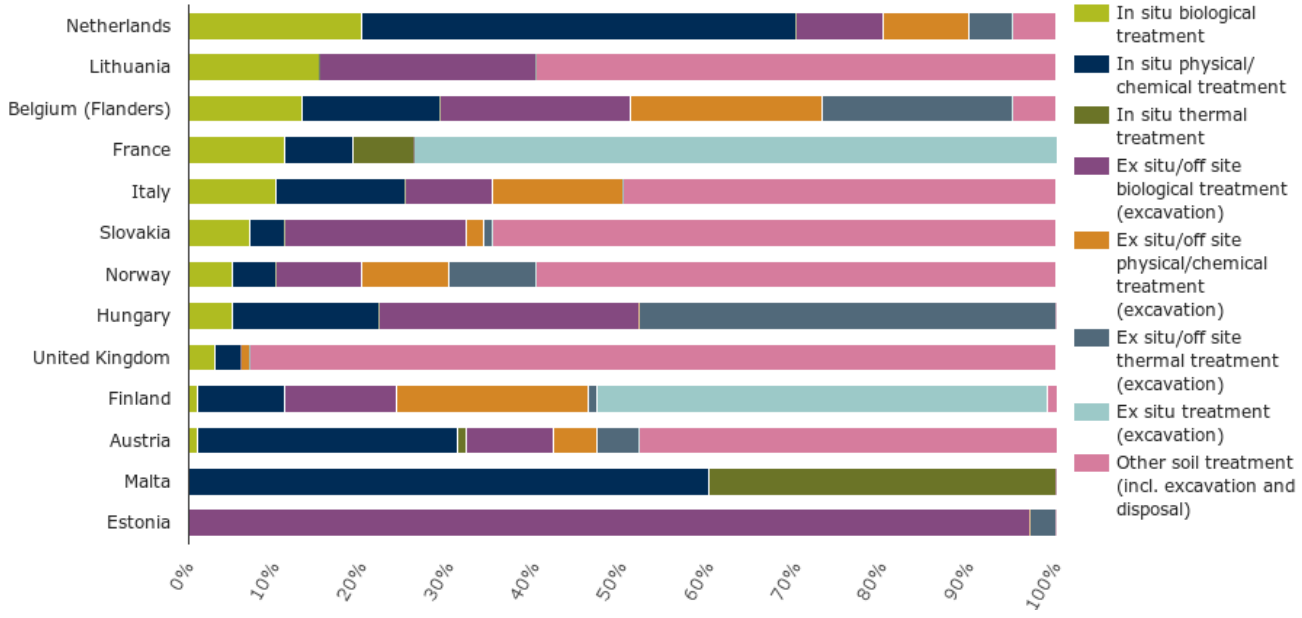


**Figura 1.4.** Algunas técnicas de remediación de suelos contaminados más utilizadas en algunos países europeos. Fuente: Agencia Europea de Medio Ambiente (EEA), 2015.

Las técnicas de remediación más comúnmente utilizadas se pueden dividir en físicas, químicas y biológicas. A continuación se muestran algunas de ellas (Ali et al., 2013; Khan et al., 2004; Mulligan et al., 2001; Yao et al., 2012):

- Físicas: Encapsulación (impermeabilización), desorción térmica, excavación y reposición del suelo, separación mecánica de partículas, etc.
- Químicas o físico-químicas: lavado del suelo con disolventes, extracción con fluidos (*flushing*), extracción con vapores, lixiviación de metales, fijación química mediante reacciones de oxidación/reducción, precipitación o neutralización, vitrificación, técnicas electrocinéticas, etc.
- Biológicas: biorremediación (biodegradación, biolixiviación, utilización de biorreactores *ex situ*, biosorción), fitorremediación.

La remediación de un suelo puede necesitar de la aplicación simultánea de varios de estos procedimientos para reducir la contaminación a un nivel aceptable y seguro, ya que es poco probable que una sola tecnología sea universal y pueda utilizarse para contaminantes de cualquier naturaleza (Khan et al., 2004; Virkutyte et al., 2002).

La selección de un proceso de descontaminación ha de hacerse para una zona determinada, ya que es importante tener un modelo conceptual de ésta, conociendo las características geológicas y físico-químicas del suelo, el uso que se le vaya a dar, las características de los contaminantes presentes, así como sus posibles transformaciones a formas más o menos tóxicas, los riesgos asociados a la contaminación y el coste de implementar una tecnología de remediación (Caliman et al., 2011).

Las técnicas convencionales de remediación de suelos, como son la excavación, el lavado o la vitrificación, son por lo general costosas y alteran de forma irreversible las propiedades del suelo, perturbando la microflora nativa. Por ello es necesario hacer esfuerzos en buscar técnicas que sean respetuosas con el medioambiente y de bajo coste (Ali et al., 2013). Un proceso de remediación debe ser sostenible y positivo para las funciones del suelo, entendidas en sus dimensiones tanto ecológicas como socio-económicas. Por ello la técnica que se seleccione debería mejorar aspectos del suelo como la producción de biomasa, los ciclos biogeoquímicos de nutrientes, su función como fuente de carbono, la biodiversidad o la producción de materias primas, entre otros. Por tanto, cuando se evalúe un proceso remediador, deberían tenerse en cuenta parámetros de la calidad del suelo, tanto físicos y químicos como biológicos (Volchko et al., 2013).

En las últimas décadas se han hecho esfuerzos por desarrollar e investigar tecnologías de remediación que sean respetuosas con el medioambiente a la vez que logren disminuir los riesgos asociados a la contaminación de suelos y aguas. Estas tecnologías (que en la literatura habitualmente se definen como *Gentle Remediation Options (GRO)*), son menos invasivas y preservan o mejoran la estructura del suelo y sus funciones, basándose principalmente en el empleo de plantas (fitorremediación), hongos y microorganismos, y en ocasiones el uso de enmiendas, para eliminar o estabilizar los contaminantes del suelo (Mench et al., 2010; Cundy et al., 2013; Kumpiene et al., 2014).

***Fitorremediación: el uso de plantas para la recuperación de suelos contaminados***

La fitorremediación es el empleo de plantas vasculares y sus microorganismos asociados para reducir la concentración o los efectos tóxicos de los contaminantes en el medioambiente, ya sea mediante su eliminación, degradación o su estabilización en el suelo. Esta estrategia de recuperación de suelos presenta las principales ventajas de que supone un bajo coste de implementación y que es respetuosa para el medioambiente, además de que el establecimiento de una cobertura vegetal mejora de por sí las propiedades físico-químicas del suelo y reduce la erosión y la dispersión de los contaminantes (Salt et al., 1995; Cunningham et al., 1995; Chaney et al., 1997; Barceló y Poschenrieder, 2003; Alkorta et al., 2004).

La fitorremediación se ha empleado para suelos contaminados tanto por metales como por sustancias orgánicas (hidrocarburos poliaromáticos, bifenilos policlorados, pesticidas, etc.). Sin embargo, a diferencia de los contaminantes orgánicos, los metales no se pueden degradar, por lo que la estrategia utilizada debe basarse en reducir su concentración en el suelo mediante la absorción por la planta, o su disponibilidad y toxicidad, mediante su estabilización en la raíz.

Las estrategias de fitorremediación que se emplean más habitualmente en suelos se pueden dividir en dos grupos: fitoextracción y fitoestabilización, aunque también se utilizan otras técnicas.

- **Fitoextracción**. Se basa en el empleo de plantas capaces de acumular metales en su parte aérea para así reducir su concentración en el suelo. Muchas de estas especies son endémicas de suelos ricos en minerales metálicos (metalofitas), aunque también existen otras especies que han desarrollado esta capacidad

como mecanismo de resistencia ante altas concentraciones de metales en el suelo (pseudometalofitas) (Becerril et al., 2007). Muchas de las plantas utilizadas para fitoextracción son hiperacumuladoras, es decir, especies capaces de concentrar metales en sus tejidos en mayor cantidad que otras especies, sin mostrar signos de toxicidad (Baker y Brooks, 1989; Chaney et al., 1997; Padmavathiamma y Li, 2007). Baker y Brooks (1989) establecieron que para que una planta se pueda considerar hiperacumuladora debe concentrar en sus tejidos un mínimo del 0,1% de su peso seco de Co, Cu, Cr, Pb o Ni o un 1% de Zn o Mn. Las plantas empleadas en esta técnica deben combinar tanto la bioacumulación de metales como una elevada producción de biomasa (Barceló y Poschenrieder, 2003; McGrath y Zhao, 2003). La eficiencia de la fitoextracción se puede mejorar mediante prácticas agronómicas, mediante el empleo de aditivos que aumenten las biodisponibilidad de los metales (agentes quelantes, agentes desorbentes, microorganismos, etc.) o mediante la aplicación de fertilizantes y enmiendas orgánicas que aumenten la producción de biomasa, entre otros (Tassi et al., 2004; Luo et al., 2005; Sessitsch et al., 2013; Kidd et al., 2015; Álvarez-López et al., 2016).

- **Fitoestabilización**. La finalidad de esta técnica, a diferencia de la fitoextracción, no es eliminar el contaminante del suelo, sino reducir su movilidad y biodisponibilidad y en consecuencia su toxicidad (Ali et al., 2013; Barceló y Poschenrieder, 2003; Cunningham et al., 1995; Ghosh y Singh, 2005). Para lograr este objetivo se utilizan plantas capaces de acumular metales en sus raíces y su rizosfera y transferirlos en pequeña proporción a su parte aérea (mecanismo exclusor). Los mecanismos de estabilización de los contaminantes pueden implicar la acumulación en la raíz y/o reacciones de complejación, precipitación u óxido-reducción en la rizosfera (Cunningham et al., 1995; Wong, 2003; Ghosh y Singh, 2005; Mendez y Maier, 2008; Martínez-Alcalá et al., 2012). La Figura 1.4 muestra una representación esquemática de algunos mecanismos implicados en la fitoestabilización. Esta técnica se utiliza habitualmente cuando la fuente de contaminación es difusa y la concentración de contaminantes elevada, lo que complica su eliminación (Wong, 2003). Como ventaja adicional frente al uso de plantas (hiper)acumuladoras, el empleo de plantas exclusoras reduce la introducción de los contaminantes en la cadena trófica al no acumularlos en su parte comestible o cosechable. Además de la estabilización de los contaminantes, el empleo de las plantas por lo general supone una mejora en las propiedades físico-químicas y bioquímicas del suelo (Martínez-Alcalá et al., 2012; Moreno-

Jiménez et al., 2012). Del mismo modo que ocurre con la fitoextracción, la eficiencia de la fitoestabilización de un suelo se puede mejorar mediante la adición de enmiendas que, en este caso, reduzcan la movilidad de los contaminantes (fitoestabilización asistida), además de actuar como fuente de nutrientes para las plantas y mejorar las propiedades del suelo (Mench et al., 2006; Park et al., 2011; Touceda-González et al., 2016).

Otras técnicas posibles, aunque menos utilizadas son (Cunningham et al., 1995; Ghosh y Singh, 2005; Sakakibara et al., 2007; Anawar et al., 2008):

- **Fitofiltración (rizofiltración)**. Se refiere al uso de plantas, tanto terrestres como acuáticas, para adsorber, concentrar o precipitar en la raíz contaminantes presentes en medios acuosos. Para una mayor eficiencia se utilizan plantas terrestres crecidas en hidroponía, ya que desarrollan un mayor sistema radicular que las especies acuáticas.

- **Fitovolatilización**. Esta técnica es una variante de la fitoextracción, pero en este caso las plantas utilizadas absorben el contaminante y lo transforman en formas volátiles para después expulsarlo a la atmósfera. Aunque se emplea más para la eliminación de contaminantes orgánicos, también se puede utilizar para metales y semi-metales que formen compuestos volátiles como el Hg, el Se o el As.

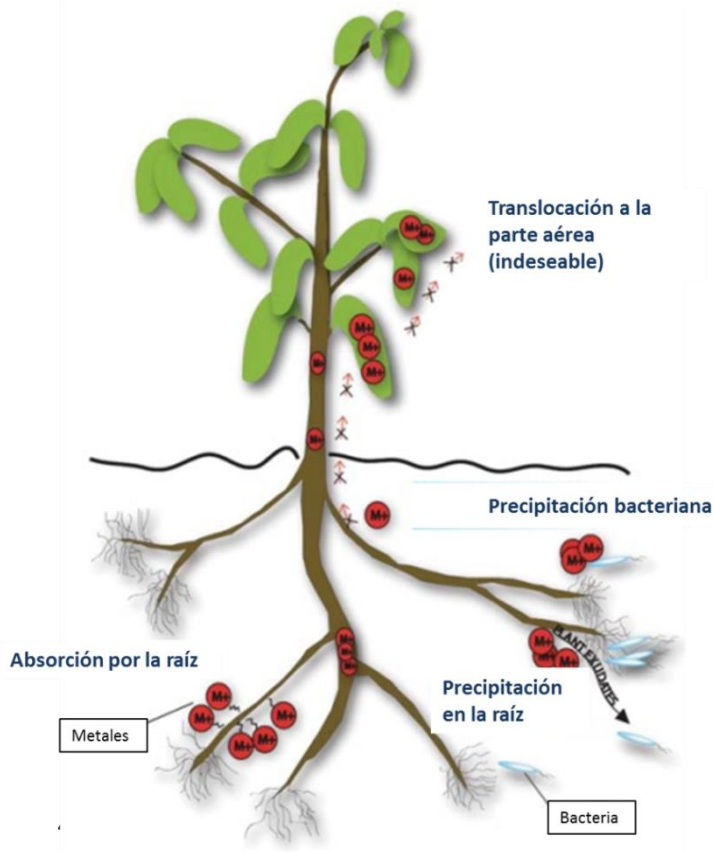


**Figura 1.4.**
Representación esquemática de algunos procesos implicados en la técnica de fitoestabilización. La utilización de plantas exclusoras se fundamenta en evitar la translocación del contaminante a la parte aérea. Modificado de Mendez y Maier (2008).

***Fitoestabilización asistida por enmiendas***

Como se ha indicado anteriormente, la técnica de fitoestabilización se utiliza para reducir la movilidad de los contaminantes hacia aguas subterráneas y limitar su dispersión e introducción en la cadena trófica, ya sea mediante procesos de absorción, precipitación o complejación en la raíz y la rizosfera de las plantas, o por la mitigación de la erosión del viento y la migración de los contaminantes a través del agua gracias al establecimiento de una cobertura vegetal (Mench et al., 2006; Vangronsveld et al., 2009; Mench et al., 2010).

En ocasiones, la elevada contaminación del suelo complica el establecimiento de una cobertura vegetal, aun cuando las especies utilizadas sean tolerantes a los contaminantes presentes. Para hacer frente a este problema, la fracción más lábil de los contaminantes se puede reducir mediante la adición al suelo de productos (enmiendas) que promuevan procesos de adsorción, precipitación y complejación de los metales en la matriz del suelo, reduciendo así su biodisponibilidad y toxicidad. En este caso hablamos de fitoestabilización asistida (Adriano et al., 2004; Mench et al., 2010; Bolan et al., 2014).

Los óxidos metálicos son componentes naturales del suelo, donde juegan un papel fundamental en la dinámica de metales y metaloides. Debido a su elevada capacidad de adsorción y a su carácter anfótero, los óxidos de metales, en especial los de hierro, se ha estudiado ampliamente como potenciales enmiendas para suelos contaminados con metales (Komárek et al., 2013; Kumpiene et al., 2008; Miretzky y Fernandez Cirelli, 2010; Bolan et al., 2014). La adsorción de los metales sobre la superficie de óxidos del suelo se puede dar mediante adsorción específica, en la que se forman complejos de esfera interna, o mediante adsorción inespecífica. La coprecipitación de metales con óxidos cobra especial importancia cuando se forman óxidos secundarios que pueden coprecipitar con los metales y metaloides presentes en el suelo (p. ej. como $FeAsO_4 \cdot 2H_2O$, $MnPbO_3$, metal-ferrihidrita), disminuyendo así su solubilidad (Komárek et al., 2013).

Los efectos de enmiendas de hierro sobre la movilidad de As han sido quizás los más estudiados, debido a la alta afinidad de este elemento por los óxidos del suelo, especialmente los de hierro.

La utilización de las sales de hierro presenta la ventaja de la formación *in situ* de óxidos de hierro en el suelo, lo que puede dar lugar a la coprecipitación de metales y metaloides además de procesos de adsorción. Hartley et al. (2004) destacaron en su estudio la mayor eficacia de los sulfatos de hierro (II) y (III), frente a otras enmiendas

como goetita y partículas de hierro, en la inmovilización de As. Tras la aplicación de $FeCl_3$ y $FeSO_4$, ambos en combinación con cal hidratada, a un suelo contaminado con As, Cutler et al. (2014) observaron que la mayor parte del Fe aplicado pasó a formar óxidos de hierro reactivos, tipo ferrihidrita, capaces de adsorber As, lo que dio lugar a una reducción en su biodisponibilidad. En este caso todo el arseniato se encontraba asociado a estos óxidos, sin evidencia de la formación de arseniato de hierro. Otros autores han destacado la eficacia de la aplicación de sulfato de hierro frente a la aplicación directa de óxidos en la estabilización de As y metales (Moreno-Jiménez et al., 2016b). Sin embargo, la adición de sulfato de hierro provoca la acidificación del suelo por la formación de ácido sulfúrico, lo que puede dar lugar a la movilización de metales y a cierta fitotoxicidad. Por ello, la coaplicación de productos encalantes se hace necesaria cuando se emplea esta sal (Komárek et al., 2013; Kumpiene et al., 2008).

Las enmiendas basadas en la aplicación de hierro elemental, Fe(0), en forma de partículas, nanopartículas, láminas, arena, etc., reducen el efecto de la acidificación del suelo. Además, estos materiales contienen una mayor cantidad de hierro por unidad de masa que las sales, por lo que al disminuir la cantidad de material empleado se pueden reducir efectos indeseables sobre la estructura del suelo. Numerosos estudios evidencian la efectividad de estos materiales en la inmovilización de metales y As (Kumpiene et al., 2006; Mench et al., 2006; Sneath et al., 2013; Cutler et al., 2014; Oustriere et al., 2016; Tiberg et al., 2016). Sin embargo, la formación de óxidos de hierro a partir de estos materiales es más lenta que cuando se aplican sales (Komárek et al., 2013). Además, los riesgos medioambientales asociados al uso de nanopartículas aún se están estudiando (Grieger et al., 2010).

Un asunto que se debe tener en cuenta al utilizar óxidos metálicos como enmiendas es la estabilidad de su reactividad a largo plazo, ya que los cambios en su estructura pueden afectar a su superficie específica y por tanto a su reactividad frente a As y metales. En este sentido existe una cierta controversia en la literatura, ya que factores como la presencia de inhibidores de la cristalización, el pH o la temperatura pueden afectar a la velocidad de cristalización de los óxidos. Por ejemplo, Kumpiene et al. (2012) observaron diez años después de la adición de una enmienda compuesta por partículas de hierro elemental, compost y cenizas de la combustión de carbón, un aumento en la lixiviación y la disponibilidad de As, lo que atribuyen en parte a un aumento en la cristalinidad de los óxidos de hierro formados. Estos resultados contrastan con los obtenidos por Tiberg et al. (2016), quienes comprobaron la eficacia de la aplicación de hierro elemental en la estabilización de As y Cu tras 6 y 15 años de su aplicación, o al efecto duradero de la estabilización y reducción de la disponibilidad

de As que observaron Cutler et al. (2014) tras dos años de la aplicación de sales de hierro a un suelo.

Otras enmiendas de óxidos metálicos son las que constan de residuos ricos en estos compuestos, que además presentan el valor añadido de la reutilización de estos residuos. Por ejemplo, se han realizado estudios en los que se añaden lodos rojos (procedentes de la fabricación de alúmina) o aguas del tratamiento de residuos ricas en hierro donde se muestran resultados prometedores relativos a la inmovilización de As y metales (Lombi et al., 2002; Garau et al., 2014; Manzano et al., 2016). Sin embargo, estos materiales por lo general dan lugar a un aumento del pH del suelo, por lo que su aplicación debe hacerse con cautela y teniendo siempre en cuenta las características del suelo.

La adición de fósforo como enmienda en suelos contaminados se realiza mediante diversos compuestos como ácido fosfórico, sales fosfatadas, roca fosfórica o fertilizantes fosfóricos, entre otras. Estas enmiendas han mostrado una especial efectividad en la inmovilización de Pb. La formación de minerales tipo piromorfita [$Pb_5(PO_4)_3X$] (X = Cl, F, B, OH) se presenta en la literatura como uno de los principales mecanismos de inmovilización de este metal. Además, la adsorción de fosfato sobre los (hidr)óxidos metálicos presentes en el suelo aumenta la capacidad de éstos para adsorber Pb (Cao et al., 2003; Wang et al., 2008; Cui et al., 2010; Park et al., 2012). La efectividad de la inmovilización de Pb aumenta con la mayor solubilidad del P aplicado, y en general los mejores resultados se obtienen con sales de fosfato, más solubles, que con roca fosfórica, más insoluble (Kumpiene et al., 2008). La inoculación de bacterias solubilizadoras de fosfato puede aumentar la eficacia de la roca fosfórica en cuanto a la inmovilización de Pb (y otros metales) y la reducción de su toxicidad hacia diferentes organismos (Park et al., 2012). También se han obtenido interesantes resultados en la reducción de las fracciones más lábiles de Cd y la disminución de su concentración en la paja y el grano de trigo (Ghafoor et al., 2008) y en la reducción de la movilidad y fitodisponibilidad de Zn y Cu (Cao et al., 2003; Liu y Zhao, 2007; Wang et al., 2008). Aunque más aplicado a la fitoextracción, la adición de compuestos de P puede aumentar la fitodisponibilidad de As para especies hiperacumuladoras como *Pteris vittata* (Fayiga y Ma, 2006), aunque también se ha propuesto para incrementar la capacidad extractora de otras especies no acumuladoras pero tolerantes a As, como *Lupinus albus* (Tassi et al., 2004).

Los materiales encalantes se han utilizado históricamente para corregir la acidez del suelo. Sin embargo, existe evidencia de que su aplicación puede provocar la

inmovilización de metales, reduciendo así su toxicidad. Algunos materiales estudiados como enmiendas encalantes en suelos contaminados son el carbonato cálcico, la piedra caliza y la cal hidratada (Gray et al., 2006; Tlustoš et al., 2006; Houben et al., 2012; Moreno-Jiménez et al., 2012; Pardo et al., 2014a). La utilización del residuo de la producción del azúcar de remolacha como enmienda en suelos ácidos contaminados ha dado lugar a una reducción de la movilidad de metales como Cd, Zn y Cu, además de aportar materia orgánica y nutrientes al suelo (Madejón et al., 2006; Pérez de Mora et al., 2006; Clemente et al., 2015). Los lodos residuales de la industria papelera se han estudiado en ocasiones como posibles enmiendas debido a su elevada proporción de carbonato cálcico y a que también aportan cierta cantidad de materia orgánica. En general pueden ayudar a reducir la movilidad de metales, pero su capacidad encalante puede dar lugar a una movilización de As (Galende et al., 2014; Manzano et al., 2014a; Zhang et al., 2015).

Como otro ejemplo de potencial enmienda inorgánica, se ha observado que la adición de azufre a suelos contaminados con Cd y Zn provoca una aumento en la solubilidad de estos metales localizado en la rizosfera, mejorando la capacidad extractora de *Salix smithiana* sin suponer un riesgo de lixiviación, lo que la hace una enmienda apta para técnicas de fitoextracción (Iqbal et al., 2012; Hoefer et al., 2015).

La utilización de enmiendas basadas en materiales orgánicos ha sido estudiada en numerosos trabajos. Las reacciones implicadas en la inmovilización de metales y metaloides son variadas y dependen tanto de la composición de la enmienda empleada como de la naturaleza de los contaminantes presentes en el suelo.

Las enmiendas orgánicas pueden ayudar a incrementar la estabilidad de metales mediante su efecto encalante y el aumento de la capacidad de intercambio catiónico del suelo (Alvarenga et al., 2009; Bolan et al., 2014). Además, estos materiales presentan grupos funcionales como carboxilo o fenol, capaces de complejar los metales y disminuir su solubilidad (Tsang et al., 2014). También pueden dar lugar a procesos redox que alteren la movilidad de algunos metales y metaloides como Hg, As, y Cr, ya que son una fuente de donadores de electrones y además aportan sustrato orgánico para los microorganismos que contribuyen a estos procesos (Bolan et al., 2003; Park et al., 2011). Sin embargo, se ha observado en varias ocasiones que la adición de enmiendas con alto contenido en materia orgánica provoca la movilización de metaloides como As y Sb y otros elementos como Se (Clemente et al., 2010; Moreno-Jiménez et al., 2013; Beesley et al., 2014; Arco-Lázaro et al., 2016), lo

que implica que su utilización en suelos con multi-contaminación deba hacerse con cautela y siempre conociendo las características del suelo a tratar.

Una de las principales ventajas que ofrece el uso de estas enmiendas es que por lo general ayudan a mejorar las propiedades físico-químicas y la funcionalidad del suelo, corrigiendo la acidez, aumentando la capacidad de intercambio catiónico y aportando nutrientes esenciales para las plantas (Alvarenga et al., 2009; Touceda-González et al., 2016). Sin embargo, dependiendo del material utilizado, podemos encontrar ciertas desventajas de su aplicación, como salinización, un aporte excesivo de N, la incorporación de microorganismos patógenos o la presencia de metales pesados en elevada concentración (Alvarenga et al., 2015).

Algunos ejemplos de materiales orgánicos empleados en estudios de recuperación de suelos son los lodos de depuradora (Alvarenga et al., 2009; Alvarenga et al., 2009); residuos orgánicos compostados, como los de residuos municipales y restos de poda (Mench et al., 2003; Alvarenga et al., 2009; Hartley et al., 2009; Clemente et al., 2010; Tsang et al., 2014; Gil-Loaiza et al., 2016) o compost de alperujo, un residuo de la extracción del aceite de oliva (Alburquerque et al., 2011; de la Fuente et al., 2011; Beesley et al., 2014; Pardo et al., 2014).

El biocarbón o biochar es un material carbonáceo producido por la pirolisis de residuos vegetales y animales. A pesar de que se ha utilizado ampliamente para mejorar las propiedades físico-químicas del suelo y por su labor en el secuestro de carbono en suelos, su elevada superficie específica y su elevado contenido en materia orgánica reactiva lo convierten en un material ampliamente estudiado en las últimas décadas como enmienda para suelos contaminados.

Lu et al. (2012), tomando como el ejemplo el Pb ($Pb^{2+}$), resumen los principales mecanismos de interacción entre los metales y el biochar en: (1) intercambio entre el metal y cationes presentes en el biochar, lo que da lugar a su precipitación o a la formación de complejos de esfera interna con grupos funcionales y óxidos minerales superficiales; (2) Formación de complejos en la superficie entre el metal y grupos funcionales hidroxilo y carboxilo; (3) precipitación en la superficie y adsorción física (Fig. 1.5).

La capacidad de adsorción del biochar depende de sus características, en las que influyen principalmente la materia prima que se utilice para su fabricación y la temperatura de pirolisis (Joseph et al., 2010; Bolan et al., 2014). Del mismo modo, su efecto sobre la capacidad de adsorción del suelo también depende de las características de éste. Por ejemplo, Uchimiya et al. (2011) describen diferentes mecanismos de adsorción de Cu sobre un biochar añadido a dos suelos diferentes. En

un suelo arenoso y alcalino, con poca capacidad de adsorción de metales, el Cu es retenido en las partículas de biochar principalmente mediante intercambio catiónico, mientras que en un suelo más arenoso y con menor capacidad de adsorción, los autores proponen la interacción electrostática entre el Cu y la superficie cargada negativamente del biochar, la formación de complejos con grupos funcionales presentes en la superficie, la adsorción sobre componentes minerales y la precipitación como los principales mecanismos por los que el Cu se retiene en el biochar.

Dada la variedad de materiales con diferentes características que se engloban en el término biochar, el efecto de esta enmienda sobre la movilidad de metales/metaloides puede variar dependiendo de qué, dónde y cómo se aplique. Diferentes trabajos han mostrado la estabilización de metales como Cd, Zn y Pb tras la aplicación de biochar al suelo, debido tanto a un aumento de la capacidad de adsorción del suelo como por la adsorción de metales en las propias partículas del biochar (Beesley and Marmiroli, 2011; Beesley et al., 2010; Forján et al., 2016; Karami et al., 2011). Sin embargo, los resultados obtenidos en cuanto a la inmovilización de Cu son más diversos; mientras varios autores indican una disminución en la movilidad de este metal tras la adición de biochar al suelo (Karami et al., 2011; Brennan et al., 2014; Sneath et al., 2013; Jones et al., 2016), Beesley et al. (2010) observaron un aumento en la solubilidad de Cu, atribuyéndolo principalmente al aporte de carbono orgánico soluble.

De forma similar a lo que ocurre con otras enmiendas como el compost, el uso de biochar en suelos contaminados con As da lugar en ocasiones a un aumento en la movilidad de éste, propiciado por el aumento del pH del suelo y el aporte de C orgánico soluble y, en ocasiones, de P disponible (Beesley et al., 2013, 2011; Beesley et al., 2013; Brennan et al., 2014).

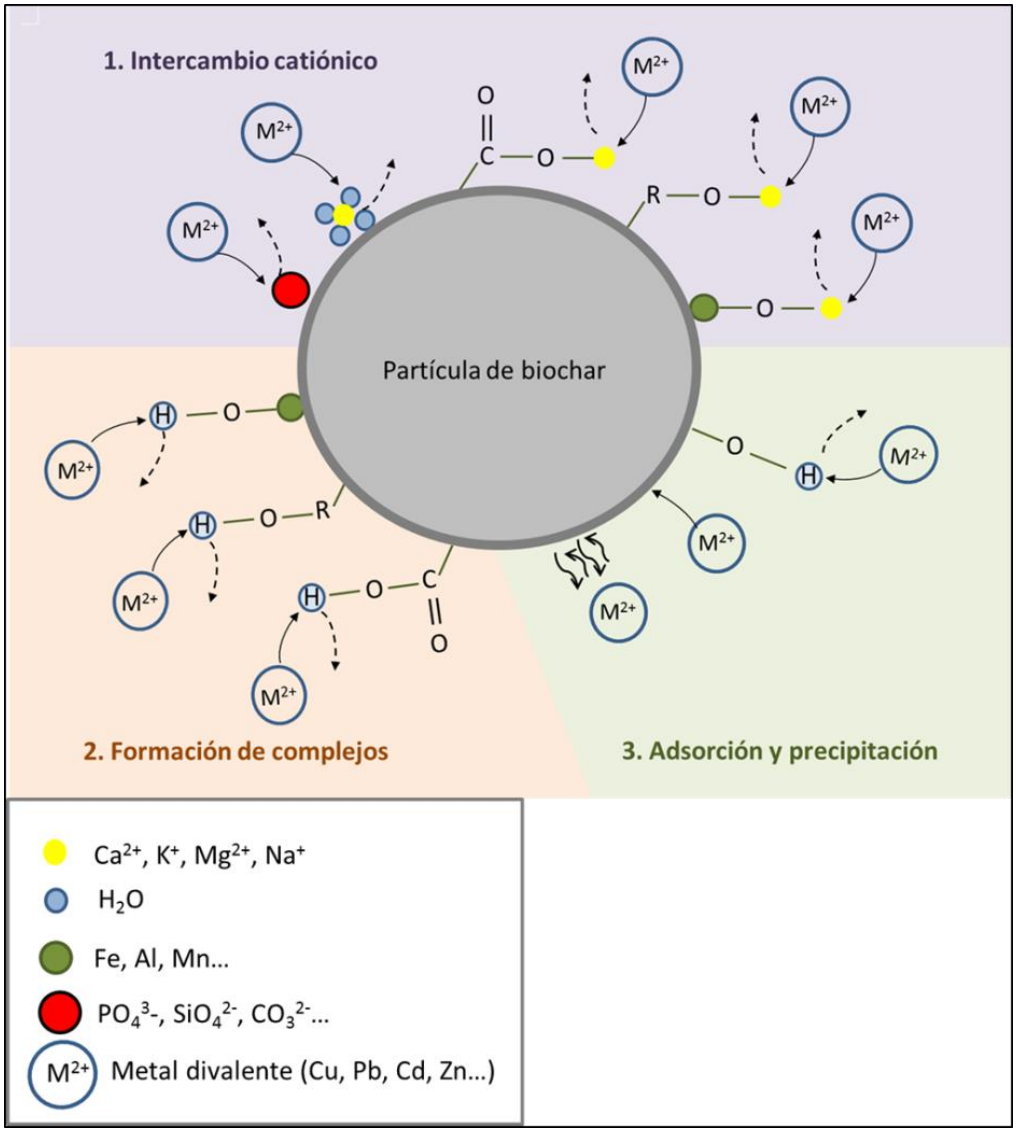


**Figura 1.5.** Representación esquemática de algunos mecanismos de interacción entre metales y biochar. Modificado de Lu et al. (2012).

***Influencia de la rizosfera en procesos de fitorremediación***

La rizosfera se define como el volumen de suelo que rodea a la raíz y que está sometido a la influencia directa de la actividad radicular (Hinsinger et al., 2005).

La biodisponibilidad de los contaminantes es el resultado de la interacción entre diversos factores asociados a las propiedades del suelo, las características de los contaminantes y los efectos de la actividad de las raíces de las plantas y la comunidad microbiana asociada a ellas. Por tanto, la eficiencia de un proceso de fitorremediación depende de la biodisponibilidad del contaminante y de los cambios en su especiación y solubilidad producidos por las plantas y los microoganismos asociados. Las plantas pueden modificar y controlar la biodisponibilidad de metales y metaloides en su rizosfera mediante mecanismos de absorción y mediante la actividad de las raíces (Wenzel, 2009). De entre los factores que más afectan a la biogeoquímica de los metales en la rizosfera se encuentran los cambios producidos en el pH y el potencial redox del suelo rizosférico y los compuestos (fitosideróforos, ácidos orgánicos, etc.) exudados por las raíces o por microorganismos asociados (Kidd et al., 2009; Wenzel, 2009).

En la literatura existen numerosos trabajos en los que se tratan de dilucidar los mecanismos que utilizan las plantas (hiper)acumuladoras para aumentar la disponibilidad de metales y alcanzar tan altos niveles de acumulación en sus tejidos. Wenzel et al. (2003) y Puschenreiter et al. (2005) sugirieron en sus estudios que los ácidos orgánicos exudados por las raíces de la hiperacumuladora de Ni *Thlaspi goesingense* induce la disolución de minerales de Ni, aumentando así la fracción soluble de este metal en su rizosfera. De forma similar, Gonzaga et al. (2006) relacionaron la eficiencia en la acumulación de As de los helechos hiperacumuladores *Pteris vittata* y *Pteris biaurita* con su habilidad para solubilizar As en la rizosfera a través de los exudados de la raíz.

Aunque se encuentran menos publicaciones referentes a la influencia de la rizosfera en procesos de fitoestabilización, varios autores han descrito algunos mecanismos de movilización e inmovilización de metales en la rizosfera de plantas no acumuladoras y exclusoras (Kidd et al., 2009). Por ejemplo, Tao et al. (2003) observaron una movilización de Cu desde fracciones menos lábiles a otras más disponibles en la rizosfera de maíz, lo que los autores relacionan con los cambios inducidos por la actividad de las raíces en el potencial redox del suelo, el C orgánico soluble y la actividad microbiana. Por su parte, Bravin et al. (2008) asocian los cambios en la especiación de Cu, y por tanto en su disponibilidad, a la alcalinización o

acidificación de la rizosfera de trigo, independientemente del pH del suelo no rizosférico (*bulk*). Martínez-Alcalá et al. (2009 y 2010) sugieren que la reducción en la solubilidad y disponibilidad de ciertos metales como Mn, Zn, Cu y Pb en la rizosfera de *Lupinus albus,* crecida tanto en un suelo relativamente ácido como en uno calcáreo, se debe a las condiciones más oxidantes en la rizosfera. Panfili et al. (2005) mostraron la precipitación de Zn en forma de fosfato o Zn-filosilicato en la rizosfera de varias especies tras dos años de cultivo.

Cuando la estrategia de fitoestabilización está unida al uso de enmiendas, la influencia de los procesos rizosféricos en la eficacia de las enmiendas para inmovilizar metales también debería tenerse en cuenta. En este sentido, Hashimoto et al. (2011) mostraron que la actividad en la rizosfera de varias especies podría interferir en la capacidad de algunas enmiendas de P para inmovilizar Pb.

La manipulación de la rizosfera se puede utilizar para optimizar las técnicas de fitorremediación. Por ejemplo, la inoculación de bacterias promotoras del crecimiento vegetal puede ser una eficaz estrategia para mejorar la eficiencia de la fitoestabilización. Estos microorganismos pueden, por un lado, mejorar la absorción de nutrientes, favoreciendo así el establecimiento de una cobertura vegetal y aliviando la fitotoxicidad de los contaminantes, y por otro pueden inducir la precipitación de metales en la rizosfera, reduciendo así su biodisponibilidad (Burd et al., 2000; Wenzel, 2009).

# Capítulo 2. Objetivos

# *Chapter 2. Objectives*

# Objetivos

El principal objetivo de esta Tesis Doctoral es evaluar la combinación de sulfato de hierro con diferentes materiales orgánicos como única enmienda para la recuperación de suelos contaminados con As y Cu, investigando su efecto tanto en la movilidad de estos elementos en el suelo y la rizosfera como en la mejora de la calidad del suelo.

Para ello se establecieron los siguientes objetivos parciales:

- Estudiar el efecto de las enmiendas sobre la inmovilización de As y Cu y la mejora de la calidad del suelo.

- Evaluar la influencia de las enmiendas en la producción de biomasa de especies con potencial valor agro-ambiental en suelos contaminados.

- Estudiar la interacción entre las enmiendas de hierro y el As en la rizosfera de altramuz y su efecto sobre la disponibilidad de As.

# Objectives

The main objective of this PhD thesis is to evaluate the combination of iron sulfate and organic materials as single amendment for the remediation of a soil contaminated with As and Cu, assessing the influence of the amendments on As and Cu mobility in soil and the rhizosphere and the improvement of soil functions.

For that, several sub-objectives were established:

- To study the effect of the amendments on As and Cu immobilisation and the improvement of soil quality.

- To evaluate the influence of the amendments on biomass production of species with potential agro-environmental value in contaminated soils.

- To study the interaction of iron treatments and As in the rhizosphere of white lupin and its effect on As phytoavailability.

# Chapter 3

# Assessment of iron sulfate combined with organic materials as amendments for an As- and Cu-contaminated soil

## Introduction

Within the 34,2000 contaminated sites recently identified in Europe, industrial activities, including mining, have been reported to represent 33% of the contamination sources (Panagos et al., 2013). Contaminated soils generally present high contents of potentially toxic metal/metalloids affecting connected environmental compartments and are usually characterized by poor physico-chemical properties and loss of vegetation (Vangronsveld et al., 1994). To overcome this problem, *gentle* remediation strategies, based on plants and amendments, have been developed not only to act on metal/metalloids mobility but also on the recovery of soil functions (Alvarenga et al., 2009).

Iron oxides naturally existing in soils are well known to be important scavengers for As and hence their incorporation to soils has been shown to efficiently immobilise As in short and long time scales. The use of Fe(0) as a precursor of iron oxides results in a reduction in As mobility, but generally the application of Fe(II) and Fe(III) salts is proposed as a better alternative for amending As-contaminated soils than iron oxides, as it has shown to immobilize As more efficiently through inducing chemical reactions in soil (*e.g.* co-precipitation) than iron oxides do through adsorption (Hartley et al., 2004; Komárek et al., 2013). Within iron salts, agricultural grade $FeSO_4$ is recommended over $FeCl_3$ due to its lower cost and ease of application (Cutler et al., 2014).

The use of industrial and municipal by-products as soil amendments is gaining more importance, as it entails the additional environmental and economic benefit of an appropriate disposal and recycling of these materials (Garrido et al., 2003; Galende et al., 2014; Clemente et al., 2015). The application of organic by-products as amendments in contaminated sites has often shown to improve soil physico-chemical properties and soil functions, supporting the establishment of a plant cover (Alvarenga et al., 2009a, 2009b; Galende et al., 2014; Pardo et al., 2014b; Gil-Loaiza et al., 2016). Within a soil recovery strategy, the establishment and development of a plant cover is essential, as plants improve soil physical properties and help to mitigate the dispersion of contaminants. For this purpose, the addition of organic amendments provides a suitable substrate for plant growth (Vangronsveld et al., 1995; Wong, 2003).

Olive mill waste or *alperujo* is a semi-solid by-product generated in the two-phase extraction of olive oil. It is rich in organic matter and essential nutrients, although its high content in phenolic compounds limits its direct application to soils as it shows

some phytotoxicity that may be reduced by composting (Alburquerque, 2004; Alburquerque et al., 2006). Olive mill waste compost has been proved to enhance metals immobilization while alleviating soil toxicity and promoting plant growth (Walker and Bernal, 2008; Clemente et al., 2015). However, its application to As-contaminated soils is less recommended, because it often leads to As mobilization (Moreno-Jiménez et al., 2013; Beesley et al., 2014).

Green waste compost has been widely investigated as soil amendment. Its addition has often shown an improvement of soil physico-chemical characteristics and a decrease in metals mobility (Alvarenga et al., 2009a; Jones et al., 2016; Tsang et al., 2014). However, similar to olive mill waste compost, its application to multi-contaminated soils may be an issue, as this material may enhance metalloids mobility (Clemente et al., 2010; Hartley et al., 2009).

Biochar is a carbonaceous material obtained by the pyrolysis of biomass under limited supply of oxygen; its production is relatively inexpensive and brings the recycle of organic wastes and energy. Biochar application as a soil amendment may give rise to several benefits such as enhancement of C sequestration, improvement of soil physical properties and, in some cases, recycling of valuable nutrients (Novak et al., 2009)(Biederman and Stanley Harpole, 2013). Many studies have shown the efficiency of biochar on heavy metals retention in soils and the reduction in their availability and phytotoxicity (Sneath et al., 2013; Moreno-Jiménez et al., 2016a). But, as it happens with compost, biochar addition to As-contaminated soils may pose a risk due to a potential increase in As solubility (Hartley et al., 2009b; Beesley et al., 2013).

Paper mill sludge is a waste product generated in the pulp and paper industry. Its relatively high content of organic matter and carbonates suggests its potential use as an amendment for metal-contaminated soils. It has been proven to rise soil pH and reduce metals availability but it also solubilizes some As when applied as a single amendment (Galende et al., 2014; Manzano et al., 2014b).

The stabilization of trace metals and the reduction of associated risks, involving their transfer to the food chain and the groundwater, is one of the main targets of aided phytostabilisation technologies. The success of a remediation process can be evaluated using different methods, but changes in the bioavailable and mobile (or soluble) metal(loid)s pools should be investigated over their total content, since these pools offer more reliable information about ecotoxicological risks (Mench et al., 2009; Moreno-Jiménez et al., 2010; Manzano et al., 2014). The knowledge of how (in which forms) trace elements are distributed in soils, which can be achieved by sequential

extractions, is a useful tool to make predictions about the stability of *in situ* immobilisation events (Vangronsveld et al., 2009; Rodríguez-Vila et al., 2016). Since a soil remediation strategy should need little maintenance, if not be definitive, long-term studies should be also carried out to evaluate the sustainability of the stabilization process (Mench et al., 2006; Bidar et al., 2016).

However, monitoring of a soil remediation process should not only integrate changes in trace elements mobility, but also biological tests that provide comprehensive information on improving soil quality and providing ecosystem functions (Alvarenga et al., 2009b). Some of these tests include direct and indirect assays using living organisms, such as germination tests or the response of aquatic microorganisms to soil leachates, and the evaluation of enzymatic activities related to the biogeochemical cycles of nutrients (Alkorta et al., 2003; Girotti et al., 2008; P Alvarenga et al., 2009b; Pardo et al., 2014a).

One of the main goals of the application of soil amendments is the establishment of a plant cover. For that, other parameters such as plants nutritive status can be evaluated besides effects on plant biomass development. There are other biological processes associated to plant growth that can serve to evaluate an improvement in soil functions as, for instance, the appearance of root nodules (due to the symbiotic relationship between leguminous plants and nitrogen-fixing soil *rhizobium* bacteria), whose formation and functionality have shown to be negatively affected by high concentration of trace elements (Vazquez et al., 2006; Pajuelo et al., 2008; Sánchez-Pardo et al., 2013).

In the studies described in the following three sub-chapters, the efficacy of an aided-phytostabilisation strategy based on the combination of iron sulfate and the previously described organic materials was assessed. For that, different size and time scales and different methods were used to study As and Cu stabilisation and the improvement of soil functions, paying special attention to the development of a plant cover.

# Chapter 3.1

# Assessment of iron sulfate combined with organic materials as amendments for an As- and Cu-contaminated soil: effects on As and Cu fractionation and soil toxicity

## 3.1.1. Introduction and objective

The evaluation of a soil remediation process should not only integrate changes in trace elements mobility, but also biological tests that provide comprehensive information on improving soil quality and providing ecosystem functions (Alvarenga et al., 2009a). Additionally, toxicity bioassays may assess potential risks associated not only to the soil, but also to other associated ecosystems such as the groundwater (Alvarenga et al., 2009b; Pardo et al., 2014a).

This work evaluates the co-application of iron sulfate and different organic materials as a suitable strategy to remediate As- and Cu-contaminated soils. For this purpose, the effects on As and Cu fractionation and mobility as well as the effects on soil quality and ecotoxicological parameters in the short-term were investigated in a pot experiment using a soil contaminated with As and Cu.

## 3.1.2. Materials and methods

### *Soil and amendments characterization*

An As and Cu rich material mainly composed of arsenopyrite-scorodite masses (Recio-Vazquez et al., 2010) was collected from the spoil heaps of El Verdugal, an ancient smelting factory located in the North of Madrid (Spain). The waste heaps contain large amounts of arsenopyrite and scorodite and the soils directly affected by As contamination have been classified as *Spolic Technosol (Toxic)* according to the FAO guidelines (Recio-Vazquez et al., 2010).

Soil was collected from the surroundings of this area and was not directly affected by the contaminated spoil heaps. The soil was air-dried, sieved (<4 mm) and gently mixed with the contaminated material (<2 mm, air dried) in a ratio 90:10 (w:w).

The following materials were selected as amendments:

- Iron sulfate ($FeSO_4 \cdot 7H_2O$, PRS, Panreac);
- $CaCO_3$ (PRS, Panreac);
- Paper mill sludge (PS), obtained from the company Holmen Paper S.L. (Madrid, Spain);
- Olive mill waste compost (OMWC), prepared from solid olive mill waste (alperujo) and cow manure at CEBAS-CSIC (Murcia, Spain);
- Olive tree pruning biochar (BC) was obtained by slow pyrolysis at 450 ºC in the University of León (Spain) (Brennan et al., 2014b).

PS, OMWC and BC were all considered as organic materials (organic amendments). The main characteristics of the soil and the organic materials are shown in Table 3.1.1. All the amendments were air-dried, homogenised and sieved (<2 mm) before mixing with the soil.

***Experimental design***

The following mixtures were applied as treatments in a dry soil weight basis:

(1) **Control**: non-treated soil
(2) $FeSO_4$ (1% w:w) + $CaCO_3$ (1% w:w) (**Fe+lime**);
(2) $FeSO_4$ (1% w:w) + Paper mill sludge (1% w:w) (**Fe+PS**);
(3) $FeSO_4$ (1% w:w) + olive mill waste compost (3% w:w) (**Fe+OMWC**);
(4) $FeSO_4$ (1% w:w) + biochar (olive tree pruning) (3% w:w) (**Fe+BC**).

The soil and the amendments were individually manually mixed and 600 g of each treatment were placed in methacrylate cylinders, which were used as pots, over a 2 cm layer of sand; the bottom of the pots was covered with a cloth to prevent soil loss. Four replicates were established for each treatment. The mixtures were moistened to achieve ~70% of the soil water holding capacity (WHC) and were incubated for 15 days in a growth chamber under controlled conditions (day/night: 13/11 h; 25/20 ºC; 40/60% of relative humidity (RH) and a photon flux of 520 µmol $m^{-2}$ $s^{-1}$). Soil humidity was maintained at ~70% of the WHC by weighing and adding water losses every two days.

After 15 days of incubation, a soil sample was taken from each pot (D15) and four one week old seedlings of *Arrhenatherum elatius*, previously germinated in peat, were transplanted to each pot. Pots were watered slightly over the WHC to collect

leachates once a week. The volume of the leachates was recorded and pH and EC were immediately measured. The leachate samples were filtered through 0.45 µm filters and stored at 4ºC until their analysis.

After 45 days of soil incubation, shoots of *A. elatius* were cut above the soil surface, washed, dried with tissue paper and weighed. The plant material was oven-dried for three days at 60 ºC and weighed again for dry weights recording and As and Cu analysis. Soils from each pot were homogenised and part of sample was air-dried, sieved (<2 mm) and stored for further analysis (D45).

***Toxicity bioassays***

Fresh and dry weights of *A. elatius* were recorded to evaluate the shoot growth response to the treatments. Arsenic and Cu concentrations in *A. elatius* shoots were used to evaluate risk of transfer to plant shoots.

A germination test was performed using *Lactuca sativa* (lettuce) seeds. Ten grams of dried soil from each pot (D45) were placed in petri dishes and hydrated to ~80% of their WHC. Then, 25 lettuce seeds were placed in each petri dish and kept for two days in the darkness (25/25 ºC, 40/60% RH) and for three more days with a photoperiod of 13/11 h (day/night). Germination success was calculated as the percentage of seeds germinated from those placed in each petri dish.

The effect of soil extracts on the bioluminescence of the bacteria *Vibrio fischeri* was tested according to the standardised method ISO 11348-2. Soils were mixed with water in a ratio 1:10 (w:v) and shaken for 24 h at 140 rpm and room temperature. The extracts were diluted with NaCl 2% (w:v) to achieve concentrations of 0, 6.25, 12.5, 25, 50 and 100% (v/v). The luminescence of the bacteria was measured using a luminometer (Optocom I, MGM Instruments) after the diluted extracts were in contact with a suspension of the bacteria for 30 min at 15 ºC. All the measurements were performed in duplicate. $EC_{50}$ was calculated as the percentage of soil extracts that caused a 50% reduction in the luminescence of the bacteria.

***Analytical procedures***

Soil particle distribution was determined using the densimeter method. The organic matter (OM) content of soil and amendments was determined by loss on ignition at 550 ºC. Total carbonates content was measured by $CO_2$ release after addition of HCl using a calcimeter. Pseudo-total element concentration in soils and organic materials was analysed after acid digestion with $HNO_3$+$H_2O_2$. For plant analysis, all replicates

from each treatment were merged into a composite sample and acid digested with $HNO_3$+$H_2O_2$ (Lozano-Rodríguez et al., 1995).

Soil pH was measured in 1:2.5 (w:v) D15 and D45 soil:water extracts, while in the organic amendments it was measured in 1:10 (w:v) water extracts and directly in the sampled leachates. Plant available Cu and As were determined in D15 and D45 by extraction with 0.1 M $(NH_4)_2SO_4$ (1:10, w:v). Exchangeable Ca, Mg and K in soils were extracted in D45 with 1 M ammonium acetate (pH 7) and available P with 0.5 M $NaHCO_3$ (P-Olsen). For total organic carbon (TOC) and total nitrogen (TN) content determination, D45 samples were pre-treated with diluted HCl in order to eliminate inorganic C contained in carbonates.

Copper sequential extraction was performed in D45 soil samples using the following extraction steps: 0.1 M $CaCl_2$ (1:10 w:v; 16 h) (exchangeable forms); 0.5 M NaOH (1:10 w:v, 16 h) followed by acid digestion of the extracts (associated with OM); 0.05 M $Na_2H_2EDTA$ (1:10 w:v; 1 h) (carbonates fraction) and digestion with $HNO_3$+$H_2O_2$ (residual fraction).

Arsenic sequential extraction was carried out by the slightly modified method especially developed for As by Larios et al. (2012), which involves the following steps: $H_2O$ for 24 h (F1, readily soluble); 0.5 M $Na_2HPO_4$ for 8 h (F2, strongly adsorbed onto mineral surfaces); 0.5 M $NH_4F$ for 15 h (F3, associated with Al oxyhydroxides); 0.1 M $Na_4P_2O_7$ for 16 h (F4, bound to organic matter); 0.2 M ammonium oxalate/oxalic acid (pH=3, dark) for 2 h+2 h (F5, incorporated into amorphous Fe oxyhydroxides; 0.2 M sodium citrate+0.6 M sodium bicarbonate+0.4 M ascorbic acid (pH=8) for 21 h, twice (F6, associated with poorly crystalline Fe (hydro)oxides); digestion with $HNO_3$+$H_2O_2$ (FR, residual fraction).

Arsenic was analysed in all the extracts, digests and leachates by hydride generation atomic fluorescence spectroscopy (HG-AFS) (PS Analytical 10.055, Millennium Excalibur) and Cu by atomic absorption spectroscopy (AAS) (Analyst 800, Perkin Elmer). Ca, Mg, and K in ammonium acetate extracts and P in $NaHCO_3$ extracts were analysed by ICP-OES (ICAP 6500 DUO, Thermo Scientific). Ca and Mg were analysed by AAS and K by atomic emission spectroscopy (EAS) in the organic materials digests (Analyst 800, Perkin Elmer). TOC and TN contents were measured in HCl-pre-treated D45 samples with a LECH CHNS-932 Analyser.

***Data analyses***

Statistical analyses were performed using IBM SPSS 21.0. Normality of the data was tested with the Shapiro-Wilk test. When homogeneity of variances and a normal distribution of the data were confirmed, a one-way ANOVA test was performed to determine whether there were differences among treatments, followed by Tukey´s HSD test to assess differences between groups. Games-Howell test was performed for non-homoscedastic cases.

**Table 3.1.1.** Main characteristics of the contaminated soil and the organic materials used in this experiment. Mean (n = 3) ± standard error (SE).

| | **Soil** | **PS** | **OMWC** | **BC[a]** |
|---|---|---|---|---|
| pH | 5.07 ± 0.03 | 8.69 ± 0.04 | 9.39 ± 0.01 | 9.34 ± 0.05 |
| EC (dS $m^{-1}$) | 1.85 ± 0.04 | 0.54 ± 0.02 | 6.98 ± 0.11 | 2.43 ± 0.03 |
| Clay (%) | 14.4 | - | - | - |
| Silt (%) | 22.6 | - | - | - |
| Sand (%) | 63 | - | - | - |
| OM (%) | 2.35 ± 0.01 | 31.9 ± 0.1 | 71 ± 2 | 90 ± 10 |
| Carbonates (%) | - | 36.9 ± 5.7 | 2.7 ± 0.6 | 8.0 ± 0.4 |
| Pseudo-total element (mg $kg^{-1}$) | | | | |
| As | 5046.3 ± 178.9 | 0.0095 ± 0.0004 | 0.0071 ± 0.0007 | 6 ± 2 |
| Cu | 299.1 ± 36.2 | 125.5 ± 2.3 | 24.8 ± 0.7 | 114 ± 3 |
| Zn | 40.4 ± 9.1 | 46.7 ± 0.7 | 128.2 ± 4.8 | 24 ± 1 |
| Fe | 26140.6 ± 1648.6 | 716.7 ± 22.9 | 819.7 ± 8.0 | 496 ± 12 |
| Mn | 82.4 ± 19.6 | 61.5 ± 2.3 | 65.6 ± 3.0 | 50 ± 1 |
| K | - | 45.8 ± 3.2 | 2098.8 ± 14.2 | 9159 ± 30 |
| Na | - | 56.6 ± 1.3 | 233.9 ± 3.0 | - |
| Ca | - | 15405.7 ±279.2 | 2944.6 ± 75.2 | 28524 ± 1070 |
| Mg | - | 256.3 ± 7.6 | 492.0 ± 20.4 | 2088 ± 73 |

[a]Determined by Brennan et al. (2014b)

## 3.1.3. Results

### *Effect of the amendments on soil pH*

Soil pH values remained similar at both samplings (D15 and D45) for all treatments (Fig. 3.1.1). However, the organic amendments applied in combination with iron sulfate differed in their ability to counteract the acidification provoked in the soils by the oxidation of Fe(II) (Eq. 3.1.1). Treatment Fe+lime (at a proportion 1:1) provoked the biggest increase of pH, which exceeded that of control soil in 2.8 units (D45). Addition of Fe+PS and Fe+OMWC resulted in a slight increase in soil pH, while Fe+BC-treated soil pH was lower ($P < 0.05$) than that of the control (Fig. 3.1.1).

$$4FeSO_4 + O_2 + 6H_2O \longrightarrow 4FeOOH + 4SO_4^{2-} + 8H^+ \qquad \text{(Eq. 3.1.1)}$$

### *Effect on As solubility and fractionation*

Treatments Fe+PS, Fe+OMWC and Fe+BC resulted in a significant decrease ($P < 0.05$) in $(NH_4)_2SO_4$-extractable As at D45, accounting for -78%, -77% and -85%, respectively, of that in the control (Fig. 3.1.1). However, addition of Fe+lime led to an increase of 26% in extractable As. A bivariate correlation analysis demonstrated a significant correlation ($P < 0.05$) between extractable As and soil pH (data not shown).

The pH of leachates was in general higher when Fe+lime was applied and was the lowest ($P < 0.05$) with Fe+BC in most fractions (Table 3.1.2). The treatments Fe+PS, Fe+OMWC and Fe+BC effectively reduced As concentration in the leachates with respect to the control, especially in the last fractions sampled, where Fe+OMWC and Fe+BC showed significantly lower ($P < 0.05$) concentration of As than in the control (Table 3.1.2). Addition of Fe+BC resulted in the greatest reduction of As solubility, as it leached only 2.3 µg As $L^{-1}$ in the last fraction sampled, compared to the control leachates with 313.8 µg As $L^{-1}$. Contrastingly, Fe+lime provoked As mobilization in the two last fractions, doubling the As leached in control pots. The total amount of As leached from the pots was calculated as the sum of the amount leached in all fractions (Table 3.1.2). The highest total As leached was found in Fe+lime, despite there were not leachates from any of the replicates from this treatment in the 30-day fraction. As expected, Fe+PS, Fe+OMWC and Fe+BC significantly reduced the amount of As leached from the pots ($P < 0.05$), reducing that in the control in 92.7% with Fe+PS, 71.8% with Fe+OMWC and 90.8% with Fe+BC.

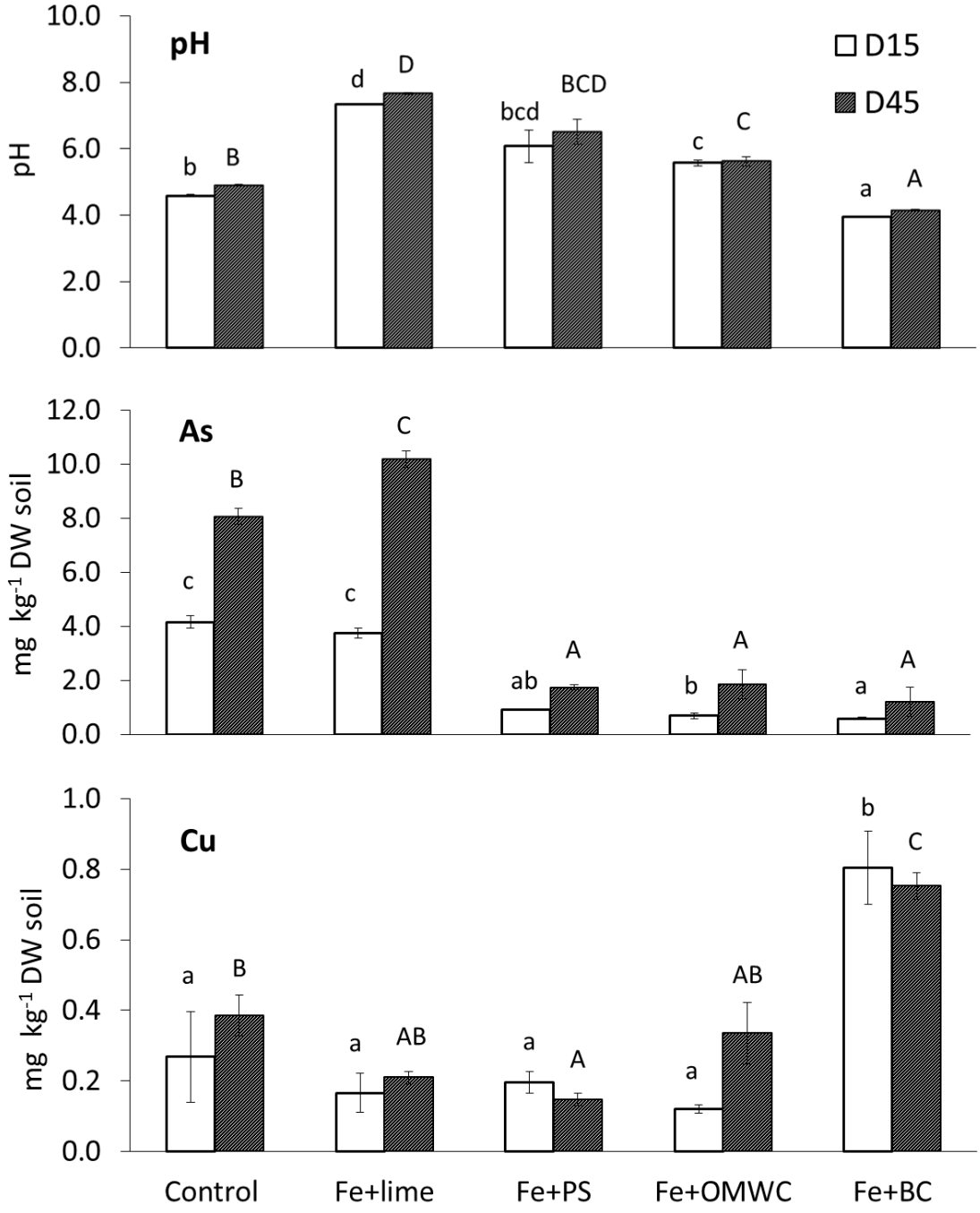


**Figure 3.1.1**. Soil pH and concentration of $(NH_4)_2SO_4$-extractable As and Cu in the control and the treated soils at 15 (D15) and 45 (D45) days of incubation. Mean (n = 4) ± SE. Different letters mean significant differences ($P < 0.05$) among treatments; lower case letters correspond to D15 and upper case letters to D45.

The distribution of As in the different fractions of control soil (Fig. 3.1.2) showed that most of the As was associated to amorphous (F5) and poorly crystalline iron oxides (F6), 52% an 23%, respectively, of the total As extracted, while the residual fraction represented only a small proportion (10%). The sum of As extracted in fractions F1 and F2, *i.e.* readily soluble and adsorbed onto mineral surfaces, represented less than 2% of the total As extracted. The effect of the amendments was more evident in these fractions. The treatment Fe+lime significantly increased ($P < 0.05$) As in F1 and F2 compared to the control. Readily soluble As (F1) was slightly decreased with Fe+PS and Fe+OMWC and was significantly reduced ($P < 0.05$) with Fe+BC, where the readily soluble fraction represented just 0.002% of the total As extracted. Most of the amendments provoked a significant increase ($P < 0.05$) in As associated to F2, *i.e.* strongly adsorbed onto mineral surfaces, which was more pronounced with Fe+lime. More As was extracted as well in F3 (associated to Al oxyhydroxides) in all the treated soils compared to the control. All the treatments resulted in a significant increase ($P < 0.05$) of As incorporated to amorphous iron oxides, that accounted >80% of the total As extracted.

**Table 3.1.2.** Arsenic concentration and pH values in the leachate samples collected throughout the experiment. The last column indicates the total amount of As leached in the whole experiment. Mean (n = 3). Different letters (normal letters for As and italics for pH) in the same column indicate significant differences among treatments ($P < 0.05$).

| Treatment | | 15 days | 23 days | 30 days | 36 days | 45 days | Total As leached (µg) |
|---|---|---|---|---|---|---|---|
| **Control** | **pH** | 6.13 *b* | 5.57 *a* | 6.74 *c* | 5.93 *bc* | 6.53 *bc* | |
| | **As (µg $L^{-1}$)** | 244.2 ab | 264.9 ab | 307.3 b | 327.9 b | 313.8 b | 28.7 ab |
| **Fe+lime** | **pH** | 7.48 *d* | 6.92 *c* | n.l. | 6.67 *c* | 6.73 *c* | |
| | **As (µg $L^{-1}$)** | 204.7 b | 393.2 b | n.l. | 753.4 c | 722.8 c | 39.6 b |
| **Fe+PS** | **pH** | 7.01 *c* | 6.81 *c* | 6.02 *b* | 6.63 *c* | 6.54 *bc* | |
| | **As (µg $L^{-1}$)** | 30.6 a | 8.4 a | 80.3 ab | 137.3 ab | 152.2 ab | 2.1 a |
| **Fe+OMWC** | **pH** | 6.89 *c* | 6.29 *b* | 5.20 *a* | 5.38 *ab* | 6.19 *b* | |
| | **As (µg $L^{-1}$)** | 10.0 a | 60.5 a | 211.2 ab | 86.4 a | 21.6 a | 8.1 ab |
| **Fe+BC** | **pH** | 4.54 *a* | 5.20 *a* | 5.07 *a* | 4.85 *a* | 4.80 *a* | |
| | **As (µg $L^{-1}$)** | 21.7 a | 0.0 a | 0.0 a | 13.0 a | 2.3 a | 2.6 a |

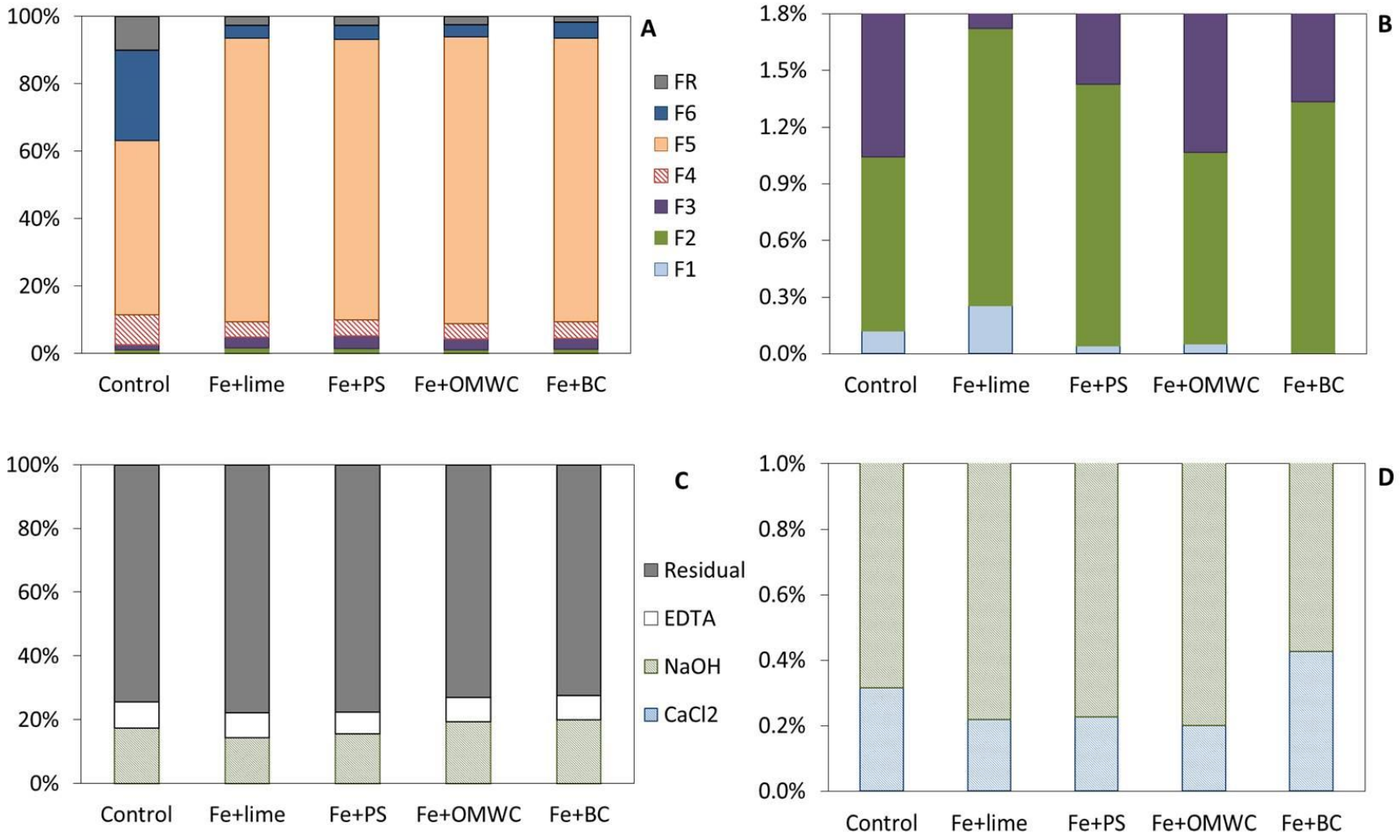


**Figure 3.1.2.** Sequential extraction of As (**A**) and detail of fractions F1 and F2 (**B**); sequential extraction of Cu (**C**) and detail of the $CaCl_2$ fraction (**D**) in the control and the treated soils at the end of the experiment. Data represent the percentages of the elements in each fraction with respect to the total extracted, mean (n = 4).
For As sequential extraction: **F1**: readily soluble; **F2**: strongly adsorbed onto mineral surfaces; **F3**: associated with Al oxyhydroxides; **F4**: bound to organic matter; **F5**: incorporated into amorphous Fe oxyhydroxides; **FR**: residual fraction.

***Effects on Cu fractionation***

No statistical differences ($P < 0.05$) were found in $(NH_4)_2SO_4$-extractable Cu at D15 between the control and most of the amendments (Fig. 3.1.1). Nonetheless, Fe+BC increased $(NH_4)_2SO_4$-extractable Cu in 200% at D15 and in 95% at D45, compared to the control. At D45, a significant reduction ($P < 0.05$) in the extractable Cu was observed in the Fe+PS-treated soil, while no differences were observed with Fe+lime and Fe+OMWC. A bivariate correlation analysis confirmed a significant negative correlation ($P < 0.01$) between soil pH and extractable Cu.

Cu distribution in the different fractions of control soil (Fig. 3.1.2-C and -D) shows that most of the Cu was present in the residual fraction (>70%). The soluble and exchangeable forms of Cu (extracted with $CaCl_2$) represented less than 0.5% (0.78 mg $kg^{-1}$). About 17% of the total copper was associated to OM (extracted with NaOH) while the carbonate fraction, extracted with $Na_2EDTA$, accounted for 8%.

No significant differences were found in $CaCl_2$-extractable Cu between the treated soils and the control, but the addition of Fe+BC increased Cu concentration in this fraction ($P < 0.05$) compared to the other treatments. Fe+OMWC and Fe+BC significantly increased ($P < 0.05$) the amount of Cu associated with OM, from 42.4 ± 1.2 mg $kg^{-1}$ in control soil to 52.0 ± 2.4 and 46.8 ± 0.7 mg $kg^{-1}$ in Fe+OMWC and Fe+BC, respectively (Fig. 3.1.2-C).

***Effects on soil nutrients***

The addition of compost (Fe+OMWC) promoted a significant increase ($P < 0.05$) of the available P pool. Remarkable differences were observed among treatments regarding extractable Ca; while Fe+OMWC and Fe+BC had no effect on it, Fe+lime and Fe+PS significantly enhanced ($P < 0.05$) exchangeable Ca in soil. Exchangeable K was significantly affected ($P < 0.05$) by the addition of compost (Fe+OMWC). TN content significantly increased ($P < 0.05$) after addition of Fe+OMWC and Fe+BC (Table 3.1.3) and the latter increased also TOC content by 450%.

**Table 3.1.3.** Concentration of ammonium acetate-extractable Ca, K and Mg (mg $kg^{-1}$); $NaHCO_3$-extractable P (mg $kg^{-1}$); total organic carbon (TOC) and total nitrogen (TN) contents (g $kg^{-1}$) in the soils after 45 days of incubation.
Mean (n = 4) ± SE. Different letters in the same column indicate significant differences among treatments ($P < 0.05$).

| Treatment | Ca | K | Mg | P | TOC | TN |
|---|---|---|---|---|---|---|
| **Control** | 1401.7 ± 149.3 $^{a}$ | 41.9 ± 0.7 $^{a}$ | 40.5 ± 1.4 $^{b}$ | 7.5 ± 0.1 $^{ab}$ | 4.18 ± 0.07 $^{a}$ | 0.45 ± 0.03 $^{a}$ |
| **Fe+lime** | 5414.1 ± 439.6 $^{c}$ | 39.5 ± 1.1 $^{a}$ | 42.7 ± 5.7 $^{b}$ | 6.7 ± 0.5 $^{a}$ | 3.33 ± 0.23 $^{a}$ | 0.41 ± 0.02 $^{a}$ |
| **Fe+PS** | 2510.1 ± 432.1 $^{b}$ | 41.5 ± 1.6 $^{a}$ | 29.0 ± 8.1 $^{ab}$ | 7.1 ± 0.3 $^{a}$ | 4.39 ± 0.21 $^{a}$ | 0.49 ± 0.04 $^{ab}$ |
| **Fe+OMWC** | 2145.0 ± 148.4 $^{ab}$ | 294.3 ± 45.4 $^{b}$ | 46.6 ± 14.7 $^{b}$ | 13.6 ± 2.1 $^{c}$ | 7.00 ± 0.39 $^{a}$ | 0.75 ± 0.05 $^{c}$ |
| **Fe+BC** | 1626.1 ± 287.8 $^{a}$ | 77.2 ± 6.8 $^{a}$ | 15.9 ± 6.7 $^{a}$ | 9.7 ± 0.3 $^{b}$ | 22.53 ± 3.41 $^{b}$ | 0.68 ± 0.05 $^{bc}$ |

***Toxicity bioassays***

The treatment Fe+OMWC significantly enhanced *A. elatius* shoot growth (Table 3.1.4), whose dry weight almost doubled that in the control. Treatments Fe+lime, Fe+PS and Fe+BC had no effect on plant biomass, while a slight reduction was observed with Fe+PS and Fe+BC. All treatments reduced As concentration in *A. elatius* shoots (Table 3.1.4) but the greatest effect was observed for Fe+BC. On the contrary, Cu concentration in leaves was higher in plants from treated soils than in those from the control, where the Cu concentration was below the limit of quantification. Due to there was a single sample per treatment, the statistical analysis of the results from element concentration in plant shoots could not be performed.

Contrary to *A. elatius* growth, Fe+BC significantly increased ($P < 0.05$) the germination index of *L. sativa* (from 64% in the control to 82% in Fe+BC treatment); Fe+lime also enhanced germination success, but to a lesser extent (Table 3.1.4).

Similar to the germination test, soil water-extracts from Fe+BC and Fe+lime did not cause any toxic effect on *V. fischeri*, as no inhibition in the luminescence was observed ($EC_{50}$ could not be calculated). 9.9%, 7.6% and 10.5% of the water-extracts of Control, Fe+PS and Fe+OMWC, respectively, were required to halve luminescence intensity after 30 minutes of exposition. Addition of compost (Fe+OMWC) led to a slight decrease in the toxicity for *V. fischeri* with respect to the control, while Fe+PS resulted to be the most toxic mixture for this organism, as shown by the lowest $EC_{50}$ value obtained (Table 3.1.4).

**Table 3.1.4.** Germination success of *L. sativa* in the non-amended and amended soils (mean (n = 4) ± SE); $EC_{50}$ values for *V. fischeri* obtained by contact with soil extracts; shoot dry weights (DW) (mean (n = 4) ± SE) and As and Cu concentrations in *A. elatius* shoots (concentration in the composite plant sample).

| | **Germination (%)** | **$EC_{50}$ $t$=30 (%)** | **Shoots DW (mg pot$^{-1}$)** | **Concentration in *A. elatius* shoots (mg kg$^{-1}$)** | |
|---|---|---|---|---|---|
| **Treatment** | ***L. sativa*** | ***Vibrio fischeri*** | ***A. elatius*** | **As** | **Cu** |
| **Control** | 64 ± 2$^{ab}$ | 9.9 | 54.0 ± 6.9$^{a}$ | 71.10 | 0.0 |
| **Fe+lime** | 77 ± 6$^{bc}$ | n.i. | 54.3 ± 9.5$^{a}$ | 40.24 | 12.8 |
| **Fe+PS** | 56 ± 2$^{a}$ | 7.6 | 40.3 ± 6.2$^{a}$ | 49.81 | 45.8 |
| **Fe+OMWC** | 62 ± 3$^{ab}$ | 10.5 | 100.5 ± 13.1$^{b}$ | 55.39 | 66.6 |
| **Fe+BC** | 82 ± 5$^{c}$ | n.i. | 29.3 ± 2.3$^{a}$ | 5.09 | 83.5 |

## 3.1.4. Discussion

***Arsenic mobility and distribution after amendments application***

The low proportion of As extracted in fraction F1 (0.12%) and F2 (0.92%) in the control soil (Fig. 3.1.2) indicates low mobility of this element. However, due to a high total As content in the contaminated soil (Table 3.1.1), this low proportion was nevertheless related to a high concentration of potentially available As (F1: 7.6 mg $kg^{-1}$; F2: 56.1 mg $kg^{-1}$).

All the treatments which combined iron sulfate and organic material (*i.e.* Fe+PS, Fe+OMWC and Fe+BC) were effective at reducing As solubility, as shown by lower concentration of leachable As, and lower water and $(NH_4)_2SO_4$-extractable fractions (Table 3.1.2, Fig. 3.1.2-B and Fig. 3.1.1). The addition of iron amendments has been reported to efficiently immobilise As in contaminated soils (Moreno-Jiménez et al., 2016b). However, addition of Fe (II) sulfate triggers soil acidification (Eq. 3.1.1), which may result in As and metal mobilisation (Manzano et al., 2014b). Therefore, liming products must be applied in combination with this ferrous salt to counteract soil acidification (Warren et al., 2003). In our experiment, all the amendments co-applied with $FeSO_4$ seemed to be effective, in a greater or lesser extent (BC had little effect), at preventing the great soil acidification expected if $FeSO_4$ would have been applied alone, at least after 45 days.

It is well known that As solubility generally increases when increasing soil pH and this pH-dependence is stronger in soils with high contents of oxide minerals, such as iron oxides (Sadiq 1997). This would explain why Fe+lime, with the highest pH (Fig. 3.1.1), increased the proportion of readily soluble As (F1; Fig. 3.1.2), $(NH_4)_2SO_4$-extractable As (Fig. 3.1.1) and total As leached (Table 3.1.2). The strong influence of pH on As mobility is evidenced by a significant positive correlation between $(NH_4)_2SO_4$-extractable As and soil pH (Spearman coeff. = 0.535, $P$ = 0.018). This would explain as well why Fe+BC efficiently immobilised As, as its addition resulted in a low soil pH (Fig. 3.1.1). In fact, the immobilization of As in biochar itself may be generally ruled out, as its sole application has no effect on exchangeable As (Brennan et al., 2014a) or even leads to As mobilisation (Brennan et al., 2014b; Beesley et al., 2014).

All the treatments resulted in a higher proportion of As associated to amorphous Fe oxides (F5; from 52% to >80%), somehow expected as the addition of iron (II) salts to soils results in fast formation of amorphous and poorly crystalline iron oxides. The

most probable mechanism of As immobilisation after addition of iron oxides or sulfates is the sorption of As on iron oxyhydroxides (Kumpiene et al., 2008; Komárek et al., 2013). Iron(III) arsenates may also precipitate, but they are too soluble to decrease As solubilisation (Miretzky and Fernandez Cirelli, 2010). The sequential scheme followed here allowed us to differentiate between amorphous (F5) and poorly crystalline iron oxyhydroxides (F6). All the amendments resulted in an increase in the As associated to amorphous iron oxides (F6), but not to the poorly crystalline oxides (F5) (Fig. 3.1.2). It has been reported that higher crystallinity of iron oxyhydroxides gives rise to lower sorption efficiency for As due to a decrease in specific surface area and sorption sites density (Dixit and Hering, 2003). Hence, the newly formation of amorphous iron oxides in soils represents a benefit for amendment-assisted As stabilisation strategies at longer time scales (Kumpiene et al., 2008).

Arsenic mobilisation after addition of organic matter to soils was consistently observed before (Hartley et al., 2009; Clemente et al., 2010; Beesley et al., 2013). However, in this work the addition of PS, OMWC and BC, differing in their OM content (Table 3.1.1), did not mobilise As due to the preventive effect of the ferrous salt application. In fact, no differences were found in the concentration of As extracted in F4, *i.e.* bound to OM (Fig. 3.1.2-A and -B), suggesting that the in situ-formed iron oxyhydroxides mainly control As distribution in soil and prevents its mobilisation.

***Effects on Cu fractionation***

The low solubility of Cu, shown by a low proportion extracted with $CaCl_2$, and its main accumulation in the residual fraction of the contaminated soil (Fig. 3.1.2-C and -D) revealed its low mobility and hence low risk of leachability (Pardo et al., 2011). However, due to high total Cu content (299.1 ± 36.2 mg $kg^{-1}$) and relatively high $(NH_4)_2SO_4$- and $CaCl_2$-extractable concentrations (0.27 ± 0.13 and 0.78 ± 0.17 mg $kg^{-1}$, respectively), its distribution in different soil fractions after the application of the amendments must be taken into consideration.

Iron amendments have been shown to not affect Cu mobility in a great extent (Hartley et al., 2004; Kumpiene et al., 2006) or even trigger its mobilisation due to soil acidification when iron (II) salts are applied (Manzano et al., 2014b). In this work, as acidification was generally avoided, little effect was observed with most of the amendments (Fig. 3.1.1).

The addition of Fe+OMWC and Fe+BC, which provided the greatest amount of organic matter (Table 3.1.1), resulted in a slight increase in the concentration of Cu

bound to organic matter. A similar result was also observed by Janos et al. (2010) after the addition of amendments rich in humic substances. In addition, in the Fe+OMWC-treated soil a slight decrease of the most labile pool was observed (Fig. 3.1.2-D), which could be a consequence of the complexation of Cu by stable organic matter and therefore a decrease in its availability. It is well known that Cu forms strong complexes with organic matter (Zhou and Wong, 2001). However, the addition of organic amendments to Cu-contaminated soils has shown controversial effects; on the one hand an increase of dissolved organic matter may lead to the formation of soluble complexes with Cu, thus increasing its mobility (Beesley et al., 2010). On the other hand, the addition of more stable and less soluble organic matter may result in Cu stabilisation (Karami et al., 2011; Beesley et al., 2014). The opposite effect was observed when Fe+BC was applied. Despite more Cu was associated to organic matter with this treatment than in the control soil (Fig. 3.1.2-C and -D), an increase in both $CaCl_2$- and $(NH_4)_2SO_4$-extractable Cu was observed (Figs. 3.1.1 and 3.1.2). Soler-Rovira et al. (2010) pointed out that the pH of an amended soil mainly governs Cu solubility and mobility. A decrease in soil pH may provoke an increase in the most easily exchangeable fractions due to release of Cu. This would explain the increase observed in the more labile Cu fractions upon addition of Fe+BC in this work (Figs. 3.1.1 and 3.1.2). Brennan et al. (2014b) also observed an increase in the leachable pool of Cu in a soil amended with olive pruning biochar (the same as used in this work), although in that case it could be rather related to an increase in dissolved organic carbon.

***Soil quality and toxicity assessment***

Fe+OMWC supplied an important pool of nutrients to soil (Table 3.1.3), which was likely reflected in the enhancement of *A. elatius* biomass production (Table 3.1.4). OMWC has been proposed by several authors as a suitable amendment to promote soil fertility and plant growth, especially due to high N, K and P supply (Walker and Bernal, 2008; Pardo et al., 2014b). In general there was not a good agreement between what was observed in extractable and soluble As and Cu in soils and their concentration in *A. elatius* shoots. However, as there were not replicates for this analysis (plants from each treatment were merged into one single composite sample), further research is needed to better evaluate the effects of the treatments on plant As and Cu uptake.

Regarding toxicity, each treatment showed different effects depending on the bioassay carried out (Table 3.1.4). For instance, while Fe+OMWC enhanced *A.*

*elatius* growth, a positive effect was not observed for lettuce germination success, which could be affected by other factors such as high salinity of the leachates from Fe+OMWC (data not shown), as *L. sativa* is moderately sensitive to salinity (Nasri et al., 2011).

The luminescent bacteria *Vibrio fischeri* seemed to be very sensitive to pollution in some cases, as shown by low $EC_{50}$ values obtained for Control, Fe+PS and Fe+OMWC (Table 3.1.4). However, a correlation could not be established between toxicity towards *V. fischeri* and the effects on As and Cu solubility. Unexpectedly, extracts from Fe+lime and Fe+BC did not provoke any toxicity to *V. fischeri* (Table 3.1.4). On the other hand, extracts from Fe+PS were the most toxic for *V. fischeri*, as they showed the lowest $EC_{50}$ value. Manzano et al. (2014a) also reported toxicity towards *V. fischeri* when the same mixture (Fe+PS, 1:1 w:w) was applied to a multi-contaminated soil, even though it reduced metals and As extractability. In addition, we observed a slight decrease in *A. elatius* shoots weight and in *L. sativa* germination index with this treatment. These findings suggest that other factors apart from metals/metalloids concentration in soil may affect toxicity towards several organisms after application of these amendments (especially PS).

All these observations show that the ecotoxicological tests performed are not conclusive and occasionally cannot be related to chemical evaluations.

## 3.1.5. Conclusions

The combination of iron, organic materials and lime efficiently immobilised As and Cu in the soil. Organic materials did not seem to interfere with the iron oxides capacity to stabilise As and Cu, but a strong correlation between the mobility of both elements and soil pH was found.

Fe+OMWC (1:3%) seemed to be the most suitable treatment for the tested soil, as it reduced As and Cu solubility while increasing soil nutrients concentrations and plant growth.

In general, the co-application of iron sulfate and organic materials is a valuable alternative for the remediation of multi-contaminated soils, as the combination of their single properties improves the whole soil recovery process. It is noteworthy that special attention must be taken to soil pH achieved after amendment application.

Future research should focus on the effect of aging on changes in oxides mineralogy and organic matter stability and their consequences on As and Cu associated risks.

# Chapter 3.2

# Assessment of iron sulfate combined with organic materials as amendments for an As- and Cu-contaminated soil: influence on As and Cu mobility and *Lupinus albus* L. trace elements and nutrients uptake.

## 3.2.1. Introduction and objective

When assessing the suitability of an "aided-phytostabilisation" strategy for a certain studied area, it is important to evaluate not only the effect of the amendments on the mobility of trace elements or contaminants, but also their effects on plant growth and plant "health", especially when the objective is creating a vegetal cover.

*Lupinus albus* L. (white lupin) has been proposed as a good candidate to use in phytostabilisation strategies on metal(loid) contaminated soils, since it is a metal(loid) exclusory species and can tolerate highly polluted and acidic soils (Vazquez et al., 2006; Martínez-Alcalá et al., 2010; Martínez-Alcalá et al., 2012).

The objective of this work was to assess the suitability of combining iron sulfate and several organic materials as single amendment for an As and Cu contaminated soil. For that, the influence of several treatments on As and Cu mobility in soil was studied, besides their effect on *Lupinus albus* L. growth and trace elements and nutrients plant uptake.

## 3.2.2. Materials and methods

### *Soil and amendments*

The uncontaminated soil and the contaminated waste material collected for this experiment was the same as those used in chapter 3.1, but in this case they were mixed in a ratio 95:5. The ratio was changed in this work because in previous experiments we observed certain As toxicity for white lupin with higher ratios, which could hide treatments effects on plant growth.

The amendments used in this work were:

- Iron sulfate ($FeSO_4 \cdot 7H_2O$, PRS, Panreac);
- $CaCO_3$ (PRS, Panreac);
- Paper mill sludge, obtained from the company Holmen Paper S.L. (Madrid, Spain);
- Olive mill waste compost (OMWC), prepared from solid olive mill waste (alperujo) and cow manure at CEBAS-CSIC (Murcia, Spain);
- Biochar (BC). In this experiment we changed the type of biochar with respect to the prior chapter. The one used in this work was produced by the pyrolysis of holm oak woodchips at 600 ºC (Moreno-Jiménez et al., 2016a).

Some characteristics of the composite contaminated soil (henceforth referred to as soil) and the organic materials are shown in Table 3.2.1.

***Experimental design***

The soil (waste material:uncontaminated soil 95:5, w:w) was manually homogenised and mixed with the corresponding amount of the amendments (calculated on a soil dry weight basis) to obtain the following treatments:

(1) **Control**, consisting of the non-amended soil;

(2) $FeSO_4$ (1%) + $CaCO_3$ (0.37%) (**Fe+lime**);

(3) $FeSO_4$ (1%) + paper mill sludge (1%) (**Fe+PS**);

(4) $FeSO_4$ (1%) + $CaCO_3$ (0.29%) + olive mill waste compost (3%) (**Fe+OMWC**);

(5) $FeSO_4$ (1%) + $CaCO_3$ (0.15%) + holm oak biochar (3%) (**Fe+BC**).

The amount of $CaCO_3$ applied in treatments (2), (4) and (5) was adjusted to the content of carbonates added when applying 1% of PS in treatment (3) and taking into account the total carbonates content of OMWC and BC for treatments (4) and (5).

The mixtures were gently homogenised and 3.5 kg were placed in pots in which a rhizobag system was placed and filled with 600 g of the same soil (same treatment) as in the pot. The rhizobag system will be explained in detail in chapter 4.2. Four replicates were established for each treatment. A rhizon sampler was inserted in each pot, outside the rhizobag, at an angle of 45º. These samplers (Rhizosphere Research Products, Wageningen, Netherlands) consist of a porous polymer tube that is inserted

in the soil and are used to obtain soil porewater by applying vacuum with syringes or vacuum tubes.

Then, a one-week-old *Lupinus albus* L. seedling previously grown in peat was transplanted to each pot, placed in the rhizobag. The plants were cultivated for 48 days in a growth chamber with controlled conditions (day/night: 13/11 h, 40/60% RH and a photon flux density of 520 µmol $m^{-2}$ $s^{-1}$). All soils were kept at 70% WHC during the whole experiment by weighing and adding water losses every two days.

Porewater was extracted 14, 28, 42 and 48 days after transplanting and the pH was immediately measured. Porewater samples were filtered through 0.45-µm syringe filters and stored at 4 ºC until their analysis.

After 48 days of plant growth, white lupin plants were harvested and separated into shoots (aboveground biomass) and roots and fresh weights were recorded. Roots were washed with tap and distilled water and sonicated for 3 minutes to remove soil particles. Plant material was stored at -80 ºC; all plant material was ground with liquid $N_2$ and a subsample was dried at 65 ºC for 3 days for its analysis.

***Plant and soil analyses***

For plant analyses, ground biomass (0.2 g) was digested with 4 mL of $HNO_3$ (65% v/v, PA, Sigma-Aldrich) and 1 mL of $H_2O_2$ (30% v/v, PA, Fluka) under a pressure of 1.5 kg $cm^{-2}$ for 30 min (Lozano-Rodríguez et al., 1995) and made up to 10 mL. The concentration of As and Cu in plant digests was analysed by ICP-MS (Elan 9000 DRCe, PerkinElmer). Ca, Mg, K and P concentrations were measured by ICP-OES (ICAP 6500 DUO, Thermo Scientific). The total content of nitrogen (TN) was directly analysed in the shoots with a LECH CHNS-932 Analyser.

The concentration of As, Cu, Fe and P in soil porewater samples was analysed by ICP-MS and dissolved organic carbon (DOC) was measured using a TOC analyser (Shimadzu TOC-V CSH).

Soil pH was measured in soil:water extracts (1:2.5 v:v).

The labile element fraction in soils was determined by soil extraction with 0.1 M $(NH_4)_2SO_4$ (1:10 w:v, shaken at 140 rpm for 4 h). Arsenic was analysed in the extracts by hydride generation-atomic fluorescence spectroscopy (HG-AFS) and Cu and Fe by atomic absorption spectroscopy (AAS).

P-Olsen was measured in 1:20 (w:v) $NaHCO_3$-extracts by ICP-OES.

Exchangeable K, Ca, Mg and Na were extracted with 1 M ammonium acetate (pH 7); the concentration of Ca and Mg in the extracts was measured by AAS and K and Na by atomic emission spectroscopy (AES).
TOC and TN contents were measured in HCl-pre-treated soil samples with a LECH CHNS-932 Analyser.

***Data analyses***

The statistical analysis of the data was performed using IBM SPSS Statistics 21. Data were checked for normality (Shapiro-Wilk test) and homoscedasticity; logarithmic transformations were applied when necessary. Differences among treatments were evaluated by analysis of variance (one-way ANOVA) followed by Tukey´s HSD test (homoscedastic data) or by Games-Howell test (heteroscedastic data). Bivariate correlations (Pearson´s coefficient) and multiple linear regression (stepwise, backward and forward methods) was carried out to assess the relationship between several variables.

Prediction of metal speciation was carried out with the software Visual Minteq.

## 3.2.3. Results

***Effect of treatments on soil porewater chemistry***

Figure 3.2.1 shows changes in As, Cu and DOC concentrations and pH in porewater throughout the experiment.

As shown in Figure 3.2.1, the treatments resulted in a significant decrease ($P < 0.05$) in the concentration of As in soil porewater with respect to the control at all sampling times. At the last sampling time (day 48) the reduction in soluble As in Fe+lime, Fe+PS, Fe+OMWC and Fe+BC accounted for 93%, 62%, 69% and 50%, respectively.

The concentration of As in the porewater significantly increased ($P < 0.05$) throughout the experiment in most of the treatments. At day 48, As concentration in porewater of the control, Fe+PS, Fe+OMWC and Fe+BC was ~24%, ~77%, ~63% and ~89% higher, respectively, than that at day 14. However, in Fe+lime we found >3-fold lower As concentration at day 48 than at day 14.

The treatments significantly reduced ($P < 0.05$) the concentration of soluble Cu after 14 days of lupin growth, with respect to the control (Fig. 3.2.1). The decrease of soluble Cu provoked by the treatments accounted for >50% and was greater in Fe+BC.

In control, Fe+lime and Fe+PS, a significant reduction ($P < 0.05$) in the concentration of soluble Cu was observed throughout the experiment, whereas in Fe+OMWC and Fe+BC, Cu concentration in porewater remained similar at all sampling times (Fig. 3.2.1).

All treatments provoked a significant increase ($P < 0.05$) in soil porewater pH, that was ≥1.4, ≥1.7, ≥2.0 and ≥2.2 pH units higher in Fe+lime, Fe+PS, Fe+OMWC and Fe+BC than in the control, respectively (Fig. 3.2.1).

Porewater pH was similar throughout the experiment in all cases, as no significant differences were found between the four sampling times for any treatment.

Most of the treatments had little effect on DOC concentration, as no differences were found between the treatments and the control and among the treatments. However, the treatment Fe+OMWC significantly increased ($P < 0.05$) the DOC concentration in porewater (Fig. 3.2.1). In this treatment, DOC varied between 93.6 and 108.6 mg $L^{-1}$ along the experiment, while in the other treatments it varied between 27.4 and 50.1 mg $L^{-1}$.

No differences in DOC were found between the sampling times in any treatment.

**Table 3.2.1**. Main characteristics of the (composite) soil and the amendments used in this experiment. Data are mean (n = 4) ± standard error (SE).

| | **Soil** | **PS** | **OMWC** | **BC (from holm oak)** |
|---|---|---|---|---|
| **pH** | 5.4 ± 0.1 | 8.69 ± 0.04 | 9.39 ± 0.01 | 9.8 ± 0.1 |
| **EC (dS $m^{-1}$)** | 1.85 ± 0.04 | 0.54 ± 0.02 | 6.98 ± 0.11 | 2.4 ± 0.2 |
| **Clay (%)** | 14.4 | - | - | 75 ± 4 |
| **Silt (%)** | 22.6 | - | - | - |
| **Sand (%)** | 63 | - | - | - |
| **OM (%)** | 2.35 ± 0.01 | 31.9 ± 0.1 | 71 ± 2 | - |
| **Carbonates (%)** | - | 37 ± 6 | 2.7 ± 0.6 | 7.4 |
| **Pseudo-total element (mg $kg^{-1}$)** | | | | |
| **As** | 2258 ± 625 | 0.0095 ± 0.0004 | 0.0071 ± 0.0007 | 0.0010 ± 0.0001 |
| **Cu** | 157 ± 33 | 125.5 ± 2.3 | 24.8 ± 0.7 | 12.4 ± 0.3 |
| **Zn** | 73 ± 11 | 46.7 ± 0.7 | 128 ± 5 | 32.6 ± 2.1 |
| **Fe** | 17058 ± 1380 | 717 ± 23 | 820 ± 8 | 1014 ± 53 |
| **Mn** | 83 ± 19 | 62 ± 2 | 66 ± 3 | 726 ± 35 |
| **K** | - | 46 ± 3 | 2099 ± 14 | 521 ± 15 |
| **Na** | - | 57 ± 1 | 234 ± 3 | <LOD |
| **Ca** | - | 15406 ± 279 | 2945 ± 75 | 5567 ± 193 |
| **Mg** | - | 256 ± 8 | 492 ± 20 | 444 ± 16 |

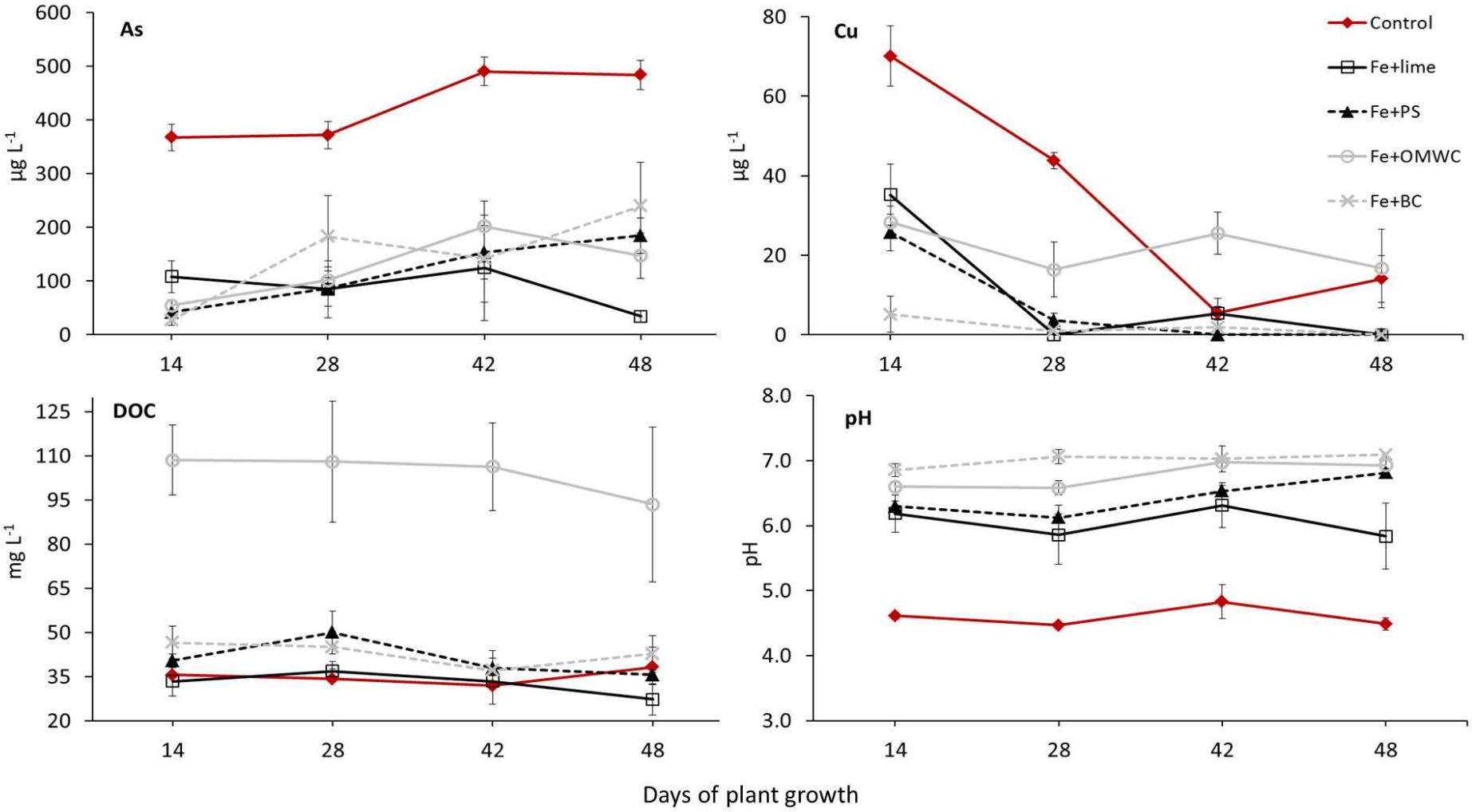


**Figure 3.2.1.** Concentration of As, Cu and DOC and pH measured in soil porewater collected after 15, 28, 42 and 48 days after white lupin transplanting. Data are mean (n = 4) ± SE.

***Effects on soil pH and extractable elements***

Figure 3.2.2 shows soil pH measured in water-extracts and the concentration of $(NH_4)_2SO_4$-extractable As, Cu and Fe in the control and the treated soils at the end of the experiment.

The addition of Fe+PS, Fe+OMWC and Fe+BC resulted in a significant increase ($P < 0.05$) in soil pH, which was 0.8, 0.9 and 1.3 pH units higher in Fe+PS, Fe+OMWC and Fe+BC, respectively, than in the control. However, no significant differences were found between Fe+lime and the control (Fig. 3.2.2).

Amongst the treated soils, significant differences ($P < 0.05$) were found between Fe+lime, Fe+PS, Fe+OMWC and Fe+BC; in these soils the pH varied in the order: Fe+BC > Fe+OMWC > Fe+PS > Fe+lime.

All treatments significantly reduced ($P < 0.05$) the concentration of $(NH_4)_2SO_4$-extractable As in soil at the end of the experiment. Arsenic concentration in the extracts of Fe+lime, Fe+PS, Fe+OMWC and Fe+BC was 89%, 81%, 74% and 79% lower than that in the control, and in all of them was lower than 1.3 mg $kg^{-1}$ (Fig 3.2.2).

Among the treatments, the concentration of extractable As was significantly higher ($P < 0.05$) in Fe+OMWC and Fe+BC than in Fe+lime, whereas no differences were found between the latter and Fe+PS.

The concentration of $(NH_4)_2SO_4$-extractable Cu was significantly higher ($P < 0.05$) in the control soil than in the treated soils and was reduced by 72% by Fe+lime, 46% by Fe+PS, 35% by Fe+OMWC and 56% by Fe+BC (Fig. 3.2.2).

When comparing the concentration of extractable Cu between the treatments, it was significantly higher ($P < 0.05$) in Fe+PS and Fe+OMWC than in Fe+lime.

P-Olsen concentration was significantly higher ($P < 0.05$) in Fe+OMWC and Fe+BC than in the control, Fe+lime and Fe+PS. The increase in Fe+OMWC and Fe+BC with respect to the control was by 2.8 and 1.9 times. No differences were found between the control, Fe+lime and Fe+PS (Fig. 3.2.2).

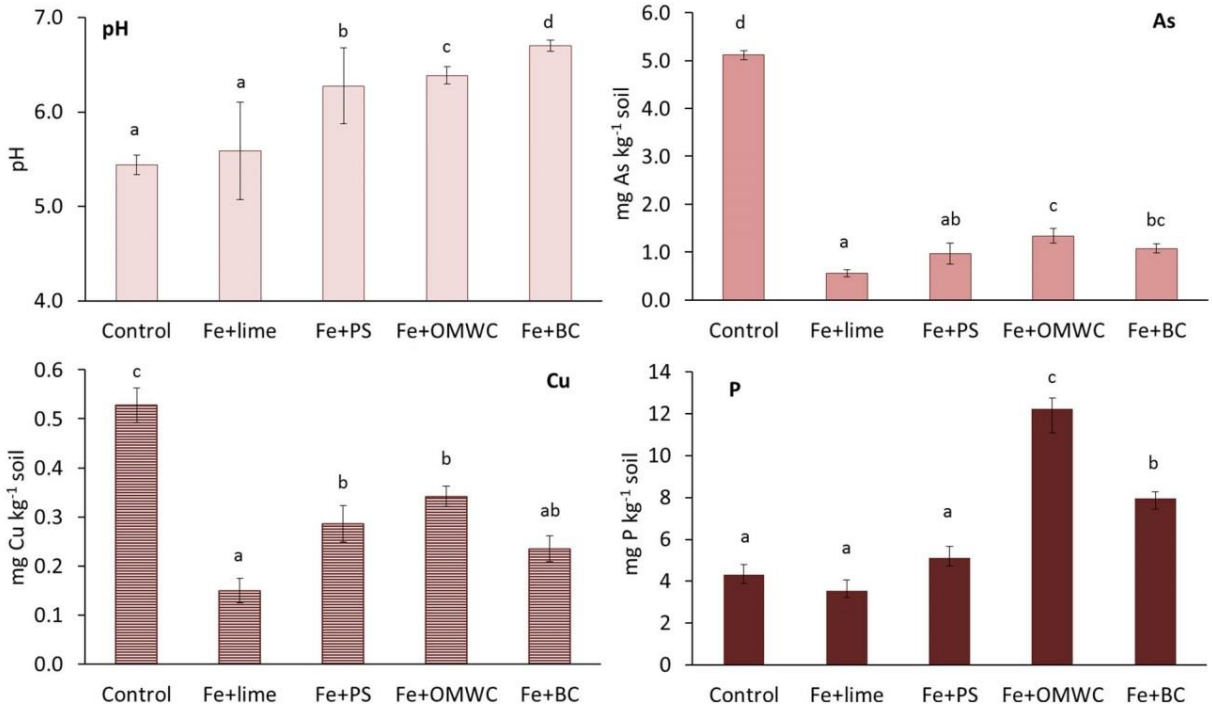


**Figure 3.2.2.** Soil pH, concentration of $(NH_4)_2SO_4$-extractable As and Cu and P-Olsen in the control and the treated soils at the end of the experiment. Data are mean (n = 4) ± SE. Different letters indicate significant differences among treatments ($P < 0.05$).

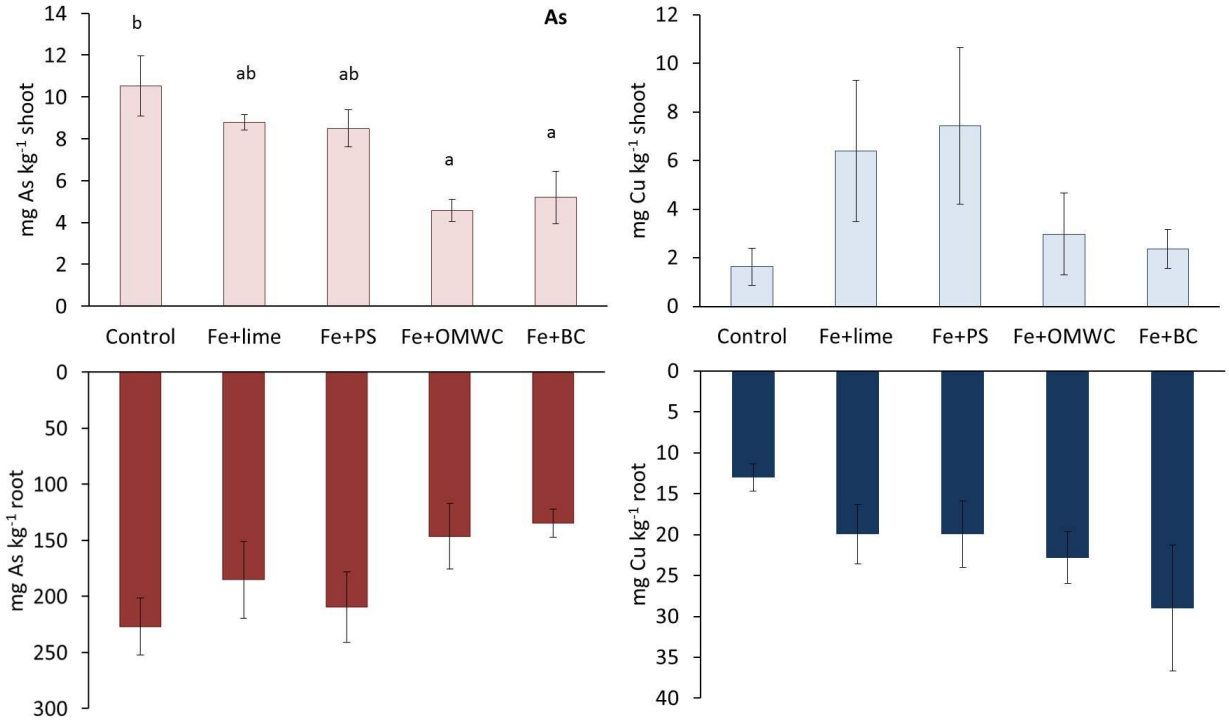


**Figure 3.2.3.** Arsenic and Cu concentration in shoots of white lupin plants grown in the control and the treated soils. Data are mean (n = 4) ± SE. Different letters above bars indicate significant differences among treatments ($P < 0.05$). Where there are no letters, no differences were found.

***Effect on As and Cu plant uptake***

The treatments Fe+OMWC and Fe+BC led to a significant reduction ($P < 0.05$) in the concentration of As in lupin shoots (Fig. 3.2.3), whereas no differences were found between Fe+lime, Fe+PS and the control plants. Nevertheless, a slight decrease in shoots As concentration was observed in all treated plants; compared to the control plants, As concentration was 1.2-fold, 1.2-fold, 2.3-fold and 2.0-fold lower in shoots from Fe+lime, Fe+PS, Fe+OMWC and Fe+BC.

The amendments did not seem to greatly affect the concentration of As in roots, as no significant differences were observed among plant roots from all soils, although, similarly to shoots, As concentration was slightly lower in roots from Fe+OMWC and Fe+BC-treated soils (Fig. 3.2.3). In any case, the total amount of As accumulated in the plants (in mg As $plant^{-1}$) did not differ among treatments (not shown).

No statistical differences were found regarding Cu concentration in white lupin shoots (Fig. 3.2.3), likely due to the high variability (standard error) of the data. However, a slight increase was observed in Fe+lime and Fe+PS with respect to the control plants, which accounted for 75% and 78%, respectively. Neither there were significant differences ($P < 0.05$) regarding Cu concentration in roots, though a slight increase was observed in the plants from all treated soils, especially Fe+BC.

***Effects on nutrients availability and on plant growth and nutrients uptake***

As could be expected due to the addition of $CaCO_3$ as amendment (and the high carbonates amount in PS), the concentration of exchangeable Ca significantly increased ($P < 0.05$) in all the treated soils (Table 3.2.2). Exchangeable K significantly increased in the Fe+OMWC and Fe+BC-treated soils, and it was more than 15 and 3 times higher, respectively, than in the control soil. The concentration of exchangeable Mg increased by 2.2-fold in Fe+OMWC and by 1.5-fold in Fe+BC, with respect to the control soil. The treatments Fe+OMWC and Fe+BC also resulted in a significant increase ($P < 0.05$) in TOC and TN contents in soil.

The application of Fe+OMWC significantly increased ($P < 0.05$) lupin shoots dry weight (Fig. 3.2.4). Similar increment was observed for Fe+BC, although in this case it was not statistically significant, likely due to the high variability (high standard error) between the replicates of this treatment. No differences were found between Fe+lime, Fe+PS and the control. Results of roots growth (dry weights) resembled those of the shoots (Fig. 3.2.4). Figure A-1 in the Annex shows how the plants looked at harvest.

**Table 3.2.2.** Concentration of ammonium acetate-extractable Ca, K and Mg (mg $kg^{-1}$), total organic carbon (TOC) and total nitrogen (TN) (g $kg^{-1}$). Data are mean (n = 4) ± SE. Different letters in the same column indicate significant differences among treatments ($P < 0.05$).

| Treatment | Ca | K | Mg | TOC | TN |
|---|---|---|---|---|---|
| **Control** | 1295 ± 19 $^{a}$ | 47.5 ± 1.9 $^{a}$ | 62.9 ± 3.7 $^{a}$ | 7.5 ± 0.6 $^{a}$ | 0.24 ± 0.04 $^{a}$ |
| **Fe+lime** | 1992 ± 119 $^{b}$ | 47.5 ± 2.8 $^{a}$ | 58.2 ± 5.0 $^{a}$ | 7.5 ± 0.4 $^{a}$ | 0.28 ± 0.03 $^{a}$ |
| **Fe+PS** | 1828 ± 69 $^{b}$ | 53.7 ± 1.4 $^{a}$ | 64.0 ± 2.7 $^{a}$ | 7.1 ± 0.7 $^{a}$ | 0.30 ± 0.05 $^{a}$ |
| **Fe+OMWC** | 2560 ± 98 $^{c}$ | 738.2 ± 33.8 $^{c}$ | 141.2 ± 9.1 $^{b}$ | 14.4 ± 1.9 $^{b}$ | 0.97 ± 0.12 $^{b}$ |
| **Fe+BC** | 2618 ± 33 $^{c}$ | 161.1 ± 4.3 $^{b}$ | 96.3 ± 1.3 $^{c}$ | 19.2 ± 1.1 $^{c}$ | 0.73 ± 0.08 $^{b}$ |

**Table 3.2.3.** Macronutrients concentration in white lupin shoots (g $kg^{-1}$ DW) after 48 days of plant growth. Mean (n = 4) ± SE. Different letters in the same column indicate significant differences among treatments ($P < 0.05$).

| Treatment | Ca | K | Mg | P | N |
|---|---|---|---|---|---|
| **Control** | 6.82 ± 0.45 $^{c}$ | 8.33 ± 1.36 $^{a}$ | 1.33 ± 0.11 $^{a}$ | 0.54 ± 0.03 $^{a}$ | 35.7 ± 2.6 a |
| **Fe+lime** | 4.86 ± 0.37 $^{b}$ | 16.67 ± 0.76 $^{bc}$ | 1.37 ± 0.11 $^{a}$ | 0.60 ± 0.02 $^{ab}$ | 37.7 ± 1.5 a |
| **Fe+PS** | 4.54 ± 0.16 $^{b}$ | 14.14 ± 0.71 $^{b}$ | 1.38 ± 0.11 $^{a}$ | 0.61 ± 0.03 $^{ab}$ | 36.7 ± 2.1 a |
| **Fe+OMWC** | 3.00 ± 0.07 $^{a}$ | 25.91 ± 0.41 $^{d}$ | 1.36 ± 0.06 $^{a}$ | 0.66 ± 0.01 $^{b}$ | 35.6 ± 0.7 a |
| **Fe+BC** | 3.09 ± 0.25 $^{a}$ | 19.06 ± 0.41 $^{c}$ | 1.89 ± 0.14 $^{b}$ | 0.59 ± 0.04 $^{ab}$ | 36.8 ± 1.6 a |

**Table 3.2.4.** Macronutrients content in white lupin shoots (mg) after 48 days of plant growth. Mean (n = 4) ± SE. Different letters in the same column indicate significant differences among treatments ($P < 0.05$).

| Treatment | Ca | K | Mg | P | N |
|---|---|---|---|---|---|
| **Control** | 5.4 ± 0.6 $^{b}$ | 6.3 ± 0.9 $^{a}$ | 1.02 ± 0.08 $^{a}$ | 0.42 ± 0.05 $^{a}$ | 28.5 ± 4.8 $^{a}$ |
| **Fe+lime** | 3.5 ± 0.5 $^{a}$ | 11.9 ± 1.1 $^{a}$ | 0.98 ± 0.13 $^{a}$ | 0.43 ± 0.04 $^{ab}$ | 27.0 ± 3.2 $^{a}$ |
| **Fe+PS** | 3.1 ± 0.2 $^{a}$ | 9.6 ± 0.7 $^{a}$ | 0.95 ± 0.15 $^{a}$ | 0.41 ± 0.03 $^{a}$ | 23.7 ± 1.9 $^{a}$ |
| **Fe+OMWC** | 3.2 ± 0.1 $^{a}$ | 27.9 ± 1.3 $^{c}$ | 1.46 ± 0.09 $^{ab}$ | 0.71 ± 0.02 $^{bc}$ | 38.4 ± 2.0 $^{a}$ |
| **Fe+BC** | 3.3 ± 0.5 $^{a}$ | 20.8 ± 4.2 $^{b}$ | 2.14 ± 0.54 $^{b}$ | 0.63 ± 0.11 $^{c}$ | 38.9 ± 5.3 $^{a}$ |

The concentration of several macronutrients in white lupin shoots are shown in Table 3.2.3 and their accumulation, calculated on a shoots dry weight basis, are shown in Table 3.2.4.

Despite the increase in the exchangeable Ca, significantly lower ($P < 0.05$) concentration and accumulation of Ca was found in plants from all treated soils.

All the amendments significantly increased ($P < 0.05$) the concentration of K in shoots compared to the control, but when its accumulation was calculated, significant differences with respect to the control were found in Fe+OMWC- and Fe+BC-treated plants.

The concentration and accumulation of Mg was significantly increased ($P < 0.05$) by Fe+BC with respect to the other treatments and the control, whereas no statistical differences were found between Fe+lime, Fe+PS, Fe+OMWC and the control.

Although only the treatment Fe+OMWC led to a significantly higher ($P < 0.05$) P concentration in shoots, P accumulation was significantly higher ($P < 0.05$) in both Fe+OMWC and Fe+BC-treated plants.

The treatments had no significant effect on N concentration and its total content in white lupin shoots, although a slight increase in the total N content was observed in Fe+OMWC and Fe+BC.

All plants presented nodules in their roots, so the nodules were separated, counted and weighed in order to evaluate a relationship between their characteristics and plant growth and N content. The number of nodules found in the roots of each treatment and the average dry weight per nodule are shown in Figure 3.2.5. The nodules seemed to be active because of their light red colour (caused by the presence of leg-haemoglobine). The number of nodules in Fe+BC-treated roots was significantly higher ($P < 0.05$) than in the control and Fe+lime, whereas no differences were found between the control and Fe+lime, Fe+PS and Fe+OMWC treatments. However, in Fe+PS- and Fe+OMWC-treated plants we found in average 11 and 13 more nodules, respectively, than in the control. The average dry weight of each nodule was significantly higher ($P < 0.05$) in the control than in the treated roots.

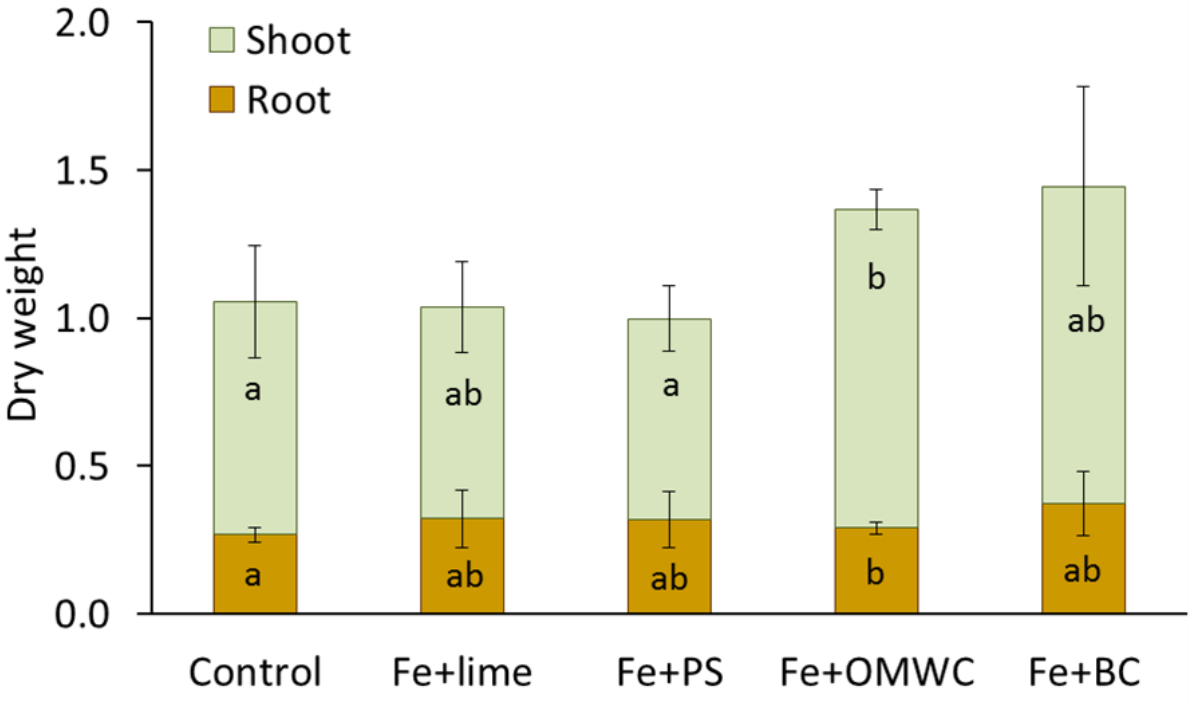


**Figure 3.2.4.** Dry weight of white lupin shots and roots grown in the control and the treated soils for 48 days. Data are mean (n = 4) ± SE. Different letters in bars indicate significant differences in shoots and roots weights among treatments ($P < 0.05$).

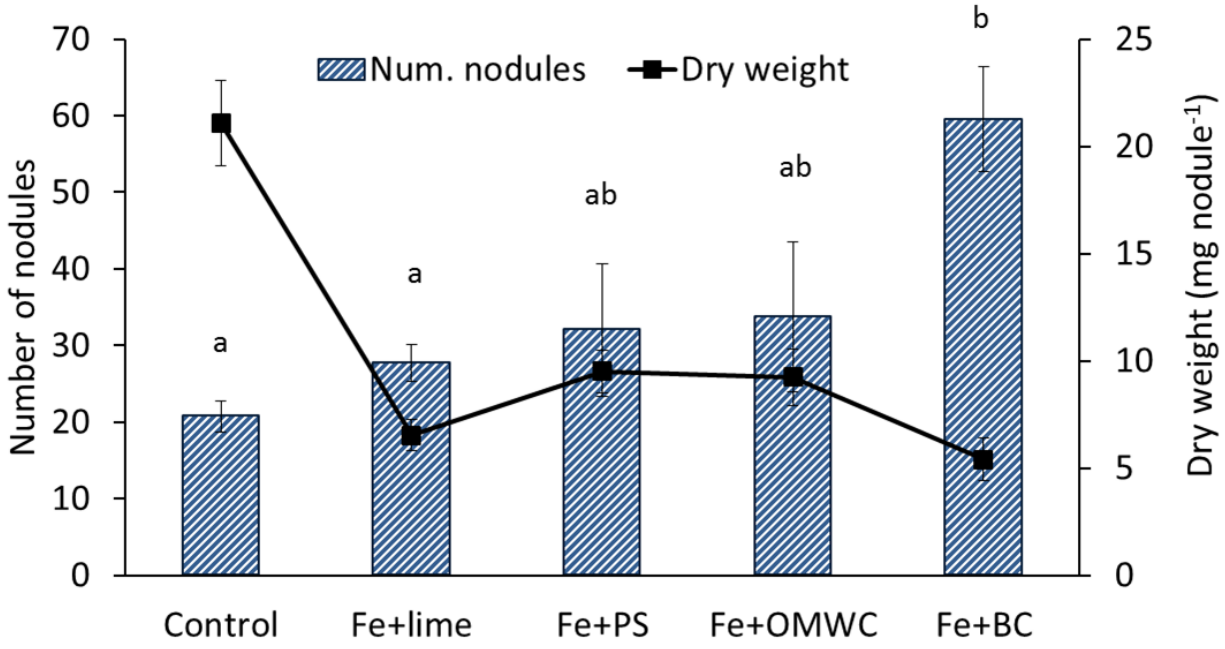


**Figure 3.2.5.** Number of nodules in white lupin roots and average dry weight per nodule. Mean (n = 4) ± SE. Different letters indicate significant differences in the number of nodules among treatments ($P < 0.05$). Nodules from all roots grown in treated soils showed significantly lower average dry weight than those from control.

## 3.2.4. Discussion

***Influence of the treatments on As and Cu mobility***

All the treatments applied effectively reduced As mobility with respect to the control, since their application resulted in a significant decrease ($P < 0.05$) in the concentration of As in soil porewater (Fig. 3.2.1) and in the concentration of $(NH_4)_2SO_4$-extractable As (Fig. 3.2.2).

Iron sulfate has been shown to be a good immobiliser of As, as its application to soils results in the *in situ* formation of "additional" iron (hydr)oxides that sequester As and stabilise it in long term (Cutler et al., 2014; Miretzky and Fernandez Cirelli, 2010). Contrastingly, As mobilisation upon addition of organic amendments, such as compost or biochar, has been previously reported (Clemente et al., 2010; Beesley et al., 2013). Since As mobility generally increases whit increasing soil pH (Fitz and Wenzel, 2002), some authors point out that the enhancement in As mobility is rather due to the increase in soil pH, as organic materials usually present an alkaline character (Beesley et al., 2010). However, competition between dissolved organic matter (DOM) and arsenate for soil surface binding sites may also occur and it would result in As release into the soil solution (Bauer and Blodau, 2006; Arco-Lázaro et al., 2016). Furthermore, the formation of soluble ternary complexes between DOM and As with iron as a bridge cation can result in As mobilisation (Ritter et al., 2006; Sharma et al., 2010; Mikutta and Kretzschmar, 2011).

In our study, the co-application of iron sulfate seemed to correct such As mobilisation derived from OM application, since we observed that, even though Fe+OMWC addition resulted in a significantly higher ($P < 0.05$) DOC concentration in soil porewater, this did not lead to an increase in the soluble As concentration (Fig. 3.2.1). Results of predicted metal speciation (Table 3.2.5) showed that, assuming $Fe^{3+}$ as the main iron species in the soil porewater, iron hydroxides would predominate in soil solution of treatment Fe+OMWC (the one with the highest DOC concentration). This, besides there was no correlation between As and DOC concentration in the porewater at any sampling time, suggests that the formation of ternary As-Fe-DOM complexes was unlikely to play a role on As solubility, in agreement with Neubauer et al. (2013), who observed that As transport in a boreal watercourse was mainly governed by colloidal iron (hydr)oxides rather than by DOM at pH >4.5.

As demonstrated in chapter 3.1 by sequential extractions, the addition of materials rich in organic matter (PS, OMWC and pine biochar) to an As-contaminated soil, applied together with iron sulfate, did not interfere with the ability of iron (oxy)hydroxides to sequester As (Fresno et al., 2016a). In addition, we found that the increase in soil pH provoked by the treatments did not lead to an increase in soluble and extractable As fractions (Figs 3.2.1 and 3.2.2). Our results suggest that the co-addition of iron sulfate increased the As sorption capacity of the soil, thus mitigating the effects of pH and DOC.

However, it is important to note that differences among treatments, both concerning soluble (Fig. 3.2.1) and extractable As (Fig. 3.2.2), were found in this work. When comparing Fe+lime, Fe+PS, Fe+OMWC and Fe+BC in the last porewater sampling, As concentration was slightly lower in Fe+lime than in the other treatments and showed a decreasing tendency that was not observed in Fe+PS, Fe+OMWC and Fe+BC (Fig. 3.2.1). This effect seemed to be related with the differences in soil porewater pH among treatments; when just considering the treated soils (*i.e.* without considering the control), we found a significant positive correlation between As concentration and porewater pH in the last sampling (day 48: r = 0.611, $P < 0.05$). Besides, significantly higher ($P < 0.05$) extractable As was found in Fe+OMWC and Fe+BC (and slightly higher in Fe+PS) with respect to Fe+lime (Fig. 3.2.2); in this case the increase in extractable As was correlated with an increase in P-Olsen (r = 0.858; $P < 0.001$).

In order to clarify the factors most affecting As mobility within the treatments, multiple linear regression was performed for soluble and extractable As. For soluble As ($[As]_{PW}$), we used total As in soil, porewater pH and the concentration of P and DOC in the porewater as variables. For extractable As ($[As]_{ext}$), the variables considered were total As, DOC concentration, P-Olsen and the pH of soil-water extracts. The following equations explained the variations in soluble and extractable As:

$$[As]_{PW} = -454 + 90.9\ pH_{PW} \qquad R^2 = 0.374;\ F_{1,15} = 8.36;\ P < 0.05 \qquad \text{(Eq. 3.2.1)}$$

$$[As]_{ext} = 0.297 + 0.087\ [P]_{ext} \qquad R^2 = 0.737;\ F_{1,15} = 39.1;\ P < 0.001 \qquad \text{(Eq. 3.2.2)}$$

Equation 3.2.1 shows that, within the treatments, As solubility was mainly affected by porewater pH, which suggests that an increase in soil pH may reduce As sorption onto iron (hydr)oxides and thus increase its release into the soil solution. Equation 3.2.2 confirms that extractable P had a significant effect on the extractable As, whereas soil pH was not considered as a significant variable. Due to phosphate and arsenate present similar behaviour in soils (Adriano, 2001a), competition between both anions for mineral sorption sites can be expected, resulting in As mobilisation from less labile soil fractions (not readily soluble) and an increase in its availability, as reported before in other studies (Clemente et al., 2012; Moreno-Jiménez et al., 2013; Beesley et al., 2014). In any case, the increase in the P-Olsen provoked by Fe+OWMC and Fe+BC did not result in an increase in extractable As with respect to the control, which suggests, again, that in the treated soils As mobility was governed by its sorption onto iron oxides.

The reduction of soluble Cu provoked by the treatments at the beginning of the experiment was likely related with the increase in porewater pH, according to the significant negative correlation between these two variables (day 14: $r = -0.884$, $P < 0.001$; day 28: $r = -0.690$, $P < 0.001$). In the case of Fe+BC, retention of Cu by biochar through electrostatic interactions or complexation by functional groups could also have affected its immobilisation (Uchimiya et al., 2011), as in this treatment very low concentration of Cu (<5 μg $L^{-1}$) was found in the soil porewater throughout the experiment.

The different behaviour observed for soluble Cu between Fe+OMWC and the other treatments (*i.e.* it remained constant in Fe+OMWC) could be explained by the positive correlation between Cu and DOC in porewater in the last two samplings (day 42: $r = 0.675$, $P < 0.01$; day 48: $r = 0.707$, $P < 0.01$). Furthermore, the prediction of Cu speciation carried out using Visual Minteq showed that up to 99.9% of Cu in the porewater of Fe+OMWC was complexed by DOM at all sampling times (Table 3.2.5). Related to this, Vargas et al. (2016) observed an increase in Cu accumulation in vetiver grass roots upon addition of humic acids to a contaminated soil, which the authors related with an increase in water-soluble Cu due to formation of soluble metal-organic complexes.

**Table 3.2.5.** Distribution of the major species of Cu, As and Fe in the Fe+OMWC-treated soil porewater.

| Sampling | Cu | | As | | Fe | | |
|---|---|---|---|---|---|---|---|
| | Cu-DOM | $Cu^{2+}$ | $HAsO_4^{2-}$ | $H_2AsO_4^-$ | Fe-DOM | $Fe(OH)_2^+$ | $Fe(OH)_{3\,(aq)}$ |
| 14 days | 99.95 | 0.05 | 29.3 | 70.7 | 4.7 | 94.9 | 0.2 |
| 28 days | 99.95 | 0.05 | 28.4 | 71.6 | 4.8 | 94.8 | 0.2 |
| 42 days | 99.96 | 0.04 | 49.8 | 50.2 | 1.3 | 98.0 | 0.5 |
| 48 days | 99.96 | 0.04 | 46.9 | 53.1 | 1.4 | 98.0 | 0.5 |

***Effects on As and Cu uptake by Lupinus albus***

Despite the great reduction that the treatments provoked on extractable As, it did not match with a great effect on plant As uptake. Although significant differences were observed between As concentration in shoots of control and the treatments Fe+OMWC and Fe+BC, these differences could be a consequence of a slight increase in biomass production (Fig. 3.2.4). Another possible explanation for this difference could be the higher P availability in these two treatments (Fig. 3.2.2) and the higher accumulation of P in shoots of Fe+OMWC and Fe+BC treated plants (Table 3.2.4), as phosphate and arsenate are known to share the same root transporters (Meharg et al., 1994) and hence higher P bioavailability can mitigate As uptake in a certain extent. Nevertheless, As accumulation in shoots was similar (data not shown), suggesting that P did not greatly affect As uptake.

Kumpiene et al., (2012) reported that the stabilisation of As due to its binding to amorphous and poorly crystalline iron oxides did not inhibit As plant uptake. Plants may alter their rhizosphere by the exudation of organic compounds, which may solubilise iron oxides and thus increase the availability of As associated to them (Jones et al., 1996; Onireti and Lin, 2016). This was also suggested by Gonzaga et al. (2006), who found that two fern species took up As from the amorphous (hydr)oxides fraction, that was the most abundant but not the most labile fraction in that soil.

Similar to that observed for As, plant Cu uptake did not reflect the results obtained by chemical extractions. Despite the lower extractable Cu in the treated soils than in

the control (Fig. 3.2.2), no differences were found regarding Cu concentration in plant tissues (Fig. 3.2.3). This could be explained by root-induced mobilisation of Cu in the rhizosphere of white lupin or by a rapid uptake, that would agree with the trend of Cu concentration in the porewater of most treatments: in control, Fe+lime and Fe+PS, soluble Cu significantly decreased throughout the experiment, which could be due to a rapid and high absorption of Cu by lupin plants. In any case, Cu concentration in shoots of any treatment was not high enough to be considered as toxic for this plant (Marschner, 2012; Sánchez-Pardo and Zornoza, 2014).

***Effects on soil nutrients availability and plant nutritive status and growth***

The treatments Fe+OMWC and Fe+BC were those that most affected nutrients concentration in the soil, as both significantly increased ($P < 0.05$) the concentration of extractable Ca, K, Mg, TN, TOC (Table 3.2.2) and P-Olsen (Fig. 3.2.2).

The great effect of OMWC addition on available K and Mg is not surprising taking into account the high total content of these nutrients in this material (Table 3.2.1). Similarly, BC supplied a high amount of Mg, which was reflected in an increase in its exchangeable fraction.

OMWC has been proposed as a useful amendment to improve plant growth in aided phytostabilisation strategies as it provides nutrients and enhances soil health (Pardo et al., 2014a; Walker and Bernal, 2008).

The mechanisms affecting nutrients by biochar can be also related with its large surface area and its high cation exchange capacity, which can reduce nutrient leaching (Laird et al., 2010). In any case, the biochar used in this study was relatively rich in some nutrients (Table 3.2.1). Our results are generally in agreement with previous studies; in a meta-analysis considering biochar application in different soils and environments, Biederman and Stanley Harpole (2013) concluded that the use of biochar as a soil amendment had in general significant positive effects on aboveground biomass growth, rhizobia nodules, plant K, available K and P, pH and total N and C contents in soil.

White lupin growth was little affected by the treatments (Fig. 3.2.4 and Fig. A-1 in the Annex). Fe+OMWC and Fe+BC slightly increased shoots biomass, likely related with the higher P, K and Mg content in shoot tissues (Table 3.2.4). In fact, the addition of OMWC and BC has been shown before to positively affect plant growth (Pardo et al., 2014b; Brennan et al., 2014).

Root nodules are formed due to the symbiotic relationship between leguminous plants and $N_2$-fixing soil *rhizobium* bacteria, and can be negatively affected by high concentration of trace elements (Pajuelo et al., 2008), although different strains of *rhizobium* have been found in soils with high levels of As (Carrasco et al., 2005).

In this work, the formation of nodules was not inhibited by the presence of As, since plants from all soils, including the control, presented active nodules in their roots. However, we found less but larger nodules in plants grown in the control soil, with the higher As availability (Fig. 3.2.5). Reichman (2007) also observed a decrease in the number of nodules in soybean as As concentration increased in the nutrient solution and suggested that this would be related to the decrease in number of root hairs, and thus a reduction in the infection site, rather than due to As toxicity. Talano et al. (2013) suggested that a reduction of nodulation in plants grown in As-contaminated soils can be also related with a reduction in the bacteria motility, which is partially responsible of the colonization of roots. Nevertheless, Pajuelo et al. (2008) observed that, once nodulation was established, nodules development and activity continued normally. Therefore, it could be hypothesised that the largest size of control-plants nodules is due to an increase in their efficiency.

Plants grown in Fe+BC-treated soil had more nodules in their roots than those grown in the other treatments (Fig. 3.2.5). This could be also explained by changes in root morphology provoked by biochar. Brennan et al. (2014) observed that biochar addition to an As- and Cu-contaminated soil enhanced the length and surface area of maize roots and the production of fine roots, so the same could be true in our study, thus leading to an increase in the number of nodules.

However, the differences in the size and number of nodules did not seem to affect N uptake, as all plants had similar N content in shoots, although it was slightly higher in Fe+OMWC- and Fe+BC-treated plants (Table 3.2.4). In any case, whether this increase was directly related with nodulation or was due to the N supplied by these treatments should be further studied.

## 3.2.5. Conclusions

The treatments Fe+lime, Fe+PS, Fe+OMWC and Fe+BC effectively reduced the mobility of Cu, and especially of As. The *in situ* formed iron (hydr)oxides controlled As mobility, thus mitigating the effect of pH and DOM. Despite this, porewater pH and available P seemed to have caused the small differences found between treated soils.

The application of OMWC and BC generally improved soil nutrients availability and nutrients content in white lupin tissues, compared to the sole application of iron sulfate and lime (Fe+lime), although little effect was observed with Fe+PS application in spite of its relatively high organic matter content. Longer term experiments should be carried out to evaluate for how long is this effect lasting.

In summary, the combination of iron sulfate and organic materials, such as olive mill waste compost and biochar, may improve soil fertility and white lupin growth at the same time as they reduce As and Cu mobility.

# Chapter 3.3

# Assessment of iron sulfate combined with organic materials as amendments for an As- and Cu-contaminated soil: effects on As and Cu stabilisation, soil quality and *Secale cereale* L. growth over two years

## 3.3.1. Introduction and objective

When an aided phytostabilisation strategy is implemented, changes in the characteristics of the amendments can occur over time that may lead to a loss of their stabilisation efficiency. In addition, organic matter mineralisation and nutrients leaching can reduce the effect of organic amendment on soil functions. Therefore, long-term evaluation of a remediation process should be carried out in addition to mesocosm experiments.

In this chapter, we aimed at assessing the effectiveness of amendments consisting on the combination of iron sulfate and organic materials on the remediation of an As- and Cu-contaminated soil in the medium/long term (22 months). We evaluated the influence of the amendments on As and Cu geochemistry, soil functions and *Secale cerelae* L. cultivation in an experiment carried out outdoors in refilled lysimeters, simulating field plots.

## 3.3.2. Materials and methods

### *Soil and amendments*

The contaminated waste material, the uncontaminated soils and the amendments $FeSO_4$, $CaCO_3$, PS, BC and OMWC were the same used in the previous chapter. In this chapter, green waste compost (GWC) was also included as organic amendments to be mixed with iron sulphate. GWC was produced by composting of green wastes from public gardens of Madrid city and was obtained from the composting facility Migas Calientes (Madrid, Spain). The main characteristics of the organic amendments are shown in Table 3.3.1.

**Table 3.3.1.**Some characteristics of paper mill sludge (PS), holm oak biochar (BC), olive mill waste compost (OMWC) and green waste compost (GWC). Data are mean (n = 3) ± standard error (SE).

| | **PS** | **BC** | **OMWC** | **GWC** |
|---|---|---|---|---|
| **pH** | 8.69 ± 0.04 | 9.8 ± 0.1 | 9.39 ± 0.01 | 9.14 ± 0.03 |
| **EC (dS $m^{-1}$)** | 0.54 ± 0.02 | 2.4 ± 0.2 | 6.98 ± 0.11 | 1.24 ± 0.04 |
| **OM (%)** | 31.9 ± 0.1 | 75 ± 4 | 71 ± 2 | 74 ± 3 |
| **Pseudo-total element (mg $kg^{-1}$)** | | | | |
| **As** | 0.0095 ± 0.0004 | 0.0010 ± 0.0001 | 0.0071 ± 0.0007 | 0.0064 ± 0.0012 |
| **Cu** | 125.5 ± 2.3 | 12.4 ± 0.3 | 24.8 ± 0.7 | 14.8 ± 0.6 |
| **Zn** | 46.7 ± 0.7 | 32.6 ± 2.1 | 128 ± 5 | 54.0 ± 2.9 |
| **Fe** | 717 ± 23 | 1014 ± 53 | 820 ± 8 | 1307 ± 67 |
| **Mn** | 62 ± 2 | 726 ± 35 | 66 ± 3 | 60.4 ± 2.8 |
| **K** | 46 ± 3 | 521 ± 15 | 2099 ± 14 | 566 ± 13 |
| **Na** | 57 ± 1 | <LOD | 234 ± 3 | 5.9 ± 1.3 |
| **Ca** | 15406 ± 279 | 5567 ± 193 | 2945 ± 75 | 2933 ± 96 |
| **Mg** | 256 ± 8 | 444 ± 16 | 492 ± 20 | 274 ± 8.4 |

<LOD: below the limit of detection

***Experimental design, soil and amendments***

A macrocosm experiment was conducted in the outer part of the *Universidad Autónoma de Madrid* greenhouse. The uncontaminated soil and the contaminated material were collected from El Verdugal (Madrid), the same area described in Chapter 3.1. Soil and contaminated material were directly placed in plastic lysimeters of 120 × 100 × 20 $cm^3$ (length × width × height) in a ratio uncontaminated soil:waste material 9:1 (v:v). The composite soil was thoroughly mixed and homogenised in the lysimeters and left to react for 9 months. The lysimeters were used to simulate field plots, so no replicates were established for each treatment; the different samples (of soil, porewater and plant) collected in each lysimeter were considered as replicates.

*September 2014:* first application of the amendments. The amount of amendment applied was calculated on a dry soil weight basis; the amount of soil in each lysimeter (~120 kg) was calculated taking into account the volume of soil and its bulk density.

The treatments at this moment were:

(1) **Control**: no amendment addition

(2) **Fe+lime**: $FeSO_4$ (1%, w:w) + $CaCO_3$ (0.4%, w.w)

(3) **Fe+PS**: $FeSO_4$ (1%, w:w) + paper mill sludge (PS) (1% w:w)

(4) **Fe+BC**: $FeSO_4$ (1%, w:w) + holm oak biochar (BC) (3% w:w)

(5) **Fe+OMWC**: $FeSO_4$ (1%, w:w) + olive mill waste compost (OMWC) (3% w:w)

(6) **Fe+GWC**: $FeSO_4$ (1%, w:w) + green waste compost (GWC) (3% w:w)

This was considered time 0 and the rest of the described actions will refer to months after this time. A second application of PS, BC, OMWC and GWC was done after 4 months, due to the low soil pH in the corresponding treated soils (Fe+PS, Fe+BC, Fe+OMWC and Fe+GWC). A schedule of the experiment and a description of the samplings and other actions carried out are shown in Table 3.3.2.

**Table 3.3.2.** Experiment schedule. Time refers to months after the treatments application.

| Time (months) | |
|---|---|
| **4** | Soil sampling.<br>The pH of soil-water extracts was analysed and, due to the low values obtained in the treatments Fe+PS, Fe+BC, Fe+OMWC and Fe+GWC (Fig. 3.3.2), a second addition of the organic amendments was planned. |
| **5** | Second addition of the organic amendments, *i.e.* PS, BC, OMWC and GWC.<br><br>The composition of the treatments after this second application was the following:<br><br>(1) **Control**: no amendment addition<br>(2) **Fe+lime**: $FeSO_4$ (1%, w:w) + $CaCO_3$ (0.4%, w:w)<br>(3) **Fe+PS**: $FeSO_4$ (1%, w:w) + paper mill sludge (PS) (2% w:w)<br>(4) **Fe+BC**: $FeSO_4$ (1%, w:w) + holm oak biochar (BC) (5% w:w)<br>(5) **Fe+OMWC**: $FeSO_4$ (1%, w:w) + olive mill waste compost (OMWC) (5% w:w)<br>(6) **Fe+GWC**: $FeSO_4$ (1%, w:w) + green waste compost (GWC) (5% w:w) |
| **7** | Soil sampling for As, Cu and pH analyses |
| **13** | Soil sampling for As fractionation (sequential extraction) |
| **14** | Soil sampling for As, Cu and pH analyses and for *Vibrio fischeri* bioassay.<br>Five rhizon samplers of 10 cm length were placed in each lysimeter. |
| **16** | *Secale cereale* cultivation. |
| **20** | First *S. cereale* harvest.<br>Soil sampling; these soil samples were used for analyses of soil pH, available As, Cu and nutrients and for ecotoxicology tests. |
| **21** | Second *S. cereale* harvest |

***Secale cereale L. cultivation and harvest***

Sixteen months after the first application of the amendments, in late December 2015, ten grams of *S. cereale* (rye) seeds were directly sown in each lysimeter and distributed homogeneously in the soils. The soils were moistened and the plants were left to grow for 6 months (further explained later).

During rye cultivation, soil porewater was collected at 17, 18 19 and 20 months (*i.e.*1, 2, 3 and 4 months after sowing). Porewater pH was immediately measured and samples were filtered through 0.45 μm syringe filters and stored at 4 ºC.

Rye plants were harvested in two moments: at 20 months (4 months after sowing, late April 16) and at 22 months (6 months after sowing, late June 2016). For that, the soil surface was divided into 8 "subplots" of ~25 × 40 cm and the borders, generally with poor vegetation, were avoided (~10 cm); the aboveground part of plants grown in four non-adjacent "subplots" was harvested 4 or 6 months after sowing. A scheme of the harvesting method is shown in Figure 3.3.1.

*First harvest (4 months after sowing)*

These plant samples were collected just before flowering, when the ear had not still developed. Rye stems from non-adjacent "subplots" were cut ~2 cm above the soil surface. The samples were weighed and dried at 65 ºC for 5 days.

*Second harvest (6 months after sowing)*

The plants remaining in the delimited area after the first harvest were collected at their physiological maturity. The harvest was made in the same way as in the first sampling, by cutting the stems ~2 cm above the soil surface. The grains were separated from the ear and weighed to calculate grain yield.

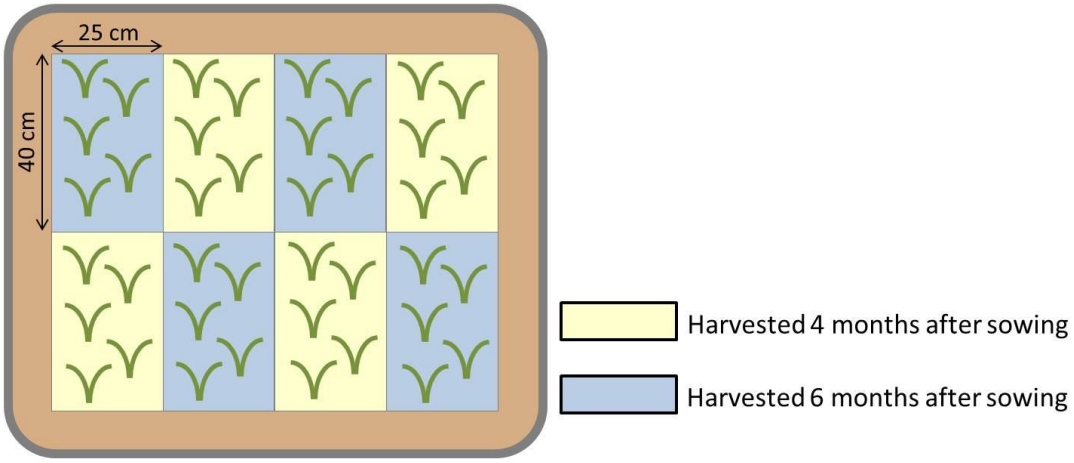


**Figure 3.3.1.** Scheme of the rye harvesting method used in the experiment

***Soil enzymatic activities and ecotoxicity bioassay***

Some soil enzymatic activities (dehydrogenase, β-glucosidase and acid phosphatase) were analysed after *S. cereale* harvest (20 months). For that, soil samples were sieved at <2 mm and stored at 4 ºC.

For the analysis of dehydrogenase activity, soil samples were incubated with 2,3,5-triphenyltetrazolium chloride for 16 h at 25 ºC and darkness. The resulting product, triphenylformazan, was measured spectrophotometrically at 546 nm (Tabatabai, 1994).

Acid phosphatase and β-glucosidase activities were determined by their reaction with the substrates *p*-nitrophenylphosphate and *p*-nitrophenyl-β-glucopiranoside, respectively. Soil samples were incubated with the corresponding substrate for 1 h at 37 ºC and the product, *p*-nitrophenol, was measured spectrophotometrically at 410 nm.

The inhibition of the luminescence of the bacteria *Vibrio fischeri* was used as an indirect exposure bioassay to evaluate the potential toxicity of soil leachates to this organism. The soil used for this assay was collected at 14 and 20 months. To obtain soil leachates, all replicates from each treatment were merged into a composite sample, mixed with deionized water in a ratio 1:10 (w:v) and shaken for 24 h (DIN 38 414-S4). The freeze-dried luminescent bacteria (Biotox$^{TM}$ Kit, Aboatox Oy, Finland) were reconstituted by suspension of an aliquot of the bacteria in NaCl 2% (w/v). Soil leachates were diluted with NaCl 2% (w/v) to achieve concentrations of 0, 12.5, 25, 50 and 100% (v/v). Then, a given volume of each diluted solution was put in contact with the bacteria at 15 ºC and the decrease in the luminescence was measured after 15 and 30 minutes using a luminometer (Optocom I, MGM Instruments).

***Soil and plant analysis***

Soil pH was measured in soil-water extracts (1:2.5 w:v) of samples collected at 4, 7, 13, 14 and 20 months and directly in all porewater samples. The electric conductivity was measured in soil-water (1:5 w:v) extracts of the samples collected at 14 months and in soil porewater samples.

The labile fraction of As and Cu was determined by extraction of soil samples collected at 7, 14 and 20 months with 0.1 M $(NH_4)_2SO_4$ (1:10 w:v).

The pseudo-total element concentration in soils was measured in the samples collected at 13 months. Arsenic fractionation in these samples was determined by a sequential extraction modified from Larios et al. (2012), that was already described in Chapter 3.1. Briefly, the fractions extracted in each step were: F1, readily soluble ($H_2O$, 24 h); F2, strongly adsorbed onto mineral surfaces ($Na_2HPO_4$, 8 h); F3, associated with Al oxyhydroxides ($NH_4F$, 15 h); F4, bound to organic matter ($Na_4P_2O_7$, 16 h); F5, incorporated into amorphous Fe oxyhydroxides (sodium citrate+$NaHCO_3$+ascorbic acid pH 8, 21 h ×2); FR, residual fraction (acid digestion).

The concentration of available nutrients was analysed after *S. cereale* first harvest, 20months. Exchangeable K, Mg, and Ca were determined by soil extraction with 1 M ammonium acetate (pH 7) and P-Olsen by extraction with 0.5 M $NaHCO_3$.

Ground rye biomass (shoots) was acid digested with 5 mL of $HNO_3$ (65% v/v) and 1 mL of $H_2O_2$ (30% v/v) at 125 ºC under a pressure of 1.5 kg $cm^{-2}$ for 30 min (modified from Lozano-Rodríguez et al. (1995)). Rye grains, previously ground into a fine powder, were left overnight with 5 mL of $HNO_3$ (65% v/v) and 1 mL of $H_2O_2$ (30% v/v) and then digested in the same way as shoots.

Arsenic was measured in the $(NH_4)_2SO_4$-extracts by HG-AFS (PS Analytica 10.055, Millenium Excallibur) and Cu by atomic absorption spectroscopy (AAS) (Analyist 800, Perkin Elmer). Cu and As concentration in porewater samples and plant digests was analysed by ICP-MS (Elan 9000 DRCe, Perkin Elmer).

The concentration of Ca, Mg, K and P in soil extracts and plant digests was measured by ICP-OES (ICAP 6500 DUO, Thermo Scientific).

Dissolved organic carbon (DOC) concentration in porewater samples collected at 15 and 20 months was analysed with a TOC analyser (Shimadzu TOC-V CSH).

***Statistical analyses***

The statistical analysis of data was carried out using the program IBM SPSS 21.0. One-way ANOVA analyses were performed upon homogeneity of variances was confirmed. *Post hoc* analyses were carried out to establish differences among treatments; Tukey´s HSD test was used for homoscedastic data and Games-Howell´s for heteroscedastic data. Bivariate correlation and multiple linear regression analyses were used to evaluate relationships between variables.

## 3.3.3. Results

### *Soil pH and As and Cu mobility along the experiment*

Some soil physico-chemical characteristics in each lysimeter are shown in Table 3.3.3.

The evolution of soil pH and the concentration of $(NH_4)_2SO_4$-extractable As and Cu along the experiment is shown in Figure 3.3.2. Soil pH was measured at 4, 7, 14 and 20 months and extractable As and Cu were analysed in samples collected at 7, 14 and 20 months. The statistics associated to these results is shown in Table 3.3.3.

Soil pH was significantly increased ($P < 0.05$) by Fe+lime after 4 months of the first amendment application, whereas no differences were found with Fe+PS and Fe+BC and Fe+GWC. The treatment Fe+OMWC resulted in a significant decrease ($P < 0.05$) in soil pH. Due to this, a second addition of PS, BC, OMWC and GWC was done, which provoked a significant increase ($P < 0.05$) in soil pH of treatments Fe+PS, Fe+BC, Fe+OMWC and Fe+GWC. In general, soil pH in each treatment did not greatly change between 7 and 20 months (Fig. 3.3.2). At 20 months, the pH of all treated soils was significantly higher ($P < 0.05$) than that of the control.

Extractable As was measured at 7, 14 and 20 months (Fig. 3.3.2). At 7 months, the concentration of extractable As was significantly reduced ($P < 0.05$) by the application of Fe+lime, Fe+PS and Fe+GWC compared to the control, whereas no differences were found with Fe+BC and a significant increase was found in Fe+OMWC. A slight decrease in the concentration of extractable As was observed from 7 to 14 months in most treatments. At 14 months extractable As was significantly lower ($P < 0.05$) in Fe+lime and higher ($P < 0.05$) in Fe+PS than in the control, while no differences were found between Fe+BC, Fe+OMWC, Fe+GWC and the control. Between 14 and 20 months all treatments except Fe+PS showed a significant increase in extractable As. No statistical differences were observed between the control and the treated soils at 20 months, likely due to the high variability of the data (high standard error), but extractable As was slightly higher in Fe+PS, Fe+BC, Fe+OMWC and Fe+GWC than in the control.

**Table 3.3.3**. Soil pH, electric conductivity (EC) and pseudototal content of As, Cu, Fe and Zn in each lysimeter (treatment) at 13 months. Mean (n = 4) ± SE.

| | Control | Fe+lime | Fe+PS | Fe+BC | Fe+OMWC | Fe+GWC |
|---|---|---|---|---|---|---|
| **pH** | 4.8 ± 0.1 | 7.10 ± 0.04 | 7.26 ± 0.04 | 7.37 ± 0.06 | 5.76 ± 0.04 | 6.38 ± 0.04 |
| **CE (dS $cm^{-1}$)** | 0.41 ± 0.04 | 0.805 ± 0.002 | 0.95 ± 0.04 | 0.80 ± 0.04 | 0.71 ± 0.05 | 0.69 ± 0.03 |
| **Pseudototal element (mg $kg^{-1}$)** | | | | | | |
| **As** | 10074 ± 733 | 14564 ± 694 | 16699 ± 1206 | 13695 ± 588 | 12211 ± 375 | 10554 ± 269 |
| **Cu** | 333 ± 31 | 528 ± 57 | 621 ± 49 | 506 ± 51 | 530 ± 54 | 367 ± 14 |
| **Fe** | 24680 ± 1189 | 31748 ± 870 | 33060 ± 1474 | 29915 ± 586 | 27940 ± 741 | 27925 ± 394 |
| **Zn** | 104 ± 6 | 126 ± 9 | 147 ± 5 | 120 ± 9 | 101 ± 4 | 127 ± 17 |

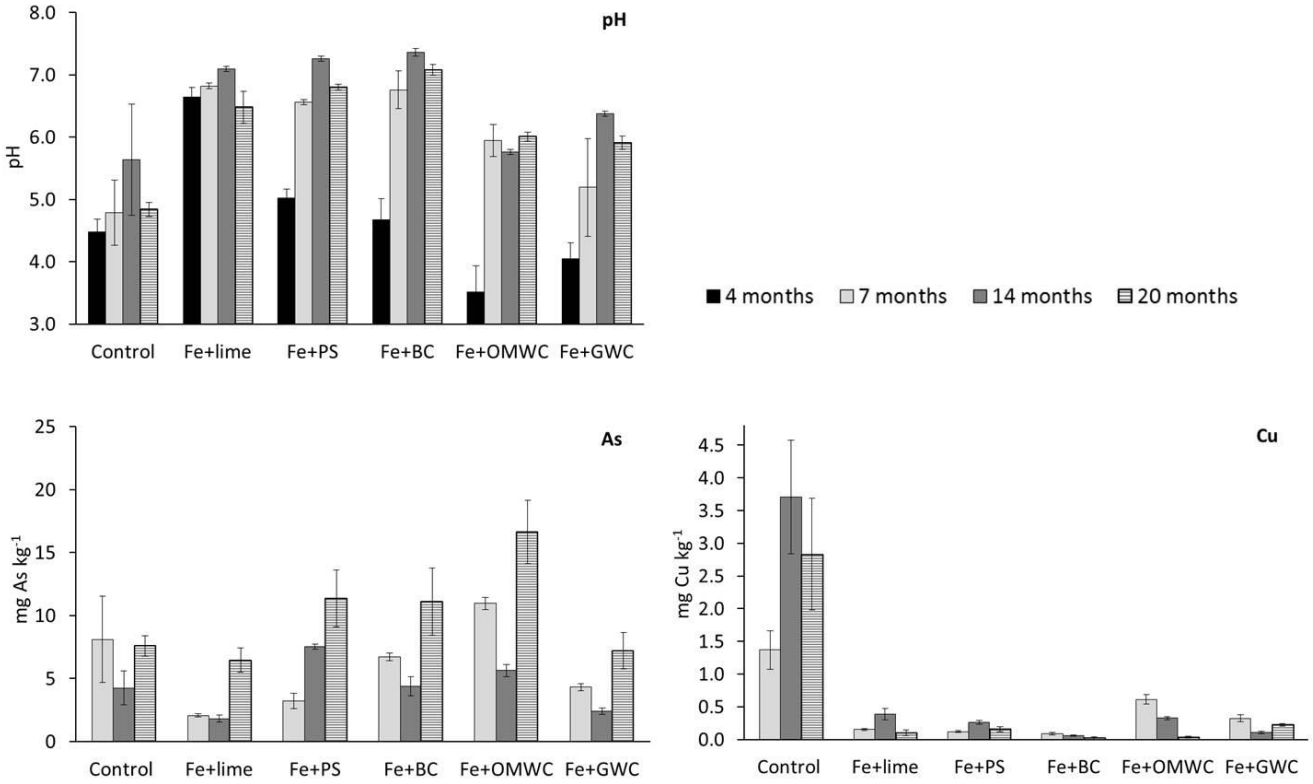


**Figure 3.3.2.** Soil pH and concentration of extractable As and Cu at the different sampling times. Mean (n = 5) ± SE.

**Table 3.3.4.** Statistics associated to soil pH and extractable As and Cu at different samplings (4, 7, 14 and 20 months), shown in **Fig. 3.3.2**. Tukey´s HSD or Games-Howell tests were performed after ANOVA. Different lower case letters in the same column are used to differentiate between treatments for each sampling ($P$ < 0.05) and upper case letters (italics) in the same raw differentiate between samplings for each treatment ($P$ < 0.05).

| | pH | | | | As | | | Cu | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **4 m** | **7 m** | **14 m** | **20 m** | **7 m** | **14 m** | **20 m** | **7 m** | **14 m** | **20 m** |
| **Control** | bc *A* | a *A* | a *A* | a *A* | d *B* | bc *A* | a *B* | e *A* | d *A* | b *A* |
| **Fe+lime** | d *A* | c *A* | b *A* | bc *A* | a *A* | a *A* | a *B* | b *A* | c *B* | a *A* |
| **Fe+PS** | c *A* | c *B* | b *C* | c *B* | b *A* | d *AB* | a *B* | ab *A* | c *B* | a *A* |
| **Fe+BC** | bc *A* | c *B* | b *B* | c *B* | d *AB* | bc *A* | a *B* | a *A* | a *B* | a *A* |
| **Fe+OMWC** | a *A* | bc *B* | a *B* | b *B* | e *AB* | c *A* | a *B* | d *C* | c *B* | a *A* |
| **Fe+GWC** | ab *A* | ab *B* | ab *C* | b *BC* | c *AB* | ab *A* | a *B* | c *C* | b *A* | a *B* |

Copper mobility was reduced ($P < 0.05$) by all the treatments (Fig 3.2.2). The significantly lower ($P < 0.05$) concentration of $(NH_4)_2SO_4$-extractable Cu in the treated soils remained along the experiment, even though there were temporal changes in the treatments. Among the treated soils, the highest Cu concentrations were found in Fe+OMWC and Fe+GWC and generally Fe+BC showed the lowest. No temporal changes in extractable Cu were found in the control, Fe+lime, Fe+PS and Fe+BC, whereas in Fe+OMWC and Fe+GWC Cu decreased over time.

The distribution of As in different soil fractions was studied in order to compare the influence of the amendments on As mobility within the soil. Figure 3.3.3 shows the percentage of As extracted in each step with respect to the total As extracted and the statistics associated to these results is presented in Table 3.3.5. The results are presented as the percentage of As instead of As concentration due to the different concentration of total As among treatments (Table 3.3.3). The recovery of the extraction method was in all cases > 90%.

The treatments only affected the first three fractions (the most available), whereas no statistical differences were found in fractions F4 (As bound to organic matter), F5 (As incorporated into amorphous Fe (hydr)oxides), F6 (As associated with poorly crystalline Fe (hydr)oxides) and FR (As co-precipitated with refractory minerals; residual fraction).

F5 was the most abundant fraction and represented up to 60% of the total As in soils. The sum of F5 and F6, both corresponding to As associated to iron oxides, accounted for more than 75% in all cases.

Similar As concentration was extracted in F1 (readily soluble As) from the control and the treated soils (Fig. 3.3.3). This fraction accounted for less than 0.1% of the total As, but the concentration of As extracted in this step in all treatments was high, between 7.6 and 21.2 mg $kg^{-1}$. The greatest differences in this fraction were observed between Fe+lime (the lowest) and Fe+PS (the highest), but they did not differ to the control.

F2 (strongly adsorbed onto mineral surfaces) represented ~1% of the total As and was significantly increased ($P < 0.05$) by Fe+lime, Fe+PS, Fe+BC and Fe+GWC, whereas Fe+OMWC led to a slight increase, though not significant, of this fraction with respect to the control.

The concentration of As released from F3, was also very low compared to the total As in the soil, as just accounted for less than 1.7% in all the cases. This As fraction was not significantly affected by any treatment, although the largest effect was observed for Fe+PS, which slightly increased this fraction, and Fe+OMWC and Fe+GWC, which led to a slight decrease in F3.

No differences were found in the concentration of As bound to organic matter (F4), even though organic matter was supplied with most of the treatments. The As fractions associated to amorphous and poorly crystalline iron (hydr)oxides, F5 and F6, did not differ among soils, and neither the residual As fraction, FR, which represented less than 15% in all cases.

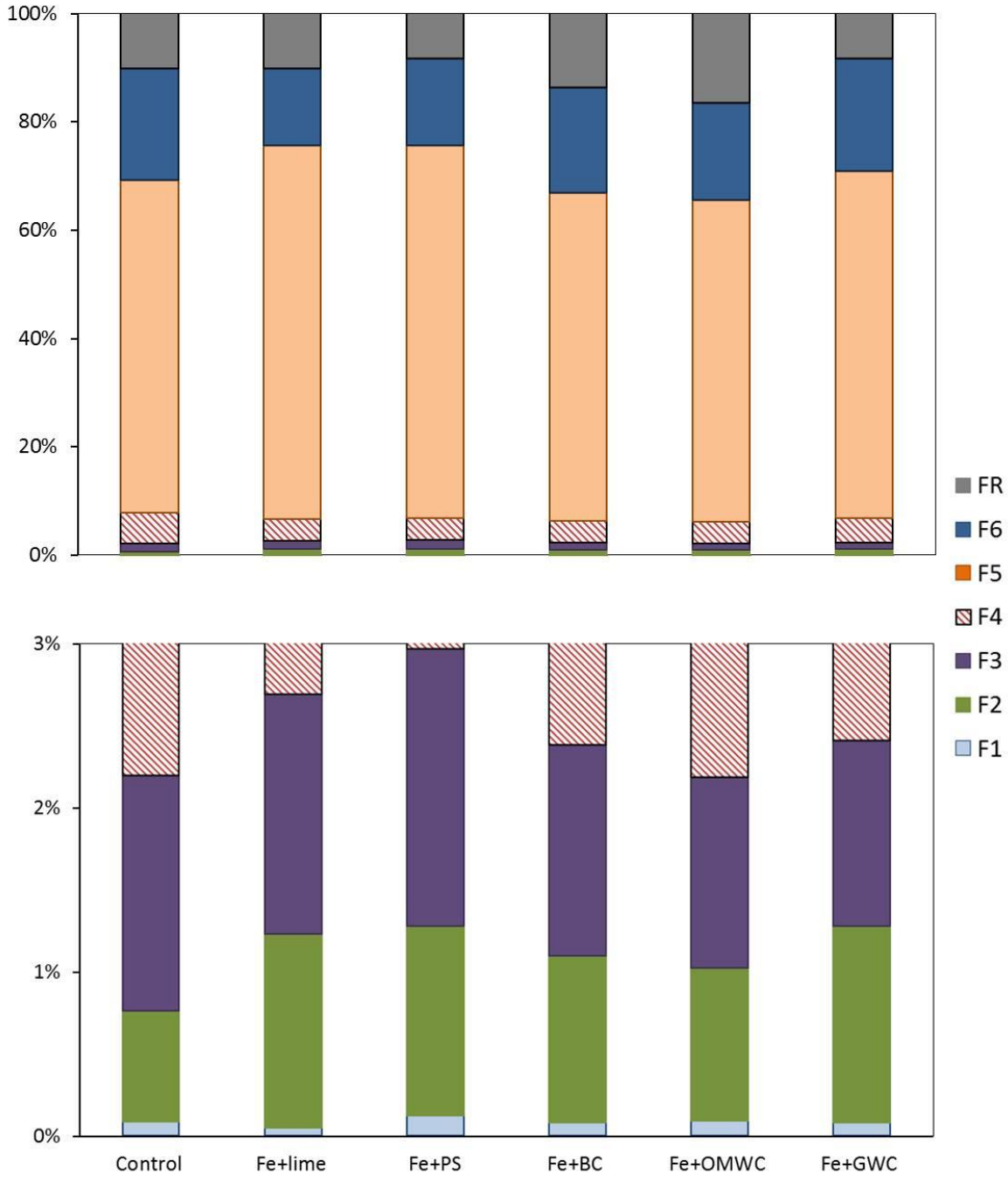


**Figure 3.3.3.** Arsenic distribution (%) within all soil fractions extracted in the sequential extraction (above) and a detail of fractions F1-F3 (below). Means (n = 5).
**F1**: readily soluble; **F2**: strongly adsorbed onto mineral surfaces; **F3**: associated with Al oxyhydroxides; **F4**: bound to organic matter; **F5**: incorporated into amorphous Fe oxyhydroxides; **FR**: residual fraction.

**Table 3.3.5**. Statistics associated to the distribution of As in each step of the sequential extraction (**Fig. 3.3.3**). Different letters in the same column indicate significant differences among treatments for each fraction (Tukey´s HSD test; $P < 0.05$).

| | F1 | F2 | F3 | F4 | F5 | F6 | FR |
|---|---|---|---|---|---|---|---|
| **Control** | ab | a | ab | a | a | a | a |
| **Fe+lime** | a | b | ab | a | a | a | a |
| **Fe+PS** | b | b | b | a | a | a | a |
| **Fe+BC** | ab | b | ab | a | a | a | a |
| **Fe+OMWC** | ab | ab | a | a | a | a | a |
| **Fe+GWC** | ab | b | a | a | a | a | a |

**Table 3.3.6**. Statistics associated to porewater pH and the concentration of As and Cu at different samplings, shown in **Fig. 3.3.4**. Tukey´s HSD or Games-Howell tests were performed after ANOVA. Different letters in the same column indicate significant differences among treatments ($P < 0.05$). Letters in green are significantly different to the control ($P < 0.05$).

| | pH | | | | | As | | | | | Cu | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **15** | **17** | **18** | **19** | **20** | **15** | **17** | **18** | **19** | **20** | **15** | **17** | **18** | **19** | **20** |
| **Control** | a | a | a | a | a | a | b | a | a | a | b | b | b | b | b |
| **Fe+lime** | b | b | b | b | abc | a | ab | a | a | a | a | a | a | a | a |
| **Fe+PS** | c | c | c | bc | d | b | c | b | b | b | a | a | a | a | a |
| **Fe+BC** | c | c | c | c | cd | a | b | a | a | a | a | a | a | a | a |
| **Fe+OMWC** | b | b | b | b | b | a | ab | a | a | a | a | a | a | a | a |
| **Fe+GWC** | b | b | b | b | ab | a | a | a | a | a | a | a | a | a | a |

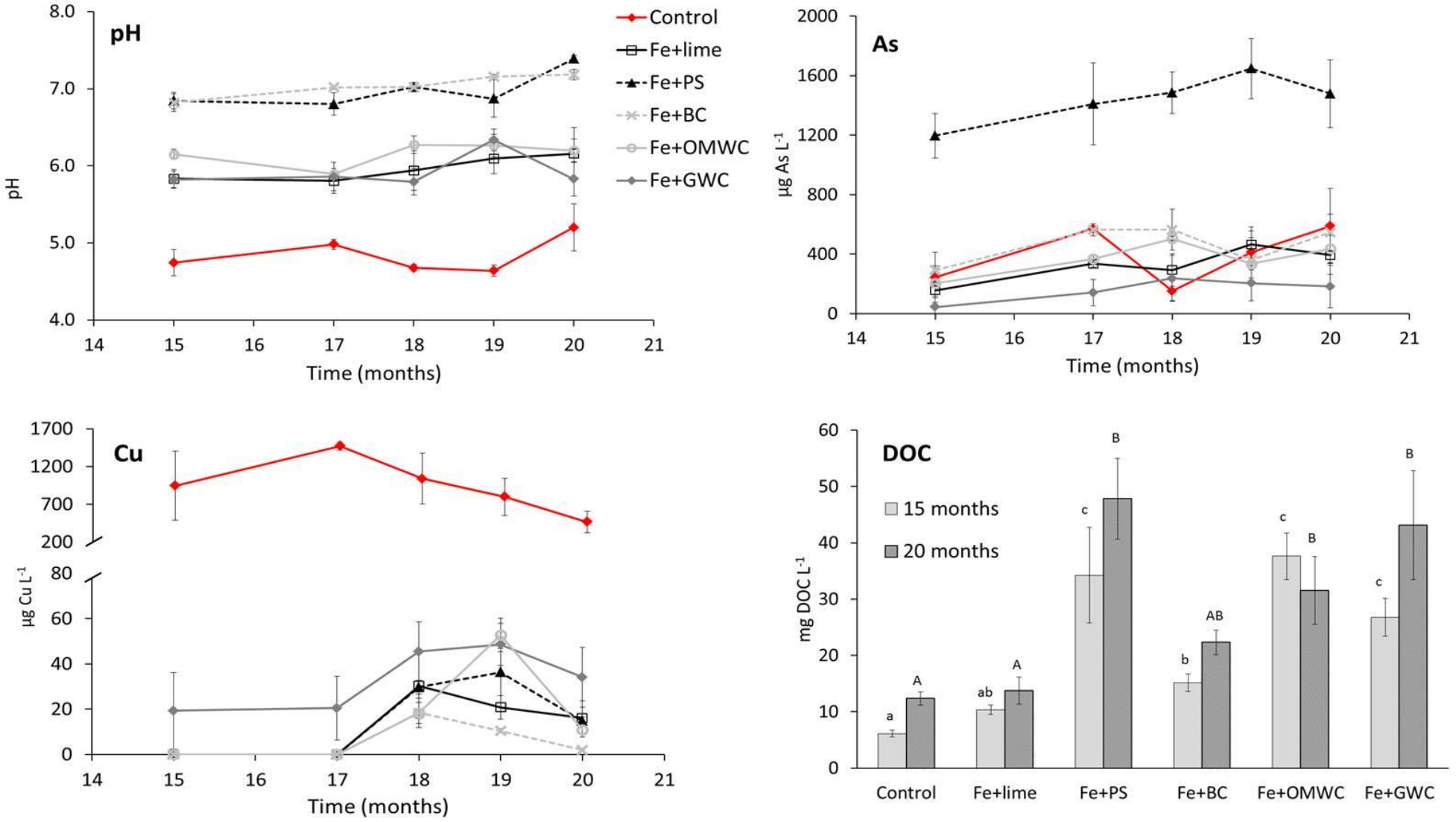


**Fig. 3.3.4.** Soil porewater pH and As and Cu concentration at 15, 17, 18, 19 and 20 months; DOC concentration in porewater at 15 and 20 months after treatments application. Means (n = 5) ± SE.

***Changes in soil porewater chemistry during S. cereale cultivation***

Soil porewater was collected at 15, 17, 18, 19 and 20 months (1 month before and 1, 2, 3 and 4 months after sowing of *S. cereale* seeds). The last samples were collected just before the second harvest. Figure 3.3.4 shows porewater pH values and the concentration of As and Cu throughout the experiment, and DOC concentration at 15 and 20 months (first and last sampling).

Porewater pH was significantly increased by all treatments. The application of Fe+lime, Fe+OMWC and Fe+GWC resulted in a porewater pH between 0.8 and 1.6 units higher than the control, whereas Fe+PS and Fe+BC provoked the greatest increase, which accounted for ~2 pH units throughout the experiment.

Arsenic solubility was not affected by most of the treatments, as only Fe+PS provoked a significant increase ($P < 0.05$) in the concentration of soluble As with respect to the control and the other treatments, that remained along the experiment. In most soils, soluble As remained similar throughout the experiment, although an increasing tendency was observed in most cases.

The concentration of Cu in porewater was significantly reduced ($P < 0.05$) by all treatments throughout the experiment. No statistical differences were found among treated soils, but we observed slightly higher soluble Cu in Fe+GWC at 15 and 17 months, while in the rest of the treatments it was very low. A slight increase in porewater Cu concentration was observed in all the treated soils from 17 months onwards.

DOC concentration in porewater was significantly increased ($P < 0.05$) by Fe+PS, Fe+OMWC, Fe+GWC, but only a slight increase was provoked by Fe+BC. Between 15 and 20 months, DOC slightly increased in all cases.

***Effects on soil fertility and on S. cereale growth and nutritive status***

The effect of the treatments on soil fertility was evaluated by the concentration of exchangeable K, Ca and Mg, extractable P (P-Olsen) and the total contents of organic carbon (TOC) and nitrogen (TN) after the first harvest of *S. cereale* (at 20 months). The results are shown in Table 3.3.7.

The addition Fe+BC, Fe+GWC and Fe+OMWC resulted in a significant increase ($P < 0.05$) in the concentration of exchangeable K, which was 2.4, 5.1 and 2.0 times higher, respectively, than in the control, whereas Fe+lime and Fe+PS had no effect on this nutrient. Exchangeable Ca was significantly increased ($P < 0.05$) by all the treatments. Compared to the control, exchangeable Mg was significantly increased ($P < 0.05$) only by Fe+OMWC, although a slight increase was also observed in Fe+PS, Fe+BC and Fe+GWC. The addition of Fe+BC, Fe+OMWC and Fe+GWC resulted in a significant increase ($P < 0.05$) in the available P concentration, whereas Fe+lime and Fe+PS had no effect. A similar effect was observed for TOC content, which was significantly increased ($P < 0.05$) by Fe+BC, Fe+OMWC and Fe+GWC. The treatment Fe+PS also resulted in a slight increase in TOC, but in a much lesser extent than the other organic amendments, which agrees to the lower OM content (Table 3.3.1). Total nitrogen (TN) content in soils was significantly higher ($P < 0.05$) in Fe+OMWC than in the control. Although Fe+BC and Fe+GWC did not show significant differences with respect to the control, likely due to the variability of the data, TN in these treatments was increased by 31- and 21-fold. Little effect on TN was observed for Fe+lime and Fe+PS.

Rye growth was evaluated by its dry weight before flowering (first harvest, 4 months after sowing) and at the plants physiological maturity (second harvest, 6 months after sowing) (Fig. 3.3.5). Figure 3.3.6 shows how plants looked in each lysimeter just before the first harvest.

As can be observed in Figure 3.3.6, the effects on rye germination success and growth differed among treatments. The amendments Fe+BC, Fe+OMWC and Fe+GWC greatly facilitated plant cover, as rye colonised almost the entire soil surface. On the other hand, Fe+lime and Fe+PS slightly enhanced seed germination, as shown by a poor vegetal cover and empty patches in these lysimeters.

At the first harvest (20 months, 4 months after sowing), Fe+BC, Fe+OMWC and Fe+GWC increased rye shoots biomass by 5.1-, 2.6- and 4.0-fold with respect to the control (Fig. 3.3.5). However, Fe+lime and Fe+PS did not affect plant growth.

All plants increased their biomass between the first and the second harvest, but the difference was greater in Fe+PS, Fe+BC and Fe+OMWC. At 22 months (second harvest), dry weight of plants from all treated soils, except Fe+lime, was higher, although in the case of Fe+GWC the difference was not statistically significant.

The concentration of several macro and micronutrients in rye shoots was evaluated after the first harvest (Table 3.3.8). In general, the treatments did not positively affect the nutrients concentration in plant tissues, as none of them led to a significant increase in the elements analysed. Due to the high variability in plant growth among treatments, the accumulation of these nutrients in shoots was calculated taking into account plants dry weight (Table 3.3.9). The greatest effect was observed for Fe+BC and Fe+OMWC, which significantly increased ($P < 0.05$) the content of K, Ca, Mg, P and Mo in the plant tissues. Fe+GWC significantly increased ($P < 0.05$) K, P, Ni accumulation in rye shoots, and slightly increased Mg and Mo content. Generally, Fe+lime and Fe+PS had little effect on the accumulation of the macro- and micronutrients analysed.

**Table 3.3.7.** Concentration of exchangeable K, Ca and Mg, P-Olsen, total organic carbon (TOC) and total nitrogen (TN) in the control and the treated soils. Mean (n = 4) ± SE. Different letters in the same column indicate significant differences among treatments ($P < 0.05$).

| Treatment | K (mg kg$^{-1}$) | Ca (mg kg$^{-1}$) | Mg (mg kg$^{-1}$) | P (mg kg$^{-1}$) | TOC (g kg$^{-1}$) | TN (g kg$^{-1}$) |
|---|---|---|---|---|---|---|
| **Control** | 48.0 ± 1.9$^{a}$ | 1187 ± 116$^{a}$ | 13.9 ± 2.1$^{ab}$ | 10.6 ± 0.5$^{a}$ | 3.7 ± 0.2$^{a}$ | 0.09 ± 0.03$^{a}$ |
| **Fe+lime** | 46.4 ± 3.0$^{a}$ | 3069 ± 247$^{b}$ | 5.1 ± 0.3$^{a}$ | 8.7 ± 0.0$^{a}$ | 4.1 ± 0.6$^{a}$ | 0.13 ± 0.01$^{a}$ |
| **Fe+PS** | 45.7 ± 2.1$^{a}$ | 4738 ± 295$^{c}$ | 16.4 ± 1.6$^{b}$ | 8.6 ± 0.8$^{a}$ | 10.1 ± 1.0$^{a}$ | 0.26 ± 0.04$^{a}$ |
| **Fe+BC** | 116.0 ± 4.8$^{b}$ | 2646 ± 204$^{b}$ | 16.2 ± 1.5$^{ab}$ | 18.1 ± 1.0$^{b}$ | 36.4 ± 1.9$^{c}$ | 2.78 ± 1.10$^{b}$ |
| **Fe+OMWC** | 243.9 ± 17.5$^{c}$ | 2646 ± 43$^{b}$ | 50.1 ± 4.8$^{c}$ | 29.1 ± 1.2$^{c}$ | 36.2 ± 4.3$^{c}$ | 2.19 ± 0.42$^{ab}$ |
| **Fe+GWC** | 96.2 ± 3.4$^{b}$ | 2918 ± 180$^{b}$ | 21.0 ± 2.7$^{b}$ | 36.1 ± 3.0$^{d}$ | 25.3 ± 3.3$^{b}$ | 1.89 ± 0.36$^{ab}$ |

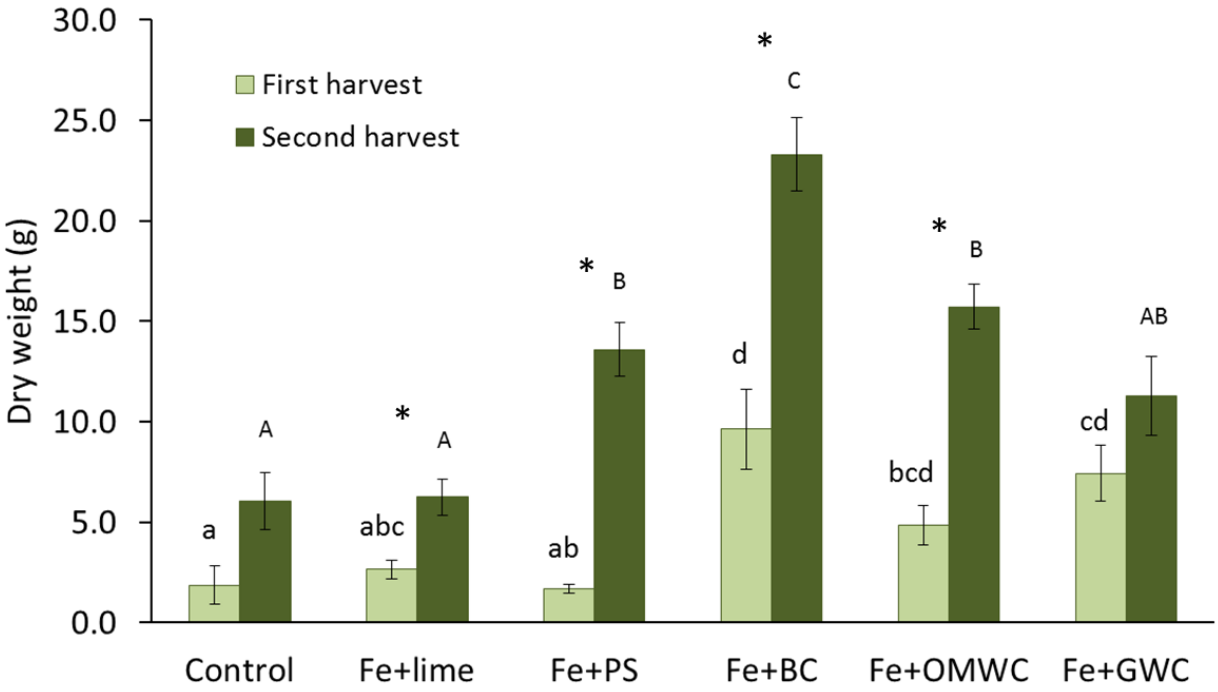


**Figure 3.3.5.** Dry weights of rye plants collected at the first and the second harvest from each lysimeter. Mean (n = 4) ± SE. Different letters indicate significant differences among treatments ($P < 0.05$); lower case letters correspond to the first harvest and upper case letters to the second harvest. The asterisks show where significant differences were found between harvests ($P < 0.05$).

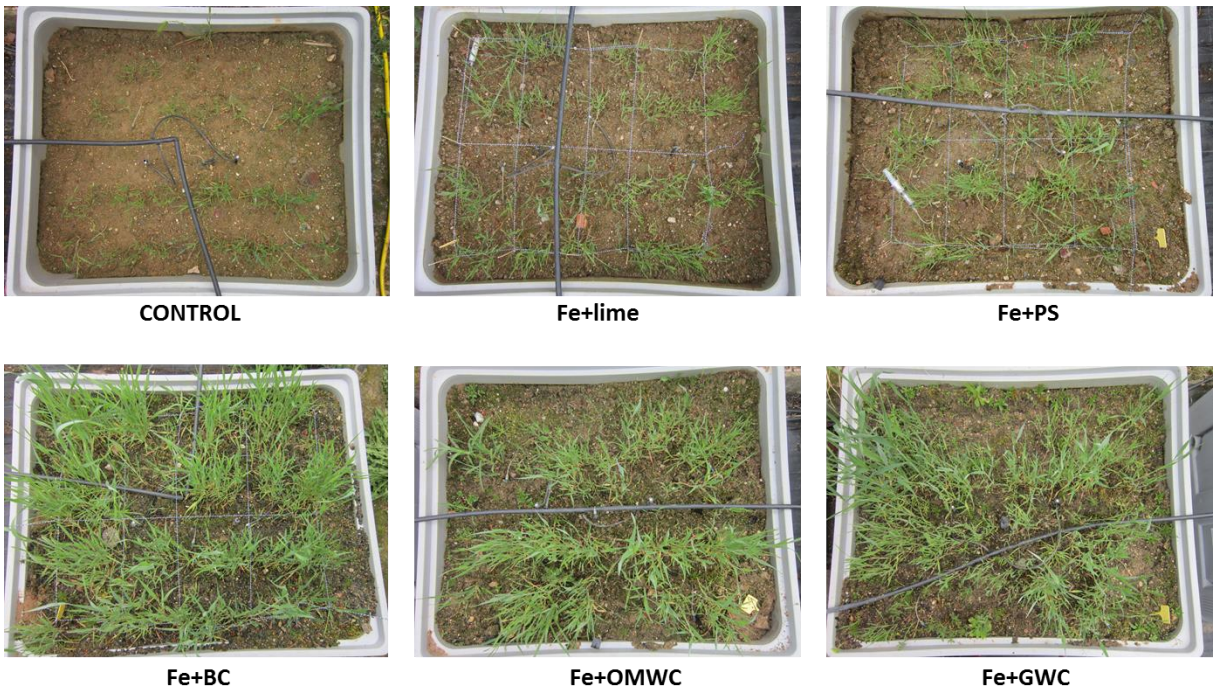


**Figure 3.3.6.** Photograph of each lysimeter before the first harvest, done 20 months after treatments application (4 months after sowing).

**Table 3.3.8.** Concentration of nutrients in *S. cereale* shoots grown in the control and the treated soils for 4 months. Mean (n = 4) ± SE. Different letters in the same column indicate significant differences among treatments ($P < 0.05$).

| Treatment | K (mg $g^{-1}$) | Ca (mg $g^{-1}$) | Mg (mg $g^{-1}$) | P (mg $g^{-1}$) | Fe (mg $kg^{-1}$) | Zn (mg $kg^{-1}$) | Ni (mg $kg^{-1}$) | Mo (mg $kg^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| **Control** | 26.2 ± 5.5$^{a}$ | 3.23 ± 0.72$^{ab}$ | 0.90 ± 0.29$^{a}$ | 2.20 ± 1.24$^{a}$ | 161.7 ± 64.4$^{bc}$ | 38.9 ± 4.2$^{d}$ | 787.3 ± 96.5$^{c}$ | 1.16 ± 0.12$^{a}$ |
| **Fe+lime** | 24.4 ± 0.6$^{a}$ | 3.17 ± 0.30$^{ab}$ | 0.71 ± 0.08$^{a}$ | 1.78 ± 0.18$^{a}$ | 82.3 ± 24.4$^{abc}$ | 17.7 ± 1.4$^{bc}$ | 196.4 ± 6.8$^{a}$ | 0.87 ± 0.09$^{a}$ |
| **Fe+PS** | 26.8 ± 0.4$^{a}$ | 4.81 ± 0.30$^{b}$ | 1.11 ± 0.22$^{a}$ | 1.91 ± 0.52$^{a}$ | 190.8 ± 41.4$^{c}$ | 21.2 ± 1.5$^{c}$ | 366.0 ± 44.4$^{ab}$ | 1.02 ± 0.21$^{a}$ |
| **Fe+BC** | 25.0 ± 1.2$^{a}$ | 2.77 ± 0.22$^{a}$ | 0.74 ± 0.06$^{a}$ | 2.40 ± 0.27$^{a}$ | 35.5 ± 12.5$^{a}$ | 10.0 ± 0.7$^{a}$ | 306.8 ± 51.1$^{a}$ | 0.78 ± 0.09$^{a}$ |
| **Fe+OMWC** | 22.7 ± 1.2$^{a}$ | 2.27 ± 0.12$^{a}$ | 0.79 ± 0.05$^{a}$ | 2.48 ± 0.30$^{a}$ | 35.9 ± 5.1$^{ab}$ | 12.3 ± 0.9$^{ab}$ | 978.2 ± 184.2$^{c}$ | 0.98 ± 0.09$^{a}$ |
| **Fe+GWC** | 24.4 ± 1.8$^{a}$ | 2.27 ± 0.17$^{a}$ | 0.77 ± 0.19$^{a}$ | 2.68 ± 0.31$^{a}$ | 120.2 ± 47.2$^{abc}$ | 12.6 ± 1.2$^{ab}$ | 674.8 ± 150.3$^{bc}$ | 0.94 ± 0.22$^{a}$ |

**Table 3.3.9.** Nutrients content in *S. cereale* shoots grown in the control and the treated soils for 4 months. Mean (n = 4) ± SE. Different letters in the same column indicate significant differences among treatments ($P < 0.05$).

| Treatment | K (mg) | Ca (mg) | Mg (mg) | P (mg) | Fe (µg) | Zn (µg) | Ni (µg) | Mo (µg) |
|---|---|---|---|---|---|---|---|---|
| **Control** | 44.1 ± 21.8 $^{a}$ | 5.3 ± 2.5 $^{a}$ | 1.6 ± 0.9 $^{a}$ | 3.7 ± 1.9 $^{a}$ | 237 ± 95 $^{a}$ | 76.8 ± 40.8 $^{a}$ | 1432 ± 761 $^{ab}$ | 2.5 ± 1.5 $^{a}$ |
| **Fe+lime** | 65.3 ± 12.0 $^{ab}$ | 8.7 ± 2.1 $^{a}$ | 1.9 ± 0.4 $^{ab}$ | 4.8 ± 1.0 $^{ab}$ | 248 ± 118 $^{a}$ | 46.2 ± 6.8 $^{a}$ | 526 ± 104 $^{a}$ | 2.4 ± 0.5 $^{ab}$ |
| **Fe+PS** | 44.5 ± 5.4 $^{ab}$ | 7.9 ± 0.8 $^{a}$ | 1.8 ± 0.2 $^{ab}$ | 3.0 ± 0.1 $^{a}$ | 296 ± 40 $^{a}$ | 35.0 ± 4.8 $^{a}$ | 585 ± 39 $^{a}$ | 3.0 ± 0.3 $^{a}$ |
| **Fe+BC** | 238.9 ± 49.6 $^{d}$ | 27.3 ± 6.9 $^{b}$ | 7.2 ± 1.7 $^{c}$ | 23.5 ± 5.8 $^{c}$ | 394 ± 208 $^{a}$ | 95.3 ± 19.8 $^{a}$ | 2879 ± 708 $^{bc}$ | 7.3 ± 1.1 $^{b}$ |
| **Fe+OMWC** | 171.1 ± 37.8 $^{cd}$ | 17.1 ± 3.7 $^{b}$ | 5.8 ± 1.0 $^{bc}$ | 18.4 ± 3.4 $^{c}$ | 268 ± 58 $^{a}$ | 90.8 ± 15.2 $^{a}$ | 7345 ± 1863 $^{c}$ | 7.0 ± 0.8 $^{b}$ |
| **Fe+GWC** | 119.5 ± 29.0 $^{bc}$ | 11.2 ± 2.5 $^{ab}$ | 3.8 ± 0.9 $^{abc}$ | 13.3 ± 3.3 $^{bc}$ | 568 ± 232 $^{a}$ | 61.8 ± 15.7 $^{a}$ | 3184 ± 745 $^{bc}$ | 4.4 ± 1.0 $^{ab}$ |

***Arsenic and copper accumulation in rye above-ground tissues and grains***

Table 3.3.10 shows the concentration and accumulation of As and Cu in the shoots of rye plants collected at the first and the second harvests, as well as their concentration in grains.

At the first harvest, As concentration in shoots was not greatly affected by the amendments, as only Fe+PS significantly increased ($P < 0.05$) As concentration with respect to the control. The highest reduction was found for Fe+BC, which reduced As concentration in shoots by half. However, due to the differences in rye growth, As accumulation (total content) in the shoots showed a different pattern. Although a significant increase ($P < 0.05$) in total As in shoots was only found in Fe+GWC, its accumulation was slightly enhanced also by Fe+PS, Fe+BC and Fe+OMWC.

The concentration of As in rye shoots did not substantially change between the first and the second harvest, as shown in Table 3.3.10. Only a significant decrease was found in plants grown in Fe+PS, likely due to the increase in their biomass. No significant differences regarding As accumulation were found between the treatments.

The concentration of As in rye grains was calculated on a dry weight basis and varied between 0.32 and 0.71 mg $kg^{-1}$ (Table 3.3.10). All the treatments had a positive effect, as they reduced As concentration in grains with respect to the control. Treatments Fe+BC, Fe+OMWC and Fe+GWC were those which showed the greatest reduction ($P < 0.05$), which accounted for -52%, -41% and -55%, respectively.

In plants collected at the first harvest, Cu concentration was significantly lower ($P < 0.05$) in Fe+BC, Fe+OMWC and Fe+GWC than in the control, although its accumulation was significantly higher in plants from these treatments (Table 3.3.10). However, at the second harvest no differences in Cu concentration were found among treatments, although its accumulation was significantly higher ($P < 0.05$) in Fe+BC and slightly higher in Fe+PS, Fe+OMWC and Fe+GWC than in the control. Compared to the control, Cu concentration in grains was significantly reduced ($P < 0.05$) by Fe+BC, Fe+OMWC and Fe+GWC, while Fe+lime and Fe+PS had no effect.

**Table 3.3.10.** Arsenic and Cu concentration in shoos and grains (mg $kg^{-1}$) and their accumulation (µg) in the shoots of *S. cereale* grown in the control soil and the treated soils for 4 (first harvest) or 6 months (second harvest). Mean (n = 4) ± SE.
Different letters indicate significant differences among treatments ($P < 0.05$); where there are no letters, no statistical differences were found.

| Treatment | First harvest | | | | Second hervest | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $[As]_{shoots}$ (mg $kg^{-1}$) | $As_{shoots}$ (µg) | $[Cu]_{shoots}$ (mg $kg^{-1}$) | $Cu_{shoots}$ (µg) | $[As]_{shoots}$ (mg $kg^{-1}$) | $As_{shoots}$ (µg) | $[As]_{grain}$ (mg $kg^{-1}$) | $[Cu]_{shoots}$ (mg $kg^{-1}$) | $Cu_{shoots}$ (µg) | $[Cu]_{grain}$ (mg $kg^{-1}$) |
| **Control** | 13.0 ± 2.0 $^{a}$ | 28.9 ± 7.2 $^{a}$ | 10.8 ± 1.3 $^{cd}$ | 34.4 ± 9.3 $^{a}$ | 12.9 ± 1.4 $^{b}$ | 75.5 ± 14.4 | 0.71 ± 0.13 $^{b}$ | 7.4 ± 0.9 | 30.3 ± 2.3 $^{a}$ | 7.3 ± 0.1 $^{c}$ |
| **Fe+lime** | 16.7 ± 6.1 $^{ab}$ | 24.0 ± 4.1 $^{a}$ | 8.5 ± 0.3 $^{bcd}$ | 88.4 ± 16.8 $^{b}$ | 11.6 ± 1.6 $^{b}$ | 70.2 ± 9.3 | 0.46 ± 0.04 $^{ab}$ | 5.0 ± 0.4 | 30.5 ± 4.0 $^{a}$ | 6.5 ± 0.3 $^{bc}$ |
| **Fe+PS** | 41.7 ± 8.6 $^{b}$ | 65.2 ± 9.3 $^{ab}$ | 12.3 ± 1.7 $^{d}$ | 82.9 ± 21.2 $^{ab}$ | 8.5 ± 1.9 $^{ab}$ | 92.2 ± 21.9 | 0.44 ± 0.06 $^{ab}$ | 6.1 ± 1.1 | 79.4 ± 8.6 $^{ab}$ | 7.1 ± 0.2 $^{c}$ |
| **Fe+BC** | 6.3 ± 1.0 $^{a}$ | 48.9 ± 9.2 $^{ab}$ | 4.6 ± 0.2 $^{a}$ | 175.0 ± 40.1 $^{b}$ | 5.2 ± 0.9 $^{a}$ | 117.6 ± 19.1 | 0.34 ± 0.01 $^{a}$ | 4.0 ± 0.6 | 95.8 ± 20.5 $^{b}$ | 5.2 ± 0.3 $^{a}$ |
| **Fe+OMWC** | 8.1 ± 1.1 $^{ab}$ | 40.0 ± 9.2 $^{a}$ | 5.8 ± 0.8 $^{ab}$ | 162.8 ± 28.4 $^{b}$ | 6.3 ± 0.8 $^{a}$ | 74.2 ± 21.3 | 0.42 ± 0.4 $^{a}$ | 5.3 ± 1.2 | 81.8 ± 17.0 $^{ab}$ | 6.0 ± 0.2 $^{ab}$ |
| **Fe+GWC** | 19.4 ± 5.5 $^{ab}$ | 135.8 ± 32.0 $^{b}$ | 7.1 ± 0.4 $^{abc}$ | 129.0 ± 17.0 $^{b}$ | 4.8 ± 0.4 $^{a}$ | 56.8 ± 13.7 | 0.32 ± 0.02 $^{a}$ | 6.6 ± 0.1 | 74.7 ± 13.6 $^{ab}$ | 5.9 ± 0.2 $^{ab}$ |

***Soil enzymatic activities and toxicity towards Vibrio fischeri***

Results of the activities of dehydrogenase, acid phosphatase and β-glucosidase in the control and the treated soils are shown in Figure 3.3.7.

Treatments Fe+lime, Fe+PS, Fe+OMWC and Fe+GWC significantly increased ($P < 0.05$) dehydrogenase activity, the two latter showing the greatest effect. However, no significant differences were found between the control and Fe+BC-treated soil.

With respect to the control, acid phosphatase activity was significantly lower ($P < 0.05$) in Fe+PS and Fe+BC and significantly higher ($P < 0.05$) in Fe+GWC, whereas Fe+lime and Fe+OMWC had little effect on this enzyme activity.

Only Fe+GWC and Fe+BC significantly affected ($P < 0.05$) β-glucosidase activity; an increase was observed in the former while the latter provoked a decrease. The addition of the other treatments did not greatly affect this enzyme activity.

The effect of soil leachates on the luminescent bacteria *Vibrio fischeri* was assessed for the control and the treated soils sampled at two moments, 14 and 20 months, the last one corresponding to the first *S. cereale* harvest. Results obtained are shown as the percentage of soil leachates that caused a reduction of 50% on the luminescence of the bacteria ($EC_{50}$, Table 3.3.11). At the first sampling time (14 months), leachates from control soil resulted to be more toxic for *V. fischeri* than those from all the treated soils, as shown by the lowest $EC_{50}$ values. Treatments Fe+lime, Fe+PS and Fe+BC had similar effect and the best results were obtained for Fe+OMWC, which increased the $EC_{50}$ by 2.4 and 2.9 times after 15 and 30 minutes of exposure, respectively. At the second sampling, at 20 months, $EC_{50}$ values obtained for control, Fe+lime and Fe+PS soils were higher than at 14 months, whereas those for Fe+BC and Fe+GWC remained similar and even decreased for Fe+OMWC.

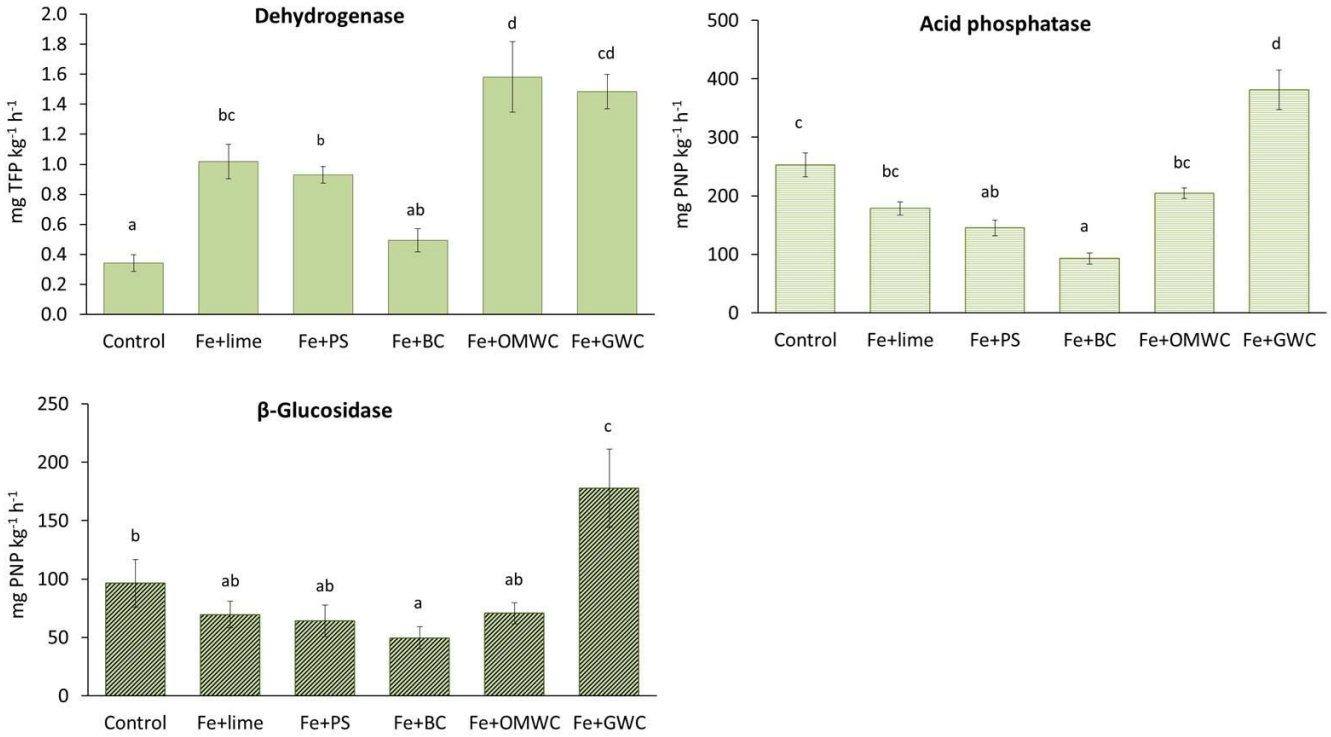


**Figure 3.3.7.** Dehydrogenase, acid phosphatase and β-glucosidase activities in the control and the treated soils, represented as the amount of product (TPF or PNP) formed after 1 hour of soil and substrate incubation. Mean (n = 5) ± SE. Different letters indicate significant differences among treatments ($P < 0.05$).

**Table 3.3.11.** $EC_{50}$ values obtained for the inhibition of the luminescence of *Vibrio ficheri* after 15 or 30 minutes of exposure to soil extracts (obtained from a composite sample prepared with all the replicates per treatment). The assay was performed with soil samples collected at 14 and 20 months.

| | 14 months | | 20 months | |
|---|---|---|---|---|
| **Treatment** | **$EC_{50}$ (%) $_{t=15}$** | **$EC_{50}$ (%) $_{t=30}$** | **$EC_{50}$ (%) $_{t=15}$** | **$EC_{50}$ (%) $_{t=30}$** |
| **Control** | 24.6 | 20.9 | 43.1 | 41.4 |
| **Fe+lime** | 41.1 | 37.3 | 54.6 | 86.7 |
| **Fe+PS** | 47.7 | 40.2 | 95.7 | 117.9 |
| **Fe+BC** | 40.1 | 37.4 | 44.4 | 45.8 |
| **Fe+OMWC** | 60.1 | 60.3 | 42.2 | 34.3 |
| **Fe+GWC** | 31.0 | 30.6 | 42.2 | 34.3 |

## 3.3.4. Discussion

***Effect of treatments and time on As mobility***

Although a stabilising effect of iron amendments on As has been repeatedly observed both in short and long term studies (Hartley et al., 2004; Kumpiene et al., 2006; Cutler et al., 2014), in this work the addition of iron sulphate to the contaminated soil in a rate of 1% (w:w), combined with other materials, did not result in a great effect on As over two years. The treatments had different effects on $(NH_4)_2SO_4$-extractable As, its distribution within several soil fractions and its soluble fraction, but in general they did not significantly altered As mobility and availability towards the end of the experiment.

Concerning the effect on the $(NH_4)_2SO_4$-extractable As fraction (Fig. 3.3.2), the treatments behaved differently over time. Whereas at 7 months several treatments significantly affected extractable As relative to the control (it was reduced by Fe+lime, Fe+PS and Fe+GWC and increased by Fe+OMWC), at 14 months only Fe+lime and Fe+PS reduced and increased it, respectively. The difference between the control and the treatments was mitigated at 20 months, when no significant differences were found among them, although a slight increase in extractable As was observed Fe+PS, Fe+BC and Fe+OMWC (Fig. 3.3.2).

One factor that might play an important role on As mobility is soil pH, and in general it can be stated that, within the common pH range found in soils (3-8), arsenate solubility increases with increasing soil pH (Smith et al., 1999; Fitz and Wenzel, 2002) .However, although most of the treatments significantly increased soil pH (Fig. 3.3.2, Table 3.3.4), it did not correlate with extractable As at any sampling time, suggesting that soil pH did not strongly influence As availability.

Other factors, such as DOC and available P, may strongly influence As mobility (Tao et al., 2006; Beesley et al., 2010; Moreno-Jiménez et al., 2013). As we only had sufficient data to establish relationships between these variables at 20 months, a linear regression was performed with data from that time in order to evaluate the influence of several factors on extractable As. For that, we selected total As, soil pH, available P (P-Olsen) and DOC in porewater as variables.

$$[As]_{ext,\ 20m} = 6.101 + 0.136\ [DOC]_{PW,\ 20m} \quad R^2 = 0.169 \quad F_{1,29} = 5.7 \quad P < 0.05 \quad \text{(Eq. 3.3.1)}$$

The regression equation obtained (Eq. 3.3.1) shows that, among all the variables tested, DOC was the factor that could explain the variations in extractable As between

treatments. This can be expected, as the addition of soluble organic carbon has been shown to provoke the release of labile As forms, even if soil presents high As adsorption capacity (Bauer and Blodau, 2006; Arco-Lázaro et al., 2016).

Results of soluble As somehow mirrored that observed in the extractable As, as most of the treatments had little effect on As concentration in porewater from 15 to 20 months. Only Fe+PS provoked a significant increase in As solubility with respect to the control and the other treatments. A significant positive correlation was found between pH and As in porewater at all sampling times (15 months: r = 0.439, $P < 0.05$; 17 months: r = 0.392, $P < 0.05$; 18 months: r = 0.651, $P < 0.001$; 19 months: r = 0.355, $P < 0.05$; 20 months: r = 0.565, $P < 0.01$), which could partly explain the effect observed for Fe+PS. Since this treatment provoked a significant increase in porewater pH relative to the other treatments (similar to Fe+BC), besides a significant increase in DOC, a synergistic effect of pH and DOC on As solubility could have occurred. An increase in porewater pH may decrease the metal oxides positively-charged surface area, thus competition of soluble organic anions with arsenate for sorption sites could be enhanced, resulting in As mobilisation (Bauer and Blodau, 2006).

One possible explanation to the lack of As stabilisation found in this experiment, in contrast to that found in previous ones (Chapters 3.1 and 3.2), could be the heterogeneous distribution of the newly formed iron oxides in the soil. Whereas in pot experiments the amendments can be well homogenised with the soil if both are thoroughly mixed, this is difficult to achieve with such large amount of soil present in each lysimeter (~120 kg).

One interesting finding regarding As mobility was the increase in extractable As observed in all treatments, including the control, between 14 and 20 months (Fig. 3.3.2). Several factors may affect the behaviour of As in the long-term when iron is used as an immobilising soil additive (Kumpiene et al., 2007).

Since generally little changes were observed in soil pH along the experiment, this did not seem to be a determining factor on the mobilisation of As found between 14 and 20 months. Kumpiene et al. (2007) also observed that pH as a single factor had small influence on As mobility over time in a zero valent iron-stabilised soil. It is known that organic matter may mobilise As associated to iron oxides (Bauer and Blodau, 2006), however in this experiment an increase in extractable As was found in all treatments, regardless of the higher or lower DOC concentration provoked by the treatments (Fig. 3.3.3). Indeed, the greatest As mobilisation between 14 and 20

months occurred in the treatment Fe+lime (extractable As increased by > 70%), which was not related to the greatest increase in DOC.

The sequential extraction performed at 13 months showed that most of the As was associated to amorphous iron oxides in the control and all treated soils (Fig. 3.3.3). The transformation of amorphous iron oxides into more crystalline phases over time results in a decrease in the density of sorption sites, which lowers the binding capacity of these soil components and may result in As mobilisation (Dixit and Hering, 2003; Kumpiene et al., 2012). In this study, the percentage of As released from amorphous (F5) and crystalline iron oxides (F6) was similar in the control and the treated soils and accounted for 59-69% and 14-20%, respectively, (Fig. 3.3.3). However, in a similar but shorter experiment (Chapter 3.1, Fresno et al. (2016)), we observed that the addition of iron sulphate increased the proportion of As associated to amorphous iron (hydr)oxides from 52% to >80% when compared to the untreated soil. This could indicate that ageing of amorphous iron oxides in the treated soils had occurred in the present study, increasing their crystallinity and leading to similar partitioning of As in both iron (hydr)oxides fractions in the control and the treated soils. Thus, it can be expected that this process would continue over time in all soils (including the control), hence inducing the mobilisation of As from this to more easily extractable forms.

In addition, other factors that are difficult to control in this type of experiments where climatic conditions are not controlled, such as changes in temperature and soil humidity (rainfall), may also affect As mobility. Indeed, Kumpiene et al. (2007) reported that the liquid-to-solid ratio was an influential factor in As release from iron-treated soil.

***Effect of treatments on Cu mobility***

Relative to the control, all the treatments effectively reduced the concentration of extractable Cu throughout the experiment (Fig. 3.3.2). Results of Cu concentration in soil porewater mirrored those of extractable Cu, as all treatments effectively reduced Cu solubility (Fig. 3.3.3).

Copper mobility in soils is highly dependent on pH and, generally, an increase in soil and porewater pH may result in precipitation of Cu hydroxides, besides increasing the negatively-charge surface area of metal oxides, which can enhance the adsorption of free and complexed Cu (Soler-Rovira et al., 2010). Based on the significant negative correlation between soil pH and extractable Cu (7 months: r = -0.729, $P <$

0.001; 14 months: r = -0.565, $P < 0.01$) and between porewater pH and soluble Cu concentration (r ≥ -0.520, $P < 0.01$ at all samplings), the increase in pH provoked by the treatments seemed to govern Cu mobility in this experiment.

Soil organic matter may also control Cu mobility through the formation of stable complexes with functional groups (Zhou and Wong, 2001; Soler-Rovira et al., 2010). If DOC concentration increases by the addition of organic amendments such as compost, it may result in an increase in Cu solubility (Beesley and Dickinson, 2009). In this study, the increase in DOC in the porewater of treatments Fe+PS, Fe+OMWC and Fe+GWC (Fig. 3.3.4) did not result in an increase in soluble Cu concentration (Fig. 4). Since metal (Fe, Al, Mn) oxides are generally good sinks for Cu (Kumpiene et al., 2008), the formation of supplemental iron (hydr)oxides upon addition of iron sulphate could have mitigated the effects of DOC by enhancing the adsorption of Cu-OM complexes onto iron (hydr)oxides surface. This would be in agreement to that pointed out by Tiberg et al. (2016), who confirmed by modelling Cu species in soil solution that the percentage of Cu complexed by metal (hydr)oxides increases over that associated to soluble organic matter at increasing soil pH in a zero valent iron-treated soil. In addition, an increase in soil pH can enhance the soil cation exchange capacity (CEC), thus favouring Cu retention.

Despite Cu concentration in porewater remained low along the experiment in all treated soils, a slight increase in Cu solubility was found during rye cultivation (Fig. 3.3.3). It is well known that graminaceous plants are able to exudate phytosiderophores to deal with micronutrients deficiency, as these compounds may form stable complexes with metals such as Fe, Zn and Cu, mobilising them from sparingly soluble pools (Kidd et al., 2009). Therefore, the increase observed in Cu solubility along rye cultivation could have been caused by the release of these compounds from rye roots. Copper is a micronutrient and, even though its concentration in the control soil porewater was high and should be lowered by applying stabilising amendments, the interaction between Cu and the amendments should be labile enough to enable its bioavailability.

***Effect of the treatments on nutrients availability, plant growth and trace elements uptake***

The best results in terms of nutrients availability were obtained in soils treated with Fe+BC, Fe+OMWC and Fe+GWC. These amendments resulted in a significant increase in available K, Ca and P and TOC and TN contents in soil (Table 3.3.7). However, generally no differences were found between the control and the Fe+lime- and Fe+PS-treated soils (Table 3.3.7). Organic amendments generally improve soil fertility due not only to the supply of available nutrients, but also to the improvement of soil physico-chemical properties such as soil CEC, porosity and water-holding capacity (Walker and Bernal, 2008; Chan et al., 2007; Biederman and Stanley Harpole, 2013; Cellier et al., 2014) and have shown even better results in terms of soil fertility and plant growth than inorganic fertilisation (Álvarez-López et al., 2016).

The enhancement of nutrients availability provoked by Fe+BC, Fe+OMWC and Fe+GWC was reflected in the nutritional status of rye plants (Table 3.3.9), and hence in a greater above-ground biomass (Figs. 3.3.5 and 3.3.6). The best results in terms of nutrients uptake by rye were obtained for Fe+BC and Fe+OMWC, which significantly increased K, Ca, Mg and P content in shoots and slightly increased some micronutrients uptake such as Zn, Ni and Mo. Nutritional status of plants grown in Fe+lime and Fe+PS mirrored that observed in terms of soil nutrients availability, as these treatments had little effect with respect to control plants. The treatment Fe+PS also provoked an increase in shoots biomass harvested at 22 months (Fig. 3.3.5), although this can hardly be attributed to a nutritional effect. Due to the treatments did not greatly affect As availability and that rye dry weights were not correlated with soluble and extractable As, the improvement in plant growth was likely rather related to the effects of the treatments on soil fertility than to As availability. The most promising results in terms of plant cover and growth were found for Fe+BC (Figs. 3.3.5 and 3.3.6).

Rye has shown low translocation of As from roots to shoots and grains (Álvarez-Ayuso et al., 2016), which makes it a suitable species for (aided) phytostabilisation strategies in As-contaminated soils.

Similar to that observed on the extractable As fraction in soil, the treatments did not have a great effect on As uptake (Table 3.3.10). At the first harvest, only Fe+PS significantly increased As concentration in shoots, which was likely related to higher As solubility in this treatment, based on the significant positive correlation between As concentration in porewater at 20 months and As shoots concentration ($r = 0.548$, $P <$

0.01). Nevertheless, the negative effect of this treatment was mitigated with time, likely due to plant growth between both harvests (Fig. 3.3.5). Results of As shoot concentration in plants collected at the second harvest showed that Fe+BC, Fe+OMWC and Fe+GWC reduced As concentration in shoots. Since phosphate can inhibit As uptake (Zhao et al., 2009a), it could be expected that the higher phosphate availability in Fe+BC, Fe+OMWC and Fe+GWC can play a role in the decrease in As concentration in plant tissues. However, as similar total As accumulation was found in all cases, the positive effect of these treatments seems to be more related to their effect on plant growth.

The addition of Fe+BC, Fe+OMWC and Fe+GWC resulted in a reduction of As concentration in grains. Since the translocation of As from the shoots to the grains seemed to be similar in all cases (data not shown) and it was not correlated neither with extractable nor soluble As, the effect on As concentration in grains is more related to As concentration in shoots than on As availability.

Rye straw can be used for animal feed and its grains are used for bread making, thus it is important to evaluate the risks associated to its consumption. In all cases, the concentration of As in the above-ground biomass exceeded the tolerable limit for animal feed established at 2 mg $kg^{-1}$ (Directive 2002/32/EC). Arsenic concentration in grains is also above the limit recently established for children feed at 0.25 mg $kg^{-1}$ in rice (Commission Regulation UE 2015/1006). Therefore, the treatments did not reduce As levels in rye above-ground organs sufficiently to meet the current legal limits.

Since Cu concentration in rye shoots did not exceed the toxic limits established for most plant species, above 20 to 30 mg $kg^{-1}$ (Marschner, 2012), As was the contaminant of major concern in this experiment. In any case, Cu concentration in shoots from the first harvest (20 months) was significantly reduced by Fe+BC, Fe+OMWC and Fe+GWC, although it was not affected by any treatment at the end of the experiment (second harvest, 22 months) (Table 3.3.10). However, the lower Cu concentration in plants from these treatments was a consequence of their positive effect on plant growth, as its accumulation in shoot tissues was higher than in control plants.

***Effects on soil health parameters***

Soil enzymatic activities are powerful tools to evaluate a soil remediation process, as they are sensitive to changes in soil management and the presence of pollutants and provide a general overview of the soil health status (Alkorta et al., 2003; Alvarenga et al., 2009; Pardo et al., 2014b).

Dehydrogenase is an intracellular oxidoreductase that reflects oxidative activities of soil microbial community and hence its activity can be used as an indicator of viable microbial activity (Alkorta et al., 2003). Many authors have shown the suitability of this enzymatic activity to evaluate the benefits of soil amendments on soil health (García-Gil et al., 2000; Pardo et al., 2014b; Manzano et al., 2014).

In this work most treatments gave rise to an increase in dehydrogenase activity (Fig. 3.3.7), which is in agreement with that observed in terms of soil pH, Cu availability and plant growth. The treatments Fe+OMWC and Fe+GWC gave rise to the greatest increase; as this enzyme is positively correlated with C mineralisation (Ouyang et al., 2014), an increase in its activity generally occurs when organic amendments are applied to soils (García-Gil et al., 2000; Crecchio et al., 2001; Pardo et al., 2014b).

One interesting finding concerning dehydrogenase activity was the difference between effects of composts and biochar: although BC, OMWC and GWC had a high and similar content of organic matter (Table 3.3.1) and significantly increased TOC (Table 3.3.7), the addition of Fe+BC had little effect on dehydrogenase activity. Some authors have reported similar results; Elzobair et al. (2016) observed a lack of microbial activity response to biochar amendment, in contrast to the enhancement observed when manure was applied. The authors suggested that either organic C supplied by biochar was not available for microbial degradation or the labile biochar C source was degraded prior sampling. Similarly, when assessing biochar as a soil amendment in several field plots, Ameloot et al. (2014) suggested that this material probably does not function as substrate for the soil microbial community in the medium to long term. Moreover, it has been pointed out that woodchip biochar, in comparison to biochar made with daily manure, generally has higher surface specific area and can sorb organic substrate, reducing its availability for microbial mineralization (low soil enzymes activity) (Ouyang et al., 2014). Besides, higher pyrolysis temperature used to produce biochar generally results in slower C mineralization in the soil (Ameloot et al., 2014). Given that we used holm oak woodchips biochar pyrolysed at 600 ºC, our results support the idea that organic C

supplied by biochar would be more stable than that supplied by compost. Yet, further research is needed to confirm and give light to such findings.

Phosphatase is a hydrolase enzyme involved in the P cycle; it releases available phosphate from organic matter and can be used as an indicator of soil health and organic matter quality (Alkorta et al., 2003; Pardo et al., 2014a). However, it has been suggested that phosphatase activity is not a good choice to assess toxic effects of trace elements, especially in As-contaminated soils, as it has shown to be stimulated with the presence of arsenate (Lyubun et al., 2013).

In this work, acid phosphatase activity was increased only by Fe+GWC, whereas it was not significantly affected by Fe+lime and Fe+OMWC, and decreased in Fe+PS and Fe+BC (Fig. 7) with respect to the control. Similar to that observed by Pardo et al. (2014a), a significant negative correlation was found between soil pH and phosphatase activity at 20 months ($r = -0.588$, $P < 0.01$), suggesting that the increase in soil pH provoked by most of the treatments could have negatively affected this soil enzyme activity (Alvarenga et al., 2008).

As has been previously reported, an increase in available P can result in a decrease in phosphatase activity (Epelde et al., 2009). This would agree with the fact that in Fe+BC and Fe+OMWC the available P pool was significantly higher in the control soil (Table 3.3.7), while phosphatase activity was lower (Fig. 3.3.7). However, as the Fe+GWC resulted in the highest concentration of available P (Table 3.3.7) but also led to a significant increase in the acid phosphatase activity, it cannot be stated that an increase in available P impaired acid phosphatase in this experiment. Nevertheless, the opposite effect could have occurred, *i.e.* a higher available P in Fe+GWC could be a consequence of the high phosphatase activity. In any case, this enzyme activity did not provide much information about the different effects of the treatments and was not well correlated to nutrients availability and plant growth.

The hydrolase β-glucosidase is related to the C cycle involved in the organic matter mineralization (Alkorta et al., 2003). The response of this enzyme activity to the treatments was similar to that of acid phosphatase. Whereas Fe+lime, Fe+PS and Fe+OMWC did not significantly affect β-glucosidase activity, Fe+BC and Fe+GWC led to a significant decrease and increase, respectively, relative to the control.

Again, Fe+BC had a negative effect on an enzymatic activity closely related to the OM. Since, as dehydrogenase, β-glucosidase has shown to be closely related to organic C mineralization (Ouyang et al., 2014), it is not surprising that both enzyme activities showed similar response to BC, suggesting again high stability of organic C

provided by biochar in a medium/long term. In contrast to that observed here, Pardo et al. (2014b) found that addition of OMWC increased β-glucosidase activity. The different results obtained for Fe+OMWC and Fe+GWC suggest either a higher stability of OM from OMWC that that from GWC, or that labile C supplied by OMWC was more rapidly available for soil microorganisms than that supplied by GWC.

Indirect toxicity assays using soil leachates, such as the inhibition of the luminescence of the marine bacteria *V. fischeri,* can provide further information about the risk of groundwater contamination (Alvarenga et al., 2008; Manzano et al., 2014a; Pardo et al., 2014b).

At 14 months, all treatments resulted in a decrease in the toxicity of soil leachates towards *V. fischeri*, showing OMWC the best results (Table 3.3.11). However, this effect changed over time. Whereas Fe+BC, Fe+GWC and Fe+OMWC had little effect on $EC_{50}$ and even reduced it, Fe+lime and Fe+PS alleviated toxicity, showing the latter even no toxicity for this organism even though it resulted in significantly higher As concentration in the porewater (Fig. 3.3.4). This evidenced this organism is more sensitive to other factors than As availability and often cannot be related to chemical parameters. Similarly, Pardo et al. (2014b) observed that the addition of organic amendments did not reduce the toxic response to *Vibrio fischeri* caused by the control, even though they mitigated trace elements mobility.

As previously suggested in Chapter 3.1 (Fresno et al., 2016), this indirect soil toxicity test is not always well correlated to chemical analysis and plant growth.

***Influence of the amendments on soil quality: multifunctional soil quality parameter (SQP)***

In order to better illustrate the overall effect that each treatment had on soil quality at the end of the experiment (20 months), a multifunctional soil quality parameter (SQP) was calculated. For that, results obtained for each treatment on several soil and plant parameters were standardised by calculating the percentage of variation (V(%)) with respect to the control:

$$V\ (\%) = \frac{(T-C)}{C} \times 100$$

where *T* is the mean value of each treatment and *C* is the mean value of the control.

The following parameters were selected:

(i) soil parameters: exchangeable-Ca, exchangeable-K, exchangeable-Mg, P-Olsen, TOC, TN, extractable-As, extractable-Cu, soluble-As, soluble-Cu

(ii) plant parameters: $DW_{20mat}$, $DW_{22mat}$, $[As]_{shoots,20mat}$, $[As]_{shoots,22mat}$, $[As]_{grain}$

Due to the difficult interpretation of differences between treatments in soil health parameters, they were not considered for the SQP calculation. Neither Cu concentration in shoots and grains was taken into account, since, as previously mentioned, it did not pose a risk in terms of plant toxicity.

Once V (%) was calculated for each parameter, SQP (%) was calculated as the average of all V (%) values. The sign of V(%) values obtained for extractable-As, extractable-Cu, soluble-As, soluble-Cu, $[As]_{shoots,20mat}$, $[As]_{shoots,22mat}$ and $[As]_{grain}$ was changed, as these parameters are negatively related with an improvement on soil quality.

SQP (%) values were:

- Fe+lime: 43
- Fe+PS: 41
- Fe+BC: 351
- Fe+OMWC: 307
- Fe+GWC: 267

In view of these results, Fe+lime and Fe+PS had similar overall effect on the improvement of soil quality, which was much lower than that showed by Fe+BC, Fe+OMWC, Fe+GWC. Among all, Fe+BC seemed to be the treatment that greater positive effect had on the enhancement of all the parameters considered here to improve soil quality and the remediation of this contaminated soil.

## 3.3.5. Conclusions

Despite the addition of organic amendments to the As and Cu-contaminated soil and the increase in soil pH, the treatments did not result in an enhancement of As mobility two years after their application, and an increase in soluble As was observed only for Fe+PS. However, iron oxides were not effective at reducing the soluble and extractable As fractions, and even an increase in the latter was found toward the end of the experiment.

All the treatments resulted in a reduction in Cu mobility, which remained low over time.

The combination of iron sulfate with biochar and compost in a rate 1:5% (w:w) improved soil properties and enhanced rye growth and its nutritional status, which in consequence reduced As concentration in shoots and grains. The best results in terms of soil quality, assessed considering several soil chemical parameters and effects on plants growth and As uptake, were obtained for Fe+BC. In addition, in view to its effects on soil enzymatic activities, this treatment seems to provide a stable source of organic C.

Although longer and larger experiments should be carried out to confirm the effectiveness of these amendments in the long-term, our results demonstrate that the positive effects of biochar and compost in terms of soil quality remains almost two years after their application. However, adjustments in the amendments application rates should be done in order to increase the As-stabilising effect of iron oxides.

# Chapter 4

# Influence of iron-based amendments on the dynamics of As in the root and rhizosphere of *Lupinus albus* L.

## Introduction

Arsenic has been identified by the WHO as one of the pollutants of major public health concern (European Comission, 2013a). Although it is naturally present in soil parent material and in groundwater, high amounts of As-containing minerals have been spread out as a consequence of mining and smelting activities (Fitz and Wenzel, 2002). As a consequence, high concentrations of arsenic (As) have been found in groundwater of several countries, such as India and Bangladesh, exceeding the limit established by the WHO of 10 µg $L^{-1}$ for drinking water.

Plants take up As from soils and accumulate it in their tissues (Zhao et al., 2009b). Since As is a main chemical contaminant in plant-based food, the European Food and Safety Authority has recommended to actively lower its level in agricultural crops (EFSA, 2009). As a consequence, there is a high demand for economical remediation techniques, such as *in situ* phytostabilisation, based on the immobilisation of contaminants in the soil using plants and soil amendments with the aim of decreasing the labile contaminant fraction (Kumpiene et al., 2012; Vangronsveld et al., 2009) Among several amendments evaluated in As-impacted soils, addition of iron oxides has been proposed as a good choice due to positive results obtained in terms of As immobilisation, both in short and long term studies (Hartley et al., 2004; Kumpiene et al., 2006; Kumpiene et al., 2012).

Since one of the major concerns of soils remediation is to mitigate the transfer of potentially toxic elements to the food chain, the behaviour of the contaminants in the rhizosphere, especially of crop plants, should be evaluated within a remediation process. Plants may induce changes in their rhizosphere directly due to nutrients uptake or due to alteration of soil chemistry to enhance nutrients availability. Some of the processes involved include acidification/alkalinisation of the rhizosphere, exudation of organic acids and other complexing compounds and variations of the redox potential (Römheld, 1987; Hinsinger, 2001). Root-induced changes may impact metal and metalloid biogeochemistry in the rhizosphere and substantially alter element mobility (Tao et al., 2003; Puschenreiter et al., 2005; Gonzaga et al., 2006; Bravin et al., 2008; Obeidy et al., 2016).

Iron plaque formation on the roots of submerged plants has been described on several occasions (Siqueira-Silva et al., 2011; Zimmer et al., 2011). In such reducing environments, Fe(III) is reduced to Fe(II). By releasing $O_2$, aquatic plants create oxidising microenvironments around their roots. Fe(II) re-oxidises to Fe(III), which

precipitates on the root surface, creating an iron plaque layer (Blute et al., 2004). The capacity of this iron plaque to sequester metals and metalloids from soil solution is well known, besides its possible role as a buffer and reservoir in nutrient uptake (Tripathi et al., 2014; Williams et al., 2014). This plaque has been predominantly studied in flooded rice; rice iron plaque sequesters As mainly in amorphous and crystalline iron (oxy)hydroxides (Liu et al., 2006). It has been considered as a mechanism to restrict As uptake and translocation to shoots (Liu et al., 2004), but also as a pool that may increase As uptake and accumulation by plants or the supply of arsenic into the soil solution (Huang et al., 2012).

Although rhizosphere processes are important in controlling the success of phytoremediation treatments, little information is available concerning the fate of As in the rhizosphere when aided phytostabilisation technologies are applied. Most research on phytostabilisation strategies focuses on the efficiency of immobilising agents in reducing metal/metalloids availability in bulk soils, but omits the direct effect of plants on the immobilising processes (Kidd et al., 2009).

*Lupinus albus* L. (white lupin) has been shown as a good candidate for phytostabilisation strategies on metal(iod) contaminated soils, as it mainly accumulates the contaminants in the roots and can tolerate highly polluted and acidic soils (Vazquez et al., 2006) (Martínez-Alcalá et al., 2010) (Martínez-Alcalá et al., 2012). White lupin is known to be highly effective in enhancing phosphorus acquisition by the development of proteoid roots under phosphorus deficiency. These cluster roots release large amounts of organic acids anions, such as citrate and malate, into the rhizosphere, increasing phosphorus solubility and availability, by desorption or solubilisation of sparingly soluble P compounds (Dinkelaker et al., 1989; Neumann, 2000). Due to the similar chemical behaviour of phosphate and arsenate in soils (Adriano, 2001b), the concomitant solubilisation of P and As in the rhizosphere of *L. albus* can be expected.

The works described in the following sub-chapters aim at evaluating the interference of iron amendments on the dynamics of As in the roots and rhizosphere of white lupin, as well as at the consequences of this interaction on As uptake. For that, both hydroponic and rhizobag/rhizotrons systems were used to investigate As distribution in *L. albus* plant tissues and its rhizosphere.

# Chapter 4.1

# Effect of iron plaque on As distribution and metabolism in *Lupinus albus* plants under aerobic conditions.

## 4.1.1. Introduction and objective

Iron (hydr)oxides are well known to be major scavengers of As in soils. However, little is known about how the addition of iron amendments affect As distribution in plants.

The objective of this work is to evaluate how different sources of iron supplied to *Lupinus albus L.* grown in hydroponic conditions influence arsenic uptake, distribution and speciation in plant tissues. Our aim was to induce iron (hydr)oxide precipitation on the root surface using continuously aerated conditions and to investigate the impact of this iron plaque on As immobilisation and transfer as well as on the As metabolism in the plant.

## 4.1.2. Materials and methods

### *Plant pre-cultivation*

*Lupinus albus* (cv. Marta) seeds were sterilized in a 1% sodium hypochlorite solution and germinated on moist paper for 3 days at 28 ºC. After germination, uniform seedlings were transferred to PVC pots (4 seedlings per pot) containing 3.5 L of a continuously aerated nutrient solution. The composition of the nutrient solution was (mM) $Ca(NO_3)_2$1.5, $KNO_3$ 1.5, $MgSO_4$ 1.0, $KH_2PO_4$ 0.1, $K_2SO_4$ 0.75; (µM) NaCl 100, $MnSO_4$ 27.3, $ZnSO_4$ 1.6, $CuSO_4$ 1.6, $NiCl_2$ 1.0, $CoCl_2$ 1.0, $(NH_4)_6Mo_7O_{24}$ 1.0, $H_3BO_3$ 20 and $Fe^{3+}$/EDDHA 53.8 (3 mg $L^{-1}$ Fe). The pH of the solution was adjusted to 5.5-6 using KOH or $HNO_3$ and the nutrient solution was changed every 7 days.

The experiment was carried out in a growth chamber under controlled conditions (night/day): T 20/25 ºC, photoperiod 11/13 h, relative humidity 60/40%. The photon flux density during the light period was 520 µmol $m^{-2}$ $s^{-1}$.

***Iron and arsenic treatments***

After four weeks of growth, the iron source was changed for half of the plants as follows: prior to the start of the iron treatment the plants were put into aerated, deionized water overnight. Then they were transferred to a nutrient solution containing 30 mg $L^{-1}$ of ferrous iron, added as $FeSO_4 \cdot 7H_2O$ (p.a. reagent, Panreac, Barcelona, Spain). This treatment was therefore called *$FeSO_4$*. The other half of the plants were kept in the same nutrient solution in which they were previously grown. This treatment was therefore called *Fe/EDDHA*.

Three days later, when a brownish coating was visible on the surface of roots treated with $FeSO_4$, plants from both iron treatments were exposed to 5 µM or 20 µM of arsenate, added as $Na_2HAsO_4 \cdot 7H_2O$, by changing the entire nutrient solution and maintaining the corresponding iron treatment (in the following these treatments will be referred to as $FeSO_4$*As5 and $FeSO_4$*As20 or Fe/EDDHA*As5 and Fe/EDDHA*As20). A control (no As) was also included for each iron treatment. Four replicates were accomplished for each treatment with 4 plants in each pot, *i.e.* four pots and sixteen plants per treatment (24 pots in total). All nutrient solutions were adjusted to pH 5.5-6, continuously aerated and changed every five days. Plants were exposed to As for ten days.

***Sampling and plant processing***

After ten days of As exposure, all plants were harvested, washed thoroughly with tap water and distilled water and separated into shoots and roots.

One plant of each pot, previously washed as explained before and separated into roots and shoots, was dried at 65 ºC for 72 h, weighed and ground using a ball mill. 0.25 g of dry, ground plant material (shoots and roots) was digested in an autoclave using 3 mL of $HNO_3$ (65%, w/w), 1.5 mL of $H_2O_2$ (30%, w/w) and 4 mL of purified water (18 MΩ cm, reagent grade type I) under a pressure of 1.5 kg $cm^{-2}$ for 30 min (Lozano-Rodríguez et al., 1995).

One intact root from each pot was sampled separately and was incubated for one hour at room temperature in 40 mL 0.03 M sodium citrate and 0.125 M sodium bicarbonate, with the addition of 3 g sodium dithionite (DCB method) (modified from Taylor and Crowder, 1983). Then DCB incubating solutions were collected and roots were washed three times with distilled water, which was added to the DCB extracts and were made up to 100 mL. The washed roots were dried at 65 ºC for three days and digested as explained above. This iron plaque-extraction (DCB method) was

applied for both iron treatments for comparing the results between $FeSO_4$- and Fe/EDDHA-treated roots. The differences in As, Fe and P concentrations between DCB-unwashed roots and DCB-washed roots were used to evaluate the distribution of these elements in the roots.

For arsenic speciation analysis in plant tissues, one plant of each pot was frozen at -80 ºC. Frozen shoots and roots were ground in liquid nitrogen using a mortar and pestle. Between 0.25 g and 0.5 g of the ground material were extracted with a phosphate buffer solution (PBS), containing 2 mM $NaH_2PO_4$ and 0.2 mM $Na_2$-EDTA (Sigma-Aldrich) at pH 6.0, for 1 h under sonication (Xu et al., 2007). The extracts were collected and filtered through 0.45 µm syringe filters and stored at -20 ºC until their analysis.

***Analytical methods***

Arsenic and Fe concentrations in plant digests were analysed by HG-AFS (PS Analytical, 10.055, Millennium Excalibur system, Kent, UK) and Atomic Absorption Spectrometry (Analyst 800, Perkin Elmer, Waltham MA, US), respectively.

Arsenic speciation in the PBS extracts was accomplished by HPLC-HG-AFS (HPLC Agilent 1260 Infinity, Agilent, Santa Clara, USA, and HG-AFS PS Analytical 10.055, Millenium Excalibur). 100 µL aliquots of the filtered extracts were injected into the HPLC system. The isocratic separation of the arsenic species was carried out with a Hamilton PRP-X100 10 µm anion-exchange column (250 x 4.1 mm) (Hamilton, Reno, USA). An aqueous solution consisting of 20 mM $Na_2HPO_4/NaH_2PO_4$ adjusted to pH 6.25 was used as mobile phase at 1 mL $min^{-1}$ flow rate. Aqueous standard solutions of As(V), As(III), DMA and MMA, prepared by dissolving appropriate amounts of $Na_2HAsO_4 \cdot 7H_2O$, $NaAsO_2$, dimethylarsinic acid (DMA) and monosodium acid methane arsonate sesquihydrate (MMA) (Sigma-Aldrich), were used for calibration in a concentration range of 0-500 µg $L^{-1}$.

P was analyzed in the digests of shoots, of intact roots and of roots subjected to DCB-extractions by ion chromatography (Compact IC plus 882, Metrohm), using a solution of $NaHCO_3$ 1 mM and $Na_2CO_3$ 3.2 mM as mobile phase at a flow rate of 0.7 mL $min^{-1}$. Phosphate and other anion standards (Sigma-Aldrich) were used to calibrate the instrument.

***Preparation of cross root sections and calibration standards for laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS)***

Four representative sections of secondary roots of each treatment were cut using a stainless steel razor blade, rinsed with reagent grade type I water and immediately subjected to a dehydration procedure that consisted of immersing the roots in 1.5 mL of the following solvents: 30% ethanol (1×1 h); 50% ethanol (1×1 h); 70% ethanol (1×overnight); 95% ethanol (3×1 h) and acetone (2×15 min). Afterwards, root sections were embedded in an epoxy resin using the following matrices: resin:acetone 1:3 (1×2 h); resin:acetone 1:1 (1×overnight); resin:acetone 3:1 (3×1 h) and resin (1×2 h). Each step of both dehydration and embedding procedure was made at room temperature while shaken at 140 rpm on a horizontal shaker. After putting the root sections into the final resin matrix, the samples were left to polymerize at 60 ºC for 24 h. The epoxy resin was prepared using an Epoxy Embedding Medium Kit (Sigma-Aldrich, Switzerland).

Calibration standards for the analysis of Fe and As concentration in the ablated area of root cross sections were prepared by dissolving the appropriate amount of $FeCl_3$ or $AsI_3$ (Sigma-Aldrich) in acetone (Merck, Germany) and diethyl ether (Merck, Germany), respectively, and mixing it quickly with known quantities of the resin matrix. The analyte-solvent-resin mixture was then heated to 60 ºC for 24 h for resin polymerisation. At that temperature, acetone and diethyl ether evaporated completely and did not affect the hardness of the resin blocks.

Cross sections of the resin blocks (1.5 mm thickness) were cut using a low speed saw (Buehler, ITW Test & Measurement GmbH, Düsseldorf, Germany) equipped with a diamond blade (Grimas GmbH, Wolfsgraben, Austria).

***LA-ICP-MS analysis***

The analysis of As, Fe and Mg distribution in the root cross sections was carried out using a UP 193-FX (ESI, NWR Division, Portland OR, USA) laser ablation system coupled to a NexION 300 ICP-MS (Perkin-Elmer). $^{13}C$ was selected as internal normalization standard, as it was homogeneously distributed in the ablated surface. Line scans across the surface area of calibration standards and the samples were performed using the following laser ablation parameters: energy output set to 50%, laser pulse frequency of 50 Hz, scan speed of 10 µm $s^{-1}$ and a laser spot size of 10 µm. The line spacing was 35 µm. Argon was used as the carrier gas at a flow rate of 1 mL $min^{-1}$. The achieved spatial resolution was 6.4 × 35 µm.

***SEM-EDX analysis.***

Selected roots of *Lupinus albus* were dried at room temperature for one week, cut with a stainless steel razor blade and covered with a gold coat with a high resolution sputter coater (Q150T-S, Quorum Tech.). Iron and arsenic distribution was evaluated using a scanning electron microscope (SEM-EDX, Hitachi S-3000N) equipped with an energy dispersive X-ray spectrometer (Oxford Instruments).

***Data analysis***.

Statistical analyses were performed using IBM SPSS Statistics 21. Normality of the data was tested using the Shapiro-Wilk test. When homogeneity of variances and a normal distribution of the data were confirmed, a one-way ANOVA test was performed to determine whether there were differences among treatments, followed by Tukey´s HSD test to assess differences between groups. Where it was not possible to use ANOVA, Kruskal-Wallis and Games-Howell tests were performed. Two-way ANOVA was used to investigate interaction between As and Fe treatments.

Chemical images of the roots were obtained using ImageJ software (http://imagej.nih.gov/ij/, National Institutes of Health, Bethesda, USA).

## 4.1.3. Results

### *Plant growth*

For none of the plants visual symptoms of As toxicity were observed. To assess plant growth in response to both Fe and As treatments, dry weights of shoots and roots were evaluated (Table 4.1.1). Fe/EDDHA-treated plants showed no significant differences in shoot dry weights between arsenate treatments. However, plants treated with $FeSO_4$*As5 and $FeSO_4$*As20 showed a ~25% increase in biomass production ($P < 0.05$) compared to the $FeSO_4$ control. $FeSO_4$ treatment significantly increased shoot biomass in comparison to Fe/EDDHA ($P < 0.001$), and enhanced shoot weight by 28% in plants exposed to 5 µM arsenate and by 22% in those exposed to 20 µM arsenate. Exposure to As reduced root dry weight in Fe/EDDHA*As20-treated plants compared to the Fe/EDDHA control. $FeSO_4$-treated roots showed a different pattern, as there was no significant difference between the control and the 20 µM As treated roots. For both Fe treatments, root dry weight was increased at the 5 µM arsenate dose ($P < 0.05$).

**Table 4.1.1.** Dry weights (DW) of shoots and roots of white lupin treated with different Fe sources and exposed to different As doses. Mean (n = 16) ± SE. Different letters indicate significant differences between As doses for each iron treatment (Tukey´s HSD test, $P<0.05$). Analysis of variance (two-way ANOVA) was performed to evaluate the effect of each factor and their interaction. N.S.: not significant; *$P<0.05$; **$P<0.01$; ***$P<0.001$.

| | **Shoot DW (g plant$^{-1}$)** | **Root DW (g plant$^{-1}$)** |
|---|---|---|
| ***Fe/EDDHA-treated plants*** | | |
| Control | 1.77 ± 0.09 $^{a}$ | 0.47 ± 0.05 $^{b}$ |
| As 5 µM | 1.70 ± 0.1$^{a}$ | 0.54 ± 0.04 $^{ab}$ |
| As 20 µM | 1.80 ± 0.1$^{a}$ | 0.36 ± 0.03 $^{a}$ |
| ***$FeSO_4$-treated plants*** | | |
| Control | 1.74 ± 0.06 $^{a}$ | 0.42 ± 0.04 $^{a}$ |
| As 5 µM | 2.17 ± 0.08 $^{b}$ | 0.58 ± 0.04 $^{b}$ |
| As 20 µM | 2.19 ± 0.09 $^{b}$ | 0.55 ± 0.04 $^{ab}$ |
| ***Two-way ANOVA*** | | |
| Fe treatment | *** | N.S. |
| As dose | * | ** |
| Fe × As | ** | * |

***Arsenic distribution in plants***

Arsenic concentration in shoots was significantly affected ($P < 0.05$) by arsenate doses in both Fe treatments (Table 4.1.2). Arsenic concentration in Fe/EDDHA*As20-shoots was 1.6-fold higher compared to Fe/EDDHA*As5 and As in $FeSO_4$*As20-shoots was 2.5-fold higher than in $FeSO_4$*As5. However, the most significant effect was related to the applied Fe treatment. Arsenic concentration was lower ($P < 0.001$) in $FeSO_4$-treated shoots (0.766 ± 0.303 µg $g^{-1}$ in $FeSO_4$*As5 and 2.01 ± 0.75 µg $g^{-1}$ in FeSO4*As20) than in Fe/EDDHA-treated shoots (10.7 ± 3.1 and 18.1 ± 1.5 µg $g^{-1}$ in Fe/EDDHA*As5 and in Fe/EDDHA*As20).

Total As concentration in the intact roots (not washed with DCB) was neither affected by arsenate treatment nor by Fe treatment. In treatments Fe/EDDHA*As5 and Fe/EDDHA*As20 As concentration was 217 ± 20 and 194 ± 97 µg $g^{-1}$, respectively, and in $FeSO_4$*As5 and $FeSO_4$*As20 treated roots was 195 ± 59 and 130 ± 18 µg $g^{-1}$.

In contrast, DCB-extraction (washing out of surface Fe in the root) led to significant differences between Fe treatments. DCB-solution removed 99% and 87% of the total As in roots treated with $FeSO_4$*As5 and $FeSO_4$*As20, but only 59% and 33% was extracted from roots treated with Fe/EDDHA*As5 and Fe/EDDHA*As20. After the DCB-extraction, the concentration of As in roots of $FeSO_4$-treated plants was therefore significantly lower ($P < 0.001$) than that found in Fe/EDDHA-treated roots.

Significantly lower ($P < 0.001$) arsenic shoot-to-root ratios (on the basis of total root As) were observed when plants were treated with $FeSO_4$, *i.e.* up to 12.5-fold lower than in Fe/EDDHA plants, even though similar As concentrations were found in the intact unwashed roots.

**Table 4.1.2**. Arsenic concentration (mg $kg^{-1}$) in shoots and roots of white lupin plants grown with two different Fe treatments and two As doses. The values are means ($n$ = 4) ± SE. Analysis of variance (two-way ANOVA) shows the effect of As dose, Fe treatment or the inter-effect of both factors. N.S.: not significant; *$P$ < 0.05; **$P$ < 0.01; ***$P$ < 0.001.

| | **As 5 µM** | **As 20 µM** | ***Two-way ANOVA*** | |
|---|---|---|---|---|
| ***Shoots*** | | | | |
| Total As concentration in shoots (mg $kg^{-1}$) | | | As dose | * |
| Fe/EDDHA-treated plants | 10.7 ± 3.1 | 18.1 ± 1.5 | Fe treatment | *** |
| $FeSO_4$-treated plants | 0.766 ± 0.303 | 2.01 ± 0.75 | As × Fe | N.S. |
| ***Roots*** | | | | |
| Total As concentration in roots (mg $kg^{-1}$) | | | As dose | N.S. |
| Fe/EDDHA-treated plants | 217 ± 20 | 194 ± 97 | Fe treatment | N.S. |
| $FeSO_4$-treated plants | 195 ± 59 | 130 ± 18 | As × Fe | N.S. |
| Arsenic concentration after DCB extraction (mg $kg^{-1}$) | | | | |
| Fe/EDDHA-treated plants | 88.6 ± 13.0 | 130 ± 21 | As dose | * |
| % As extracted | 59.1 | 33.2 | Fe treatment | *** |
| $FeSO_4$-treated plants | 1.96 ± 1.20 | 17.1 ± 5.8 | As × Fe | N.S. |
| % As extracted | 99.3 | 86.9 | | |
| ***Arsenic transfer to shoots*** | | | | |
| Arsenic shoot:root ratio | | | As dose | * |
| Fe/EDDHA-treated plants | 0.0500 | 0.120 | Fe treatment | *** |
| $FeSO_4$-treated plants | 0.00370 | 0.0160 | As × Fe | N.S. |

***Iron distribution in plants***

The effect of different iron treatments on Fe distribution in white lupin plants is shown in Table 4.1.3.

No differences in Fe/EDDHA-treated shoots were observed after exposure to different arsenate doses, while there were significant differences ($P < 0.05$) for the $FeSO_4$ treatment. Fe concentration was significantly higher in $FeSO_4$-treated shoots than in Fe/EDDHA-shoots ($P < 0.001$). Also Fe concentration in the intact roots was higher ($P < 0.001$) with $FeSO_4$ treatment than with Fe/EDDHA.

Extraction with DCB removed >80% of the Fe from $FeSO_4$-treated roots for both arsenate doses, while less homogeneous results were obtained in Fe/EDDHA-treated roots. In this treatment DCB-extraction only removed 25% of the total Fe from control roots and around 65%-66% from roots exposed to arsenate.

Differences in the shoot-to-root ratio of Fe (calculated taking into account only the concentration of Fe in the intact roots) were observed as well. This ratio was significantly higher ($P < 0.05$) in Fe/EDDHA-treated plants, 300-fold in the control roots and 10 and 6.7-fold in Fe/EDDHA*As5 µM and Fe/EDDHA*As 20 µM, respectively, in comparison with equivalent $FeSO_4$ plants. In treatment Fe/EDDHA, significant differences ($P < 0.05$) were found for Fe shoot-to-root between control and As-treated plants. Differences in $FeSO_4$-treated plants were only found between the control and the plants exposed to 20 µM arsenate.

**Table 4.1.3.** Iron concentration (mg $kg^{-1}$) in shoots and roots of white lupin plants grown with two different iron sources and exposed to different arsenic doses. The values are means (n = 4) ± SE. Different letters indicate significant differences among As doses within Fe treatments (Games-Howell or Tukey HSD test, $P<0.05$). Stars indicate differences between Fe treatments for each As dose after ANOVA or Kruskal-Wallis test ($P<0.05$). N.S.: not significant; *$P<0.05$; **$P<0.01$; ***$P<0.001$.

| | Control | As 5 µM | As 20 µM |
|---|---|---|---|
| ***Shoots*** | | | |
| Total Fe concentration in shoots (mg $kg^{-1}$) | | | |
| Fe/EDDHA-treated plants | 97.2 ± 6.4 $^{a}$ | 106 ± 9 $^{a}$ | 105 ± 18 $^{a}$ |
| $FeSO_4$-treated plants | 290 ± 33 $^{a}$ | 356 ± 21 $^{b}$ | 385 ± 15 $^{b}$ |
| *Anova* | *** | *** | *** |
| ***Roots*** | | | |
| Total Fe concentration in roots (mg $kg^{-1}$) | | | |
| Fe/EDDHA-treated plants | 70.9 ± 16.3 $^{a}$ | 154 ± 26 $^{b}$ | 175 ± 4 $^{b}$ |
| $FeSO_4$-treated plants | (5.80 ± 0.86) × $10^{3}$ $^{a}$ | (5.16 ± 0.51) × $10^{3}$ $^{a}$ | (4.29 ± 0.11) × $10^{3}$ $^{a}$ |
| *Anova* | *** | *** | *** |
| Fe concentration after DCB extraction (mg $kg^{-1}$) | | | |
| Fe/EDDHA-treated plants | 52.7 ± 3.6 $^{a}$ | 56.7 ± 4.4 $^{a}$ | 59.7 ± 5.7 $^{a}$ |
| % Fe extracted | 25.7 | 64.2 | 65.9 |
| $FeSO_4$-treated plants | 927 ± 208 $^{a}$ | 559 ± 91 $^{a}$ | 524 ± 37 $^{a}$ |
| % Fe extracted | 87.3 | 89.4 | 87.8 |
| ***Iron transfer to shoots*** | | | |
| Fe/EDDHA-treated plants | 1.47 ± 0.30 $^{b}$ | 0.69 ± 0.15 $^{a}$ | 0.596 ± 0.095 $^{a}$ |
| $FeSO_4$-treated plants | 0.0509 ± 0.0046 $^{a}$ | 0.071 ± 0.010 $^{ab}$ | 0.0880 ± 0.0056 $^{b}$ |
| *Kruskal-Wallis* | * | * | * |

***Arsenic speciation in plant tissues***

Arsenic speciation was analysed in the PBS-extracts of roots and shoots. Only As inorganic species (As(III) and As(V)) were detected in plant extracts. As(III) was the only species detected in shoots of plants treated with Fe/EDDHA (data not shown). No As was detected in the shoot extracts of $FeSO_4$-treated plants, due to the low total As concentration. Arsenic speciation in roots showed differing results depending on the Fe treatment. As(III) was the main species present in PBS-extracts of Fe/EDDHA-treated roots (up to 84%-82%), but its proportion was lower in $FeSO_4$*As5 (3% As(III)) and $FeSO_4$*As20 (30% As(III)) roots (Fig. 4.1.1).

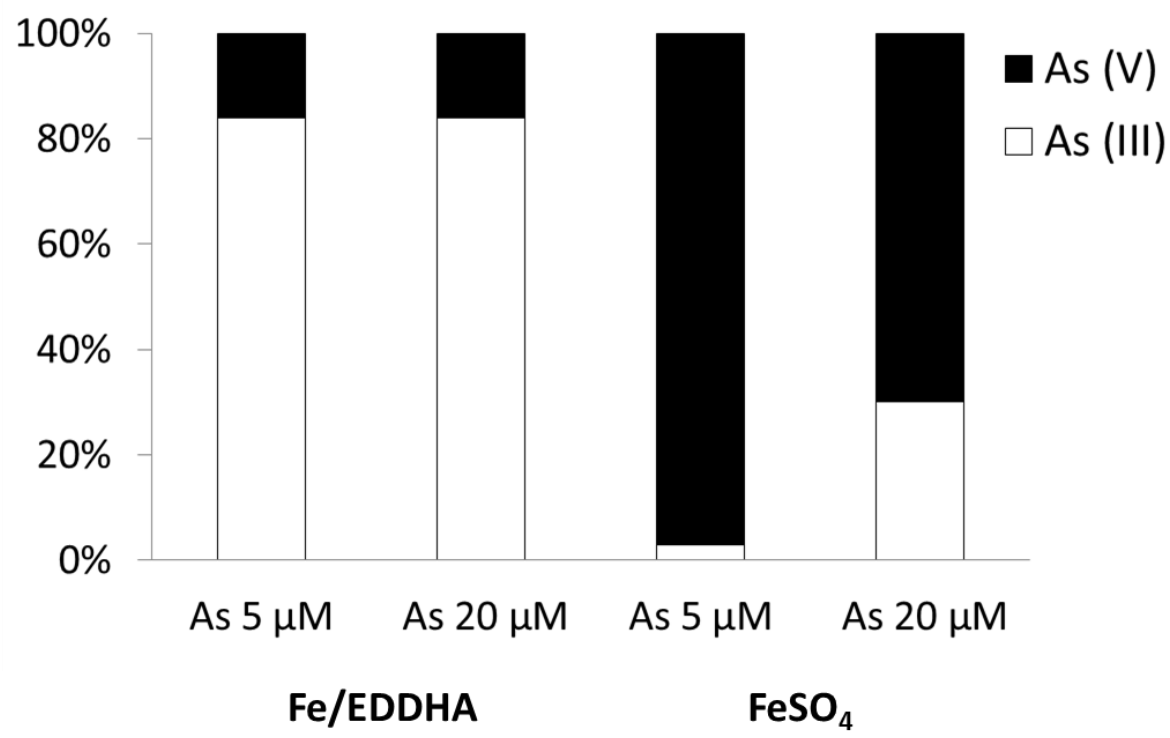


**Figure 4.1.1**. Percentage (mean, *n* = 4) of As species in the PBS-extracts of roots of white lupin. Plants were treated with Fe/EDDHA or $FeSO_4$ and exposed to arsenate 5 or 20 µM for 10 days.

***Iron and arsenic distribution in root cross sections***

The spatial distribution of Mg, As and Fe was determined in root cross sections by LA-ICP-MS (Fig. 4.1.2).

Mg was used as an indicator of the position of the root, as it was present along the whole root and was not detected in the resin matrix; therefore it is only given as signal intensity. Its distribution provides an approach to the localisation of different root tissues, *i.e.* the epidermis, the cortex and the stele (Fig. 4.1.2-A, D, G, J) after comparison with microscope images (not shown).

Fe concentrations (pg $\mu m^{-3}$) in roots treated with $FeSO_4$ were ~100-fold higher than in Fe/EDDHA-treated roots but its distribution in both treatments was similar (Fig. 4.1.2-B, E, H, K). In both cases Fe was mainly distributed along the epidermis, but also extended towards the cortex.

Arsenic concentrations (pg $\mu m^{-3}$) in sections of roots treated with Fe/EDDHA were by 10-fold lower than those in the $FeSO_4$-treated roots, but the distribution pattern was widely different. In roots treated with Fe/EDDHA, As mainly accumulated in the endodermis and the stele (Fig. 4.1.2-J, L) and no spatial correlation between Fe and As could be established. Contrastingly, a remarkable co-accumulation of As and Fe was observed in the surface and the cortex of $FeSO_4$-treated roots (Fig. 4.1.2-B, E, C, F).

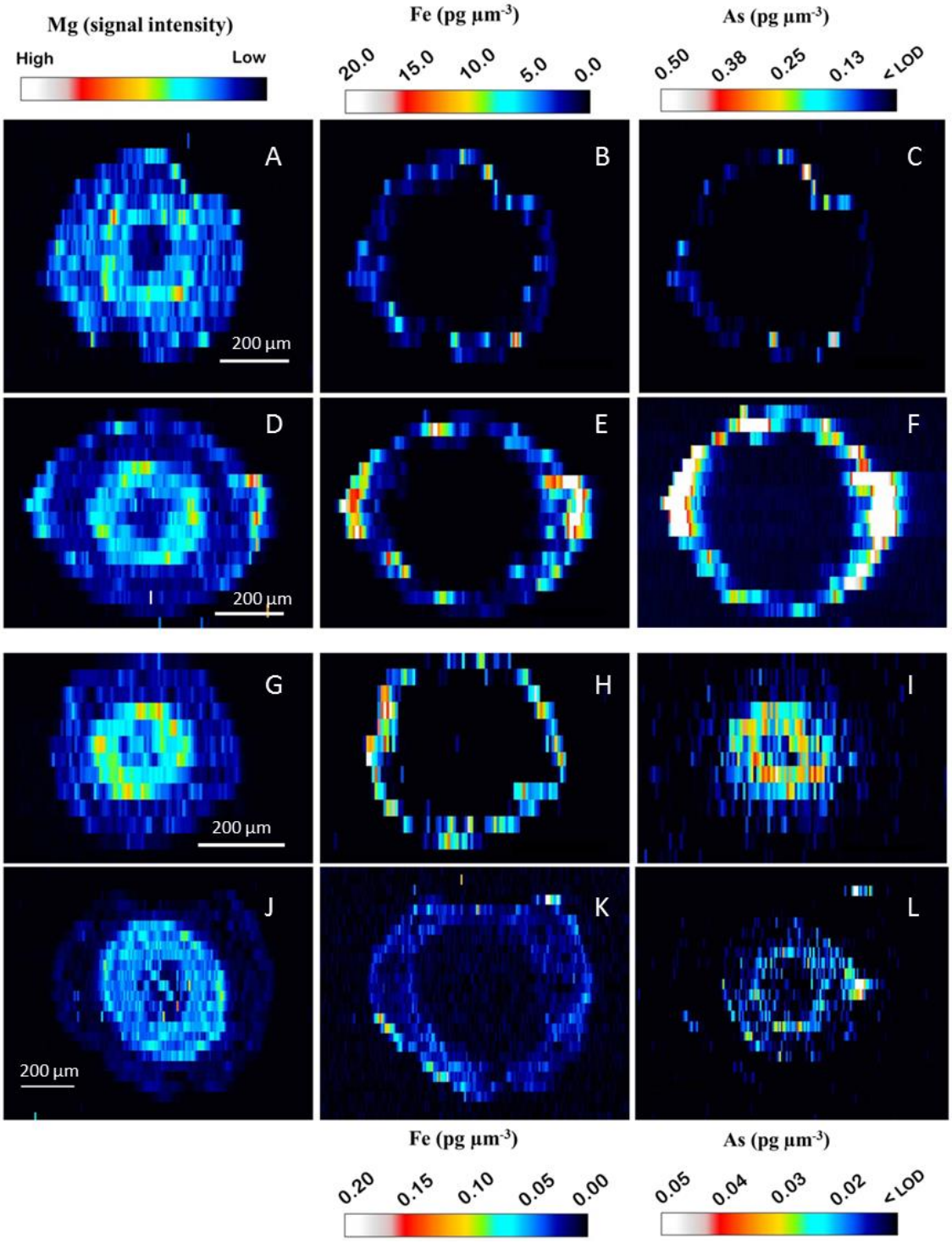


**Figure 4.1.2**. Chemical images of the distribution of Mg (left), Fe (centre) and As (right) in roots of *Lupinus albus* L. **A**, **B**, **C** images correspond to the treatment $FeSO_4$ and dose 5 µM arsenate. **D**, **E**, **F** correspond to the treatment $FeSO_4$ and dose 20 µM arsenate. **G**, **H**, **I** are images of roots treated with Fe/EDDHA and 5 µM arsenate. **J**, **K**, **L** correspond to roots treated with Fe/EDDHA and 20 µM arsenate. Top scale bars correspond to images B, C, E, F and bottom scalebars to images H, I, K, L. The Mg scalebar can be related to images A, D, G, J. Concentration units of Fe and As are in pg·µm$^{-3}$ and Mg scale is shown as the signal intensity. (Note that the color scales for the images of $FeSO_4$-treated roots are 100-by concentration of the Fe/EDDHA-treatment images for Fe and 10-by for As).

**Table 4.1.4**. Phosphorus concentration (mg $kg^{-1}$) in white lupin shoots and roots. Data are means (n = 4) ± SE. Different letters indicate differences between As doses (Tukey HSD test). Analysis of variance (ANOVA) shows the effect of iron treatment. N.S.: not significant; **P*<0.05; ***P*<0.01; ****P*<0.001.

| | Control | As 5 µM | As 20 µM |
|---|---|---|---|
| ***Shoots*** | | | |
| Total P concentration in shoots (mg $g^{-1}$) | | | |
| Fe chelate-treated plants | 1.08 ± 0.03 $^{a}$ | 1.1 ± 0.1 $^{a}$ | 1.07 ± 0.09 $^{a}$ |
| $FeSO_4$-treated plants | 0.90 ± 0.04 $^{b}$ | 0.83 ± 0.04 $^{ab}$ | 0.74 ± 0.03 $^{a}$ |
| *Anova* | * | * | * |
| ***Roots*** | | | |
| Total P concentration in roots (mg $g^{-1}$) | | | |
| Fe chelate-treated plants | 1.3 ± 0.1 $^{a}$ | 1.2 ± 0.1 $^{a}$ | 1.3 ± 0.2 $^{a}$ |
| $FeSO_4$-treated plants | 1.5 ± 0.2 $^{a}$ | 1.1 ± 0.1 $^{a}$ | 1.0 ± 0.1 $^{a}$ |
| *Anova* | N.S. | N.S. | N.S. |
| P concentration after DCB extraction (mg $g^{-1}$) | | | |
| Fe chelate-treated plants | 0.88 ± 0.03 | 0.85 ± 0.07 | 0.97 ± 0.03 |
| (% P extracted ) | 30 | 29 | 23 |
| $FeSO_4$-treated plants | 0.83 ± 0.04 | 0.72 ± 0.04 | 0.67 ± 0.04 |
| (% P extracted) | 42 | 34 | 33 |
| ***Phosphorus transfer to shoots*** | | | |
| Fe chelate-treated plants | 0.845 | 0.10 | 0.950 |
| $FeSO_4$-treated plants | 0.638 | 0.06 | 0.775 |
| *Anova* | N.S. | N.S. | N.S. |

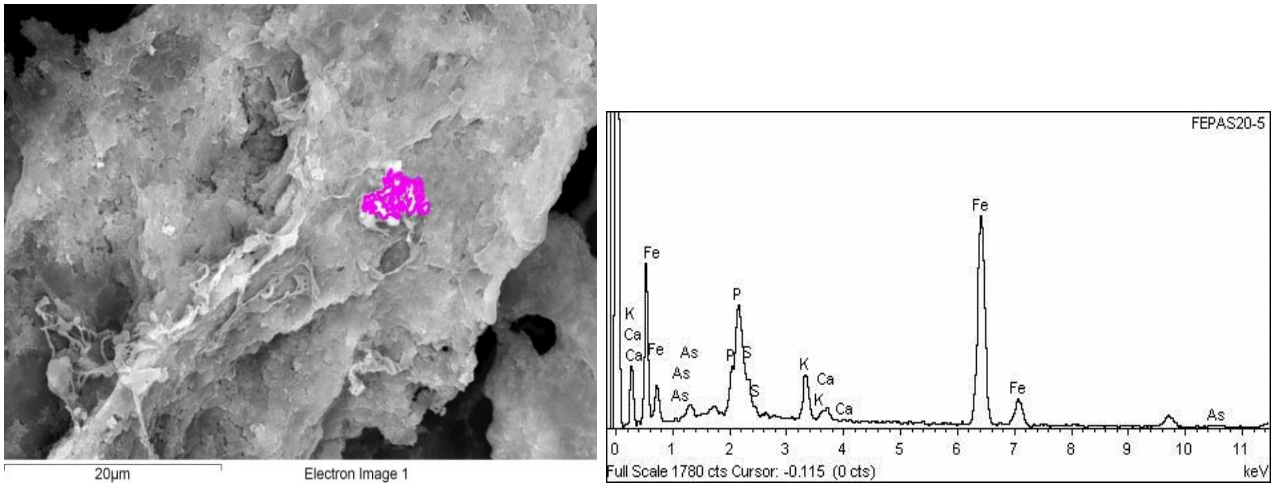


**Figure 4.1.3**. SEM image (left) and corresponding EDX spectra of the highlighted area (right) of the root surface of a white lupin plant treated with $FeSO_4$ and exposed to As 20 µM.

## 4.1.4. Discussion

Plants grown with FeSO4 showed a brownish coating on their roots (iron plaque) that was not visible in the Fe/EDDHA-treated plants (see Fig A-2 in the Annex). When exposed to As, $FeSO_4$-treated shoots had higher shoot biomass compared to Fe/EDDHA-treated ones (Table 4.1.1). This may indicate that the enhanced iron plaque promoted plant growth or ameliorated potential arsenic toxicity effects. These results also concurred with a significantly lower root-to-shoot transfer of As in $FeSO_4$-treated plants (Table 4.1.2). In contrast to previous experiments (e.g., Vázquez-Reina et al., 2005) in this experiment shoot growth was not affected by arsenate toxicity, as no decrease of shoot biomass was observed (Table 4.1.1), which was likely due to the short time of As exposure (10 d). Regardless, the absence of toxicity symptoms (growth inhibition or visual symptoms) in $FeSO_4$-treated white lupin suggests the role of iron plaque on alleviating As toxicity in plants.

Fe treatment significantly affected ($P < 0.001$) As transfer to white lupin shoots (Table 4.1.2). Although As concentrations in roots from both treatments were similar, As proportion in Fe/EDDHA-roots was lower (90-95%) than in $FeSO_4$-roots (98-99%) in relation to total As in the plants. Similarly high proportions of As in roots were reported by Vazquez et al. (2005), who pointed out that around 95% of the As taken up by the plants was retained in the root. Our study found exceptional retention of As in roots coated with iron plaque ($FeSO_4$ treatment), which can be explained by the high proportion of As extracted with DCB (Table 4.1.2). These results showed that the iron plaque efficiently immobilized As on the root surface and reduced its translocation to shoots.

Results from LA-ICP-MS analysis in roots cross sections (Fig. 4.1.2) showed a similar Fe distribution pattern in roots with contrasting Fe treatments. Although Fe concentration (pg $\mu m^{-3}$) was higher in $FeSO_4$-treated plants, in both treatments it was mainly accumulated on the surface of the roots, but in some cases it entered into the cortex (Fig. 2-B, E, H, K). Fe pools in the root apoplast have been described and mainly studied in agricultural crops as a frequent rhizospheric effect. For instance, extracellular accumulation of Fe was found at the root surface of barley with either ionic or chelated (Fe/EDDHA) Fe supplements (Clarkson and Sanderson, 1978), similar to that found in cucumber by Fodor et al., (2012). An explanation for this effect would be that only a fraction of the Fe reduced by the root iron reductases is actually translocated into the symplast (Lucena and Chaney, 2006), while the remaining Fe

may be sorbed to the cell walls or re-oxidised and precipitated in the apoplast. Although the possibility of iron deficiency due to iron plaque formation should be considered, in this work is not expected, since higher Fe concentration was found in $FeSO_4$-treated plants than in Fe/EDDHA-treated plants. In fact, apoplastic Fe may be used by the plant as a short-term storage to be used in case of deficiency, as observed in a variety of soybean (Longnecker and Welch, 1990).

The form of provided Fe had a clear impact on As distribution, as the co-association of Fe and As in root images showed differences depending on the Fe treatment (Fig. 4.1.2). In Fe/EDDHA-treated roots, As is mainly accumulated in the stele and there is generally no association with Fe distribution. Contrastingly, in $FeSO_4$-treated roots Fe and As distributions were correlated: both elements accumulated mainly in the epidermis, and to a lower extent in the cortex, and they were hardly detectable in the stele (Fig. 4.1.2-C, F). The formation of Fe deposits on the root surface was also confirmed in SEM/EDX analysis (Fig. 4.1.3), which showed surficial deposits in roots treated with $FeSO_4$*As20. These deposits were identified in the EDX spectrum as As and Fe hotspots.

Iron source also affected As speciation in lupin roots. As expected, arsenite was the main species in plants treated with Fe/EDDHA (Fig. 4.1.1), since As(III) has been found to be the main As species accumulated in plant tissues, usually accounting for >90% of the total As in plants (Zhao et al., 2009a). In contrast, As(V) was the major species in $FeSO_4$-treated roots (Fig. 4.1.1). The predominance of arsenate in roots presenting iron plaques has been previously described, accounting for 71-74% (Liu et al., 2006) or 66-89% (Seyfferth et al., 2010) of the total As present in rice roots. Yamaguchi et al. (2014) found that As sequestered in iron plaques of rice was mainly As(V) in aerated soils, while in flooded conditions the proportions of As(III) and As(V) resembled those found in the soil matrix, either As(III) or As(V) was the predominant species. Data on As speciation in plants growing in contaminated soils are not so abundant, but a predominance of As(III) would be expected in roots, as most plants quickly reduce As(V). Nevertheless, Larios et al. (2012) found that As(V) predominated in roots of plants grown in an arsenopyrite-contaminated soil, while As(III) occurred at low proportion. A possible explanation for this is that in such Fe-enriched soil plants may immobilize As(V) on roots in a similar way as we observed in our experiment.

Our findings may have a potential application in phytostabilisation of As-contaminated soils and may contribute to the mitigation of As transfer to plants in agricultural systems and thus into the food chain. The immobilisation of As in the rhizospheric iron plaque in aerobic environments may also occur naturally, but could be further enhanced by using Fe salts as soil amendments. We hypothesize that changes in the physico-chemical properties of the rhizosphere would lead to the immobilisation of As. Martínez-Alcalá et al. (2008) observed a decrease in the pH of the rhizosphere of white lupin and more oxidant conditions than in bulk soil. The authors suggested that higher redox potential would provoke Fe(III), as the predominant oxidation state, retaining metals, which is also the case for As.

Rhizosphere acidification may also contribute to the formation of an iron plaque by dissolving Fe oxides adjacent to the roots. Plant transpiration may promote water flux towards the rhizosphere, increasing Fe movement towards the roots. However, further research is needed to evaluate long-term effects of the accumulation of As in the root surface, as it can also pose a risk. Arsenic mobilisation from this iron (oxy)hydroxide plaque could occur as a consequence of solubilisation of the plaque or its transformation to more crystalline phases, that would mean a decrease of the surface specific area (Dixit and Hering, 2003).

In addition, a possible adverse effect on P nutrition should be further investigated. Phosphate is a chemical analogue of arsenate, so it may be affected in a similar way by iron plaque, as suggested before (Rahman et al. 2008). P was present in the surficial iron deposits in white lupin roots (Fig. 4.1.3), suggesting its sorption onto the induced plaque. As P was in the nutrient solution during the whole growth stage, it is difficult to explain to which extent iron plaque affected P nutrition. Nonetheless, results of P in plant tissues (Table 4.1.4) show that P concentration in shoots was lower ($P$<0.05) in plants treated with $FeSO_4$, suggesting a similar effect like for As.

## 4.1.5. Conclusions

Supplying $FeSO_4$ to the growing culture induced the formation of an iron plaque on the surface of white lupin roots. The presence of this plaque provoked a reduction in the As transfer to shoots, while showing similar As concentration in roots when Fe was applied as Fe/EDDHA.

Fe and As mapping in root cross sections showed that when Fe was applied as $FeSO_4$, both elements accumulated in the surface of the root. However, when the Fe source was Fe/EDDHA, As was mainly present in the central cylinder of the root. Furthermore, the major species in the roots treated with $FeSO_4$ was As(V), suggesting a lack of its uptake and thus its metabolism and reduction to As(III).

Our findings provided mechanistic insights on manipulating Fe biogeochemistry in the rhizosphere of white lupin for mitigating As transfer to plant tissues in aerobic soils with a mechanism similar to that occurring in the rhizosphere of flooded rice. Such mechanisms will potentially assist As phytostabilisation in soil and in lowering As in agricultural products, mainly by preventing plant uptake and by immobilising the metalloid in rhizosphere Fe oxides.

# Chapter 4.2

# Changes in the rhizosphere of *Lupinus albus* L. during iron-based amending of an As- and Cu-contaminated soil

## 4.2.1. Introduction and objective

When aided phytostabilisation is implemented in contaminated soils, the behaviour of trace elements in the plants rhizosphere should be also investigated in order to assess possible risks associated to soil treatments in terms of transfer to the food chain.

The objective of this study is to evaluate the influence of *L. albus* roots on changes in As and Cu mobility in an As- and Cu-contaminated soil and its interaction with iron-based amendments. For that purpose, we monitored changes in the soil solution chemistry of bulk and rhizosphere soil and analysed the extractable fraction of several relevant elements at the end of the experiment. We also evaluated the distribution of labile elements in the rhizosphere of white lupin grown in non-amended contaminated soil by chemical mapping performed using diffusive gradients in thin films (DGT) sampling combined with spatially resolved analysis by laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS).

## 4.2.2. Materials and methods

### *Experimental design*

Contaminated waste material was collected from the same area as the previous chapters, El Verdugal (Madrid). The contaminated material (sieved at <2 mm) was mixed with an uncontaminated soil collected from a close-by area (sieved at <4 mm) in a ratio 5:95 (contaminated:uncontaminated) to obtain a homogenous composite soil. The sandy loam (composite) soil was slightly acidic (pH 5.4) and had high concentrations of As and Cu (2258 ± 625 and 157 ± 33 mg $kg^{-1}$, respectively).

The materials used in the experiment as amendments were $FeSO_4 \cdot 7H_2O$, $CaCO_3$ (Panreac, Barcelona, Spain) and biochar produced by pyrolysis of holm oak chips at

600 ºC (Moreno-Jiménez et al., 2016a). Some characteristics of soil and this biochar are shown in Table 3.2.1.

The soil was mixed with the corresponding amount of amendments, calculated on a dry soil weight basis. The mixtures were manually homogenised, resulting in the following treatments:

(1) **Control**, consisting of the non-amended soil

(2) Soil + $FeSO_4$ (1%) + $CaCO_3$ (0.37%) (**Fe+lime**)

(3) Soil + $FeSO_4$ (1%) + $CaCO_3$ (0.15%) + biochar (3%) (**Fe+BC**)

Each treatment was replicated 4 times. The amount of $CaCO_3$ added to Fe+BC was calculated to equal the amount of $CaCO_3$ added to Fe+lime, taking into account the total carbonate content of biochar.

A rhizobag system was used to differentiate between rhizosphere and bulk soil. The rhizobags consisted of a 13 × 6.5 cm (height × diameter) cylindrical methacrylate structure covered with a nylon mesh with 30 µm pore size. Each rhizobag was placed in the centre of a plastic pot and was filled with 600 g of soil; the rest of the pot was filled with the same soil up to a total of 3.5 kg. Figure A-3 (Annex) shows some photographs of the rhizobag system.

One rhizon sampler of 5 cm length was inserted inside each rhizobag with an angle of 45° and another one of 10 cm length was inserted outside each rhizobag in the same way (see Figure A-3 in the Annex). Soils were moistened to 70% of the water holding capacity (WHC) and left to equilibrate for 15 days.

Then, one 7-days-old seedling of *L. albus* (cv. Marta), previously grown in peat, was placed in each rhizobag. Plants were grown under controlled conditions (day/night: 13/11 h, 40/60% RH and a photon flux density of 520 µmol $m^{-2}$ $s^{-1}$) for 48 days. All soils were kept at 70% WHC during the experiment by weighing and adding water losses.

Soil porewater was sampled every 2 weeks after planting, pH was immediately measured and samples were filtered through 0.45 µm syringe filters (Teknokroma, Barcelona, Spain).

At harvest, rhizobags were withdrawn from the pot to separate the soil contained in them from the bulk soil. The nylon mesh that covered the rhizobags was removed

(see Fig. A-3, Annex); the soil inside the rhizobag that was not colonised by roots was discarded and the soil adhering to the roots after gentle shaking was considered rhizosphere soil, which was homogenised and air dried. The soil outside the rhizobag, *i.e.* the bulk soil, was homogenised and a subsample was air dried for one week.

Plants were separated into shoots and roots and fresh weights were recorded. Roots were washed with tap and deionised water and sonicated for 3 minutes to remove soil particles. Then shoots and roots were frozen at -80 ºC. Plant material was ground with liquid $N_2$ using a mortar and pestle and subsamples were dried at 65 ºC for 3 days.

***Plant and soil analysis***

Ground and dried plant material (0.2 g) was digested with 4 mL of $HNO_3$ (65% v/v) and 1 mL of $H_2O_2$ (30% v/v) under a pressure of 1.5 kg $cm^{-2}$ for 30 min (Lozano-Rodríguez et al., 1995), made up to 10 mL and analysed by ICP-MS (Elan 9000 DRCe, PerkinElmer).

Biomass subsamples, stored at -80 ºC, were extracted with a phosphate buffer solution (PBS), consisting of 2 mmol $L^{-1}$ $NaH_2PO_4$ and 0.2 mmol $L^{-1}$ $Na_2$-EDTA (Sigma-Aldrich) at pH 6.0, for 1 h under sonication (Xu et al., 2007). The extracts were collected and filtered through 0.45 µm syringe filters for As speciation analysis by HPLC-HG-AFS (HPLC Agilent 1260 Infinity, Agilent, and HG-AFS PS Analytical 10.055, Millenium Excalibur) (Fresno et al., 2016b).

The labile element fraction in bulk and rhizosphere soils was determined by soil extraction with 0.1 mol $L^{-1}$ $(NH_4)_2SO_4$ (1:10 w:v, shaken at 140 rpm for 4 h). Arsenic was analysed in this extracts by hydride generation-atomic fluorescence spectroscopy (HG-AFS) and Cu and Fe by atomic absorption spectroscopy (AAS). P-Olsen was measured in 1:20 (w:v) $NaHCO_3$-extracts of bulk and rhizosphere soils by ICP-OES. The concentration of As, Cu, Fe and P in soil porewater samples was measured by ICP-MS (Elan 9000 DRCe, PerkinElmer) and dissolved organic carbon (DOC) was analysed with a TOC analyser (Shimadzu TOC-V CSH).

***Rhizotron experiment and rhizosphere chemical mapping***

A rhizotron experiment was set up in order to evaluate the distribution of several elements in the rhizosphere of white lupin grown in the non-treated soil. For that purpose, the same composite soil as in the pot experiment, but mixed in a ratio 1:9

(contaminated:uncontaminated), was used as growth medium. The rhizotrons consisted of flat perspex boxes with inner dimensions of 40 × 10 × 1.5 cm and a front plate that could be removed. The rhizotrons were carefully filled with the contaminated composite soil in order to obtain a very fine, flat soil surface, which was covered with a piece of 10 µm thick polycarbonate filter membrane (pore size 0.2 µm; Nucleopore, Whatman). The soil was moistened to 70% WHC and left to equilibrate for 3 days. White lupin seeds were germinated on moist tissue paper for 3 days and one seedling was transplanted to each rhizotron (4 replicates). The rhizotrons were angled at 18º during plant growth, so that the roots developed towards and along the removable plate of the rhizotron. Figure A-4 (Annex) shows photographs of several steps of the rhizotrons preparation and a 15-days-old lupin plant grown in a rhizotron.

High resolution diffusive gradients in thin films (HR-DGT) gels were prepared according to Kreuzeder et al. (2013). The resin gel used here is based on zirconium hydroxide and suspended particulate reagent-iminodiacetate, which allows concomitant sampling of anions and cations (Kreuzeder et al., 2013).

After 15 days of plant growth, the element distribution around lupin roots was assessed. For that, the front plate of the rhizotrons was removed and several pieces of HR-DGT gels (~3 x 4 cm) were deployed onto selected roots, with the membrane layer separating roots and DGT gels (see Figure A-5 in the Annex). The membrane served as diffusion layer instead of the typically used 0.8 mm thick diffusive gels in order to avoid image blurring (Santner et al., 2015). The gels were deployed for 24 h and then retrieved, rinsed with high quality lab water and dried in a gel dryer (Unigeldryer 3545, Laborgeräte and Vertriebs GmbH).

***Preparation of HR-DGT gels calibration standards and LA-ICP-MS analysis***

Calibration standards for the LA-ICP-MS analysis were prepared similar to (Hoefer et al., 2015).

HR-DGT gel discs were cut and placed with a 0.8 mm thick diffusive gel and a 0.45 µm pore size filter membrane in plastic DGT sampler mouldings (DGT Research Ltd., Lancaster, UK). The loaded DGT devices were immersed in 4 L of 1 mM $NaNO_3$ solutions containing different concentrations of As (0.042-1.88 mg $L^{-1}$), Cu (0.02-1.01 mg $L^{-1}$) and P (0.05-2.10 mg $L^{-1}$) and deployed for different time intervals to achieve the desired concentration of the analyte on the resin gel.

For the preparation of Fe standards, resin gels were directly soaked in $FeCl_3$ (Acros Organics, Geel, Belgium) solutions of 122, 244 and 407 mg Fe $L^{-1}$. All the standards were prepared in quadruplicate.

After deployment, resin gels were removed from the mouldings (or just removed from the solutions in the case of Fe standards). One gel was analysed by LA-ICP-MS and the other three replicates were digested using a microwave digestion system (Multiwave 3000, Anton Paar) with 5 mL of 65 % $HNO_3$ and 1 mL of 30 % $H_2O_2$ (Fluka, TraceSelect). The digests were then analysed by ICP-MS (NexION 300XX, Perkin-Elmer) to measure the amount of analyte taken up by the resin gel.

The LA-ICP-MS analysis of HR-DGT gels (samples and standards) was carried out using a UP 193-FX (ESI, NWR Division, Portland OR, USA) laser ablation system coupled to a NexION 300 ICP-MS (Perkin-Elmer). A gel area of approximately 2 × 2 cm was ablated with a line spacing of 200 µm and the following laser parameters: spot size of 100 µm, scan speed of 200 µm $s^{-1}$, energy output of 35% and a laser pulse frequency of 8 Hz. The spatial resolution achieved with this method was 76.4 × 200 µm.

***Data analyses***

Statistical analysis of the data was performed using IBM SPSS Statistics 21. All data were checked for normality and homoscedasticity. Data were analysed by analysis of variance (One-way ANOVA). Differences among treatments were established by Tukey´s HSD test or by the Games-Howell test for heteroscedastic data. Bivariate correlations between variables were evaluated with the Pearson´s test.

Chemical images of element distributions around roots were generated using the ImageJ software (http://imagej.nih.gov/ij/, National Institutes of Health, Bethesda, USA).

## 4.2.3. Results

***Treatments and plant effects on soil chemistry***

Changes in porewater composition were monitored throughout the experiment in both bulk and rhizosphere soil (Fig. 4.2.1). In addition, the treatment- and plant-induced changes in the available fraction of several elements were evaluated by different chemical extractions in bulk and rhizosphere soils at the end of the experiment (Fig. 4.2.2).

The addition of Fe+lime and Fe+BC significantly decreased ($P$ < 0.01) the concentration of As in the bulk soil porewater throughout the experiment (Fig. 4.2.1). Similarly, As concentration in the rhizosphere soil porewater was lower in the treated soils than in the control, but in this case the difference among them was less pronounced; soluble As was generally lower in Fe+BC than in control, but not significant in all cases, while in Fe+lime it was always significantly lower ($P$ < 0.01) than in the control.

In general, a slight increase in the concentration of As was observed in the rhizosphere porewater with respect to the bulk soil in all treatments (Fig. 4.2.1). In the control, this difference varied between 2 and 29%, but significant differences ($P$ < 0.05) were only found at days 14 and 42, where the increment in soluble As in the rhizosphere was 24% and 29%, respectively. In treatment Fe+lime, As concentration in the rhizosphere soil porewater was up to 50% greater than in bulk soil, however, due to the high variability of the data, significant differences ($P$ < 0.01) between soil compartments were only found at day 48, when porewater As concentration in the rhizosphere compartment was >500% higher than in the bulk soil. The concentration of As in Fe+BC-treated soil porewater was also generally higher in the rhizosphere than in the bulk soil, this difference accounted for 50-462%; similar to control, significant differences ($P$ < 0.05) were only found at days 14 and 42 in this treatment.

In the bulk soil, Cu concentration in porewater was significantly reduced ($P$ < 0.05) by both treatments with respect to the control soil (Fig. 4.2.1). In the control, soluble Cu was significantly lower in rhizosphere than in bulk soil porewater ($P$ < 0.05 at day14 and $P$ < 0.01 at days 28 and 48), but little difference between soil compartments was found in the treated soils.

At day 14, P concentration was significantly higher ($P < 0.05$) in rhizosphere than in bulk soil porewater in Control, Fe+lime and Fe+BC. From day 28 onwards, P concentration dropped to reach similar values than in the bulk soil (Fig. 4.2.1), especially in Fe+lime and Fe+BC. After this, P concentration in both compartments remained similar over the experiment.

As shown in Figure 4.2.1, a clear trend in the soluble Fe could not be established.

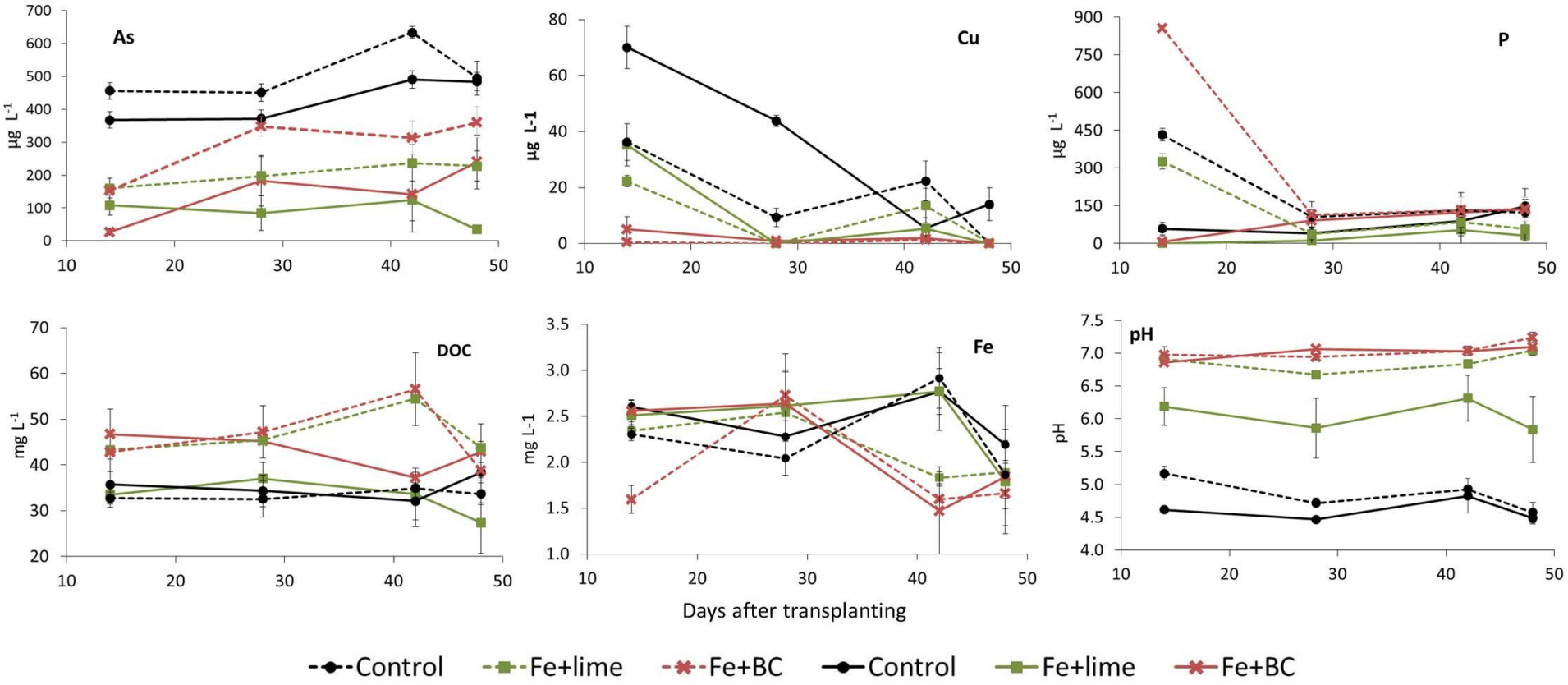


**Figure 4.2.1.** Concentration of As, Cu, DOC, P and Fe and pH in bulk (continuous lines) and rhizosphere (dotted lines) soil porewater. Samples were collected 14, 28, 42 and 48 days after white lupin was transplanted. Data are mean (n = 4) ± SE.

Both soil treatments significantly increased bulk porewater pH ($P < 0.05$) with respect to the control soil (Fig. 4.2.1). Significantly higher pH ($P < 0.05$) was observed in control-rhizosphere soil after 14 days of plant growth, although thereafter, porewater pH was similar in both soil compartments. In Fe+lime, porewater pH was between 0.5 and 1.2 pH units higher in rhizosphere than in bulk soil, but no statistical differences could be established, likely due to the high variability of the data. Similar pH was found in both soil compartments in Fe+BC.

DOC concentration in porewater did not greatly differ between rhizosphere and bulk soil and a clear trend could not be established for any treatment (Fig. 4.2.1).

Addition of Fe+lime and Fe+BC resulted in a significant decrease ($P < 0.05$) of the $(NH_4)_2SO_4$-extractable As fraction both in bulk and rhizosphere soil (Fig. 4.2.2). Extractable As was significantly higher ($P < 0.05$) in the rhizosphere of Fe+lime than in the bulk soil, but no differences were found in Fe+BC and the control between soil types.

The treatments significantly reduced the $(NH_4)_2SO_4$-extractable Cu concentration in bulk and rhizosphere soil ($P < 0.01$), with respect to control (Fig. 4.2.2). Significantly lower ($P < 0.01$) concentration of extractable Cu was found in the rhizosphere compartment of control soil, compared to bulk soil, while no differences were observed between rhizosphere and bulk soil in Fe+lime and Fe+BC.

The effect of the treatments on P-Olsen was evident in both rhizosphere and bulk soils (Fig. 4.2.2). The addition of Fe+BC significantly increased ($P < 0.05$) extractable-P in both soil compartments when compared to control and Fe+lime. No statistical differences were found between soil types for any treatment.

Even though there were no statistical differences in the $(NH_4)_2SO_4$-extractable Fe between both soil compartments of control and Fe+BC, its concentration was slightly higher in rhizosphere than in bulk soil (Fig. 4.2.2). That difference was greater in Fe+lime, where extractable Fe was significantly higher ($P < 0.05$) in rhizosphere than in bulk soil.

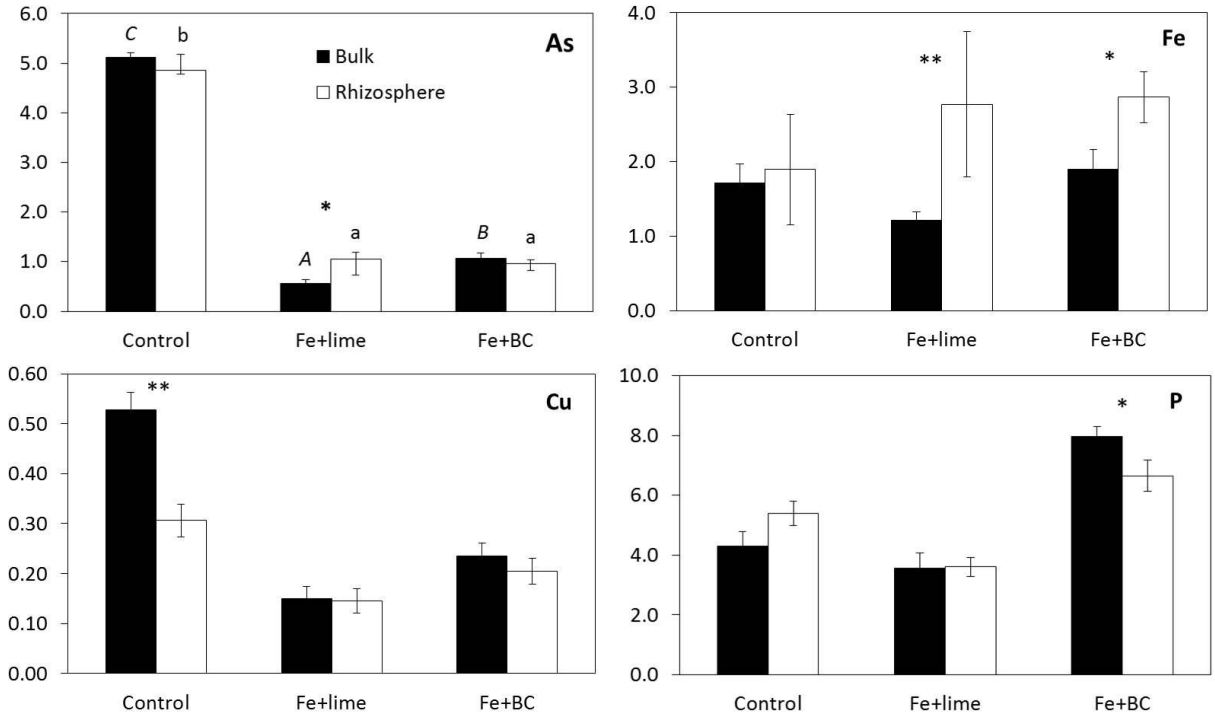


**Figure 4.2.2.** Concentration of $(NH_4)_2SO_4$-extractable As, Fe and Cu and P-Olsen (mg $kg^{-1}$ soil DW) in bulk and rhizosphere soils at the end of the experiment. Data are mean (n=4) ± SE. Different letters above bars indicate significant differences between treatments for each soil type ($P < 0.05$). Asterisks indicate significant differences between rhizosphere and bulk soil ($P < 0.05$). Where no letters or asterisks are indicated, no differences were found.

***Elemental distribution in the rhizosphere***

Figure 4.2.3 shows the distribution of labile As, Fe, P and Cu around a selected white lupin root grown in the non-treated composite soil (waste material:uncontaminated soil, 1:9) for 15 days.

For As distribution in the white lupin rhizosphere, several zones of increasing/decreasing fluxes were observed. As expected, As was depleted along the root axis. Close to this depletion zone, in the vicinity of the principal root and in several areas near root tips, a slight increase in labile As concentration was observed.

A wide Cu depletion zone (~2 mm width) was observed along the root axis. Cu solubilisation was not detected around the selected lupin root. Phosphorus was depleted near the root in a narrow area. Several zones of increased labile P concentration were also found located near the root, although in this case the increasing P-flux was very heterogeneous compared to As and Fe. Similar to As, an increase in Fe DGT-flux was observed around lupin roots. In this case, the increasing Fe-flux area extended about 1 mm from the depletion zone along the root axis.

Data of several areas in the images of As, P and Fe were evaluated in order to quantify the increase in the element fluxes observed in selected parts of the investigated soil. Therefore, three areas were selected in each image; the areas corresponded either to soil directly affected by root activities, *i.e.* rhizosphere soil (area 1, Fig. 4.2.3), a zone where we found an increase in the element DGT flux, likely due to the suspected presence of a root tip (area 2, Fig. 4.2.3) or to soil separated about 5 mm from the root surface, considered as bulk soil (area 3, Fig. 4.2.3). No areas were selected in the Cu image, as no changes were observed in the measured DGT flux. The average DGT flux (pg $cm^{-2}$ $s^{-1}$) was calculated and the maximum single pixel value (pg $cm^{-2}$ $s^{-1}$) was determined in each area. The results obtained are shown in Table 1.

Results on the average DGT-measured fluxes (pg $cm^{-2}$ $s^{-1}$) showed an increase in the As flux of 12% and 17% in As1 and As2, respectively, with respect to As3 (Table 4.2.1). A larger difference between areas was observed in the Fe image; here the average DGT-flux in both areas Fe1 and Fe2 was ~70% higher than in Fe3. The increase in P DGT-flux in P1 and P2 accounted for 10% and 12%, respectively, with respect to P3.

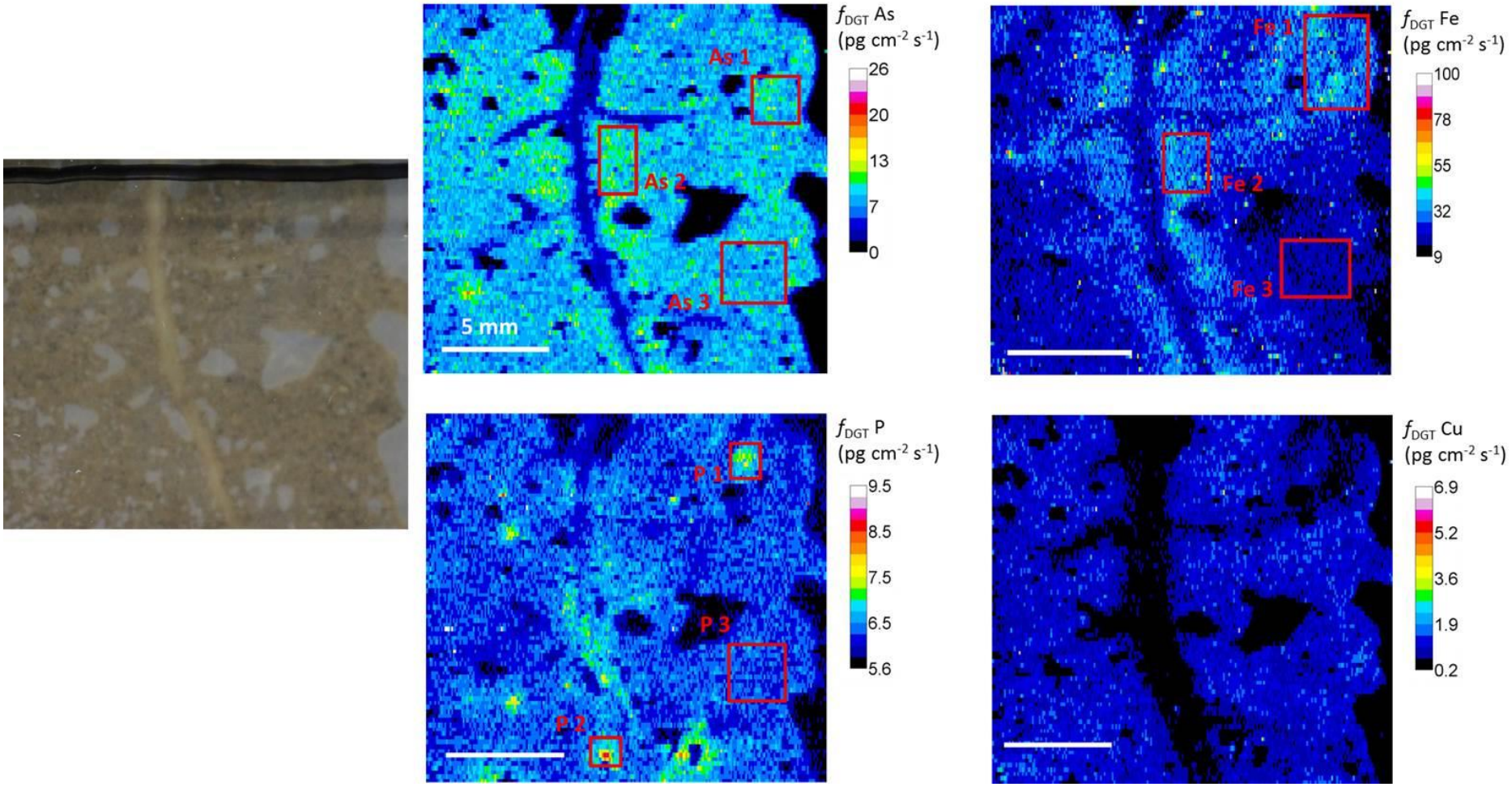


**Figure 4.2.3.** Photograph of a white lupin root selected for chemical imaging (left) and two-dimensional distribution of As, Fe, P and Cu around the root. The chemical images were obtained by applying the HR-DGT gel for 24 h after the plant was growing for 2 weeks in the non-amended soil and represent DGT-measured fluxes (pg cm$^{-2}$ s$^{-1}$).

**Table 4.2.1**. As, Fe and P DGT-fluxes in the areas selected in Figure 3. Area 1 (As/Fe/P 1) corresponds to rhizosphere soil, area 2 (As/ Fe/ P 2) is close to a suspected root tip and area 3 (As/Fe/P 3) is the bulk soil. Data for average values (pg $cm^{-2}$ $s^{-1}$) are mean (n > 30) ± standard deviation; maximum value is the maximum single-pixel value (pg $cm^{-2}$ $s^{-1}$) in each area.

| | **As** | | | **Fe** | | | **P** | | |
|---|---|---|---|---|---|---|---|---|---|
| | **As 1** | **As 2** | **As 3** | **Fe 1** | **Fe 2** | **Fe 3** | **P 1** | **P 2** | **P 3** |
| **Average (pg $cm^{-2}$ $s^{-1}$)** | 8.50 ± 0.61 | 8.91 ± 0.52 | 7.61 ± 0.31 | 30.3 ± 2.2 | 30.2 ± 2.2 | 17.9 ± 0.8 | 6.95 ± 0.12 | 7.08 ± 0.28 | 6.32 ± 0.06 |
| **Max. value (pg $cm^{-2}$ $s^{-1}$)** | 9.63 | 9.94 | 8.30 | 35.3 | 35.0 | 20.1 | 7.09 | 7.45 | 6.44 |

***Arsenic and copper plant uptake, As speciation in roots and plant growth***

Arsenic concentration in shoots was significantly lower ($P < 0.05$) in Fe+BC-treated plants than in those grown in the control soil (Table 4.2.2), while no effect was observed in As concentration in roots with any treatment. Neither total As plant uptake nor As translocation from roots to shoots was affected by the treatments. Root uptake efficiency (calculated as the total plant uptake divided by root dry weight) was only reduced in Fe+BC treatment ($P < 0.05$).

No statistical differences were observed regarding Cu concentration in both shoots and roots with any treatment (Table 4.2.2). Plant Cu uptake and root uptake efficiency were, although not significantly, slightly higher in plants grown in the treated soils than in those grown in the control soil.

The analysis of As species in PBS-extracts showed that only inorganic species were present in lupin roots, *i.e.* arsenate (As(V)) and arsenite (As(III)) (Fig. 4.2.4). In all cases As(III) was the predominant species, accounting for >70% of the total As in the extracts. The percentage of As(V) in the extracts was significantly higher ($P < 0.05$) in the control roots (30 ± 4%) than in roots treated with Fe+lime (18 ± 4%) and Fe+BC (7 ± 1%).

No significant differences in shoot and root biomass were found among treatments (Table 4.2.3), although a slight increase was observed in shoot growth when plants were grown in the Fe+BC-treated soil.

**Table 4.2.2.** Arsenic and Cu concentration in shoots and roots of white lupin, their translocation factor (calculated as the ratio shoot/root concentration); total plant uptake and root uptake efficiency. Mean (n = 4) ± SE. Analysis of variance (One-way ANOVA, *P* value); different letters in the same column indicate significant differences among treatments (Tukey HSD test at $P < 0.05$; *N.S.* not significant).

| Treatment | Shoots (mg $kg^{-1}$) | | Roots (mg $kg^{-1}$) | | Translocation factor | | Plant uptake (µg $plant^{-1}$) | | Root uptake efficiency (µg $g^{-1}$ root DW) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **As** | **Cu** | **As** | **Cu** | **As** | **Cu** | **As** | **Cu** | **As** | **Cu** |
| **Control** | 10.5 ± 1.4$^{b}$ | 1.6 ± 0.8 | 227 ± 25 | 12.9 ± 1.7 | 0.047 ± 0.008 | 0.13 ± 0.06 | 68.2 ± 6.5 | 14.6 ± 1.8 | 257 ± 28$^{b}$ | 55.8 ± 8.4 |
| **Fe+lime** | 8.7 ± 0.4$^{ab}$ | 6.3 ± 2.9 | 185 ± 34 | 19.9 ± 3.6 | 0.052 ± 0.008 | 0.32 ± 0.10 | 64.4 ± 10.1 | 26.3 ± 5.4 | 206 ± 35$^{ab}$ | 93.7 ± 29.7 |
| **Fe+BC** | 5.1 ± 1.3$^{a}$ | 2.3 ± 0.8 | 135 ± 13 | 28.9 ± 7.7 | 0.039 ± 0.012 | 0.10 ± 0.05 | 55.1 ± 9.5 | 45.8 ± 15.4 | 150 ± 14$^{a}$ | 79.5 ± 26.5 |
| **ANOVA** | 0.023 | N.S. | N.S. | N.S. | N.S. | N.S. | N.S. | N.S. | 0.050 | N.S. |

**Table 4.2.3.** Dry weights (DW) of white lupin shoots and roots. Data are means (n = 4) ± SE. Analysis of variance (One-way ANOVA) evaluates differences among treatments (*N.S.* not significant).

| Treatment | Shoots DW (g) | Roots DW (g) |
|---|---|---|
| **Control** | 0.79 ± 0.19 | 0.27 ± 0.03 |
| **Fe+lime** | 0.72 ± 0.15 | 0.32 ± 0.10 |
| **Fe+BC** | 1.07 ± 0.34 | 0.37 ± 0.11 |
| **ANOVA** | N.S. | N.S. |

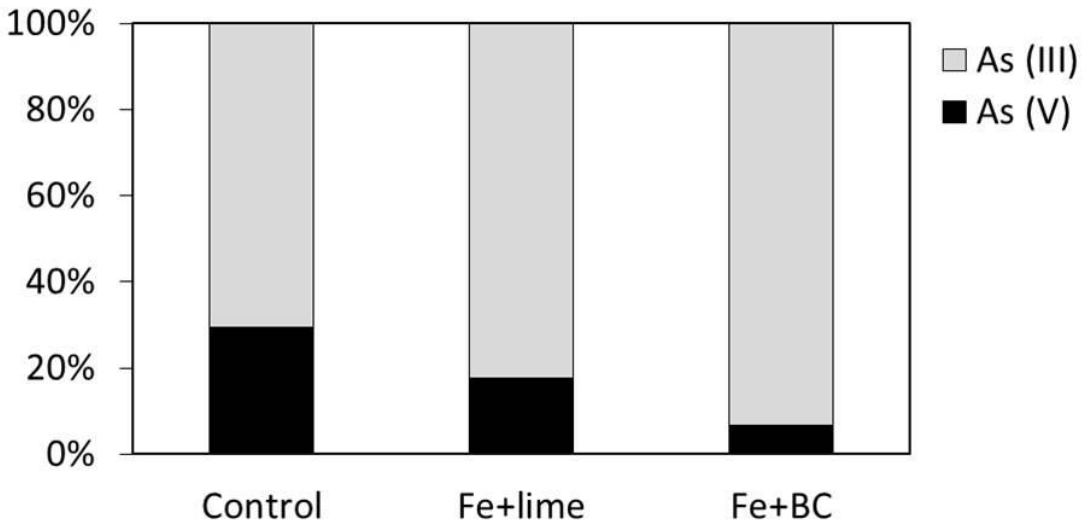


**Figure 4.2.4.** Distribution of As species in the PBS-extracts of white lupin roots. Data are means (n = 4).

## 4.2.4. Discussion

***Arsenic changes in rhizosphere and bulk soil and plant As uptake***

Both treatments were effective at decreasing the soluble (Fig. 4.2.1) and exchangeable As fractions (Fig. 4.2.2), as well as at increasing porewater pH (Fig. 4.2.1) with respect to the control. However, a slight increase in soluble As was observed in the rhizosphere soil of all treatments with respect to the bulk soil, besides slightly higher As-DGT flux around lupin roots than in the bulk soil in the chemical images (Fig 4.2.3). Arsenic solubilisation in the rhizosphere seemed to occur independently of the concentration of As in soil porewater (Fig. 4.2.1); indeed, the difference in soluble As between the rhizosphere and bulk soils was in general smaller in the control than in the treated soils.

Changes in the As biogeochemistry in the rhizosphere are known to be closely related to other elements, such as P and Fe, which are strongly affected by root exudates and plant-induced changes in soil pH (Fitz and Wenzel, 2002). Hence, the slight As solubilisation we observed in the rhizosphere of white lupin might be a consequence of co-solubilisation processes and interactions of As with other elements.

In this work, we observed higher P concentration ($P < 0.05$) in the rhizosphere porewater after 14 days of lupin growth (Fig. 4.2.1), besides several zones of enhanced P-DGT flux close to root tips of a 2-weeks old lupin plant grown the non-amended soil in rhizotrons (Fig. 4.2.3). These observations suggest that activities of roots or associated microorganisms apparently have caused P mobilisation in the rhizosphere. White lupin is well known to excrete high amounts of organic anions from roots, such as citrate and malate, mainly as a response to P deficiency (Dinkelaker et al., 1989; Jones, 1998). Therefore, it could be expected that the low P concentration measured in the bulk soil solution of all treatments in the pot experiment (6.1 to 58.7 µg $L^{-1}$) induced the release of carboxylates. Due to the similarities in P and As chemical properties, such as salt solubility and acid dissociation constants (Adriano, 2001), concomitant mobilisation of P and As triggered by organic anions released from roots can be expected.

It is important to note that, although both P and As were apparently mobilised in the rhizosphere at day 14, they behaved differently thereafter. Whereas P

concentration decreased from day 14 onwards and then remained constant, As concentration was similar or even increased along the experiment.

The reduction or increase of element concentration in soil solution depends on the ability of the plant to deplete the element from soil solution and on the capacity of the soil to replenish the soluble and exchangeable forms of the element (Hinsinger, 1998; Hinsinger, 2001). Since arsenate and phosphate share the same uptake system, which has shown higher affinity for phosphate (Meharg et al., 1994), the higher molar concentration of P than of As in porewater at day 14 (2.3-, 5.0- and 13.8-fold in Control, Fe+lime and Fe+BC, respectively), could have mitigated, although not suppressed, As uptake, thus keeping similar As concentration along the experiment.

Constant and fast replenishment of As from less labile forms, but not of P, could also explain the different behaviour of these elements. Carboxylates excreted by roots may rapidly dissolve amorphous iron oxides and form stable complexes with $Fe^{+3}$, especially in soils with pH <6.5 (Reichard et al., 2007). The mobility of As in soils is mostly controlled by co-precipitation and sorption onto the surface of Fe (Mn, Al) oxides (Fitz and Wenzel, 2002; Moreno-Jiménez et al., 2012). Therefore, dissolution of these oxides by organic anions or by reductive reactions might trigger the mobilisation of As associated to this soil fraction (Onireti and Lin, 2016). This would be in accordance with the increase of ~70% of Fe DGT-flux in the lupin rhizosphere (Fe1 and Fe2, Fig. 4.2.3, Table 4.2.1) with respect to the bulk soil (Fig. 4.2.3, Table 4.2.1) observed by chemical mapping. Although in a lower extent, a slight enhancement of labile As was also observed in the vicinity of lupin roots, where As DGT-flux was up to 12% higher close to the roots than in the bulk soil (Fig. 4.2.3, Table 4.2.1). An increase in As and Fe bioavailability due to their co-solubilisation can be expected. In this work, a significant increase ($P < 0.05$) in $(NH_4)_2SO_4$-extractable As and Fe was observed in Fe+lime rooted compartment at the end of the pot experiment, whereas no differences were found in the control and Fe+BC. The greater effect observed in Fe+lime could be explained by the large solubilisation of As found in the rooted compartment at day 48, when As concentration in rhizosphere porewater was 500% higher than in bulk soil. This suggests that plant-induced dissolution of iron oxides may have triggered As mobilisation from less available (such as As bound to iron oxyhydroxides) to a more easily exchangeable fraction, that can be extracted with $(NH_4)_2SO_4$ (Wenzel et al., 2001), in agreement to that observed by Gonzaga et al. (2006), who reported the greatest depletion in the amorphous (hydr)oxide-bound As fraction upon cultivation of As hyperaccumulator and non hyperaccumulator ferns. Lower redox potential in the rhizosphere can also induce reductive dissolution of iron

(hydr)oxides and thus As release, as suggested by Fitz et al. (2003). However, in the present work iron solubilisation was not reflected in the porewater, likely due to the relatively high Fe concentration in porewater (1-3 mg $L^{-1}$), which hinders seeing changes between soil compartments.

Several factors and mechanisms have been described in literature affecting metal(loid) mobility in the rhizosphere of accumulator and non-accumulator plants (McGrath et al., 2001; Fitz and Wenzel, 2002; Puschenreiter et al., 2005; Silva Gonzaga et al., 2006), root exudation and changes of soil pH being the most important ones.

Although acidification of the lupin rhizosphere would be expected due to its $N_2$ fixing activity (Bolan et al., 1991), in this work no acidification was observed and even alkalinisation was found in Control and Fe+lime at some sampling times (Fig. 4.2.1). The similar pH values in rhizosphere and bulk soil of Fe+BC could be related to its higher pH (6.6-7.1) or to an increase in soil cation exchange capacity caused by biochar addition (Beesley et al., 2011). The efflux of $citrate^{3-}$, which is the likely predominant species in the cytosol of root cells, might contribute to an increase in the rhizosphere pH. The $pKa_3$ of citric acid is 6.4, thus in acidic soils $citrate^{3-}$ exuded from roots would react with free $H^+$, leading to alkalinisation of the rhizosphere (Hinsinger et al., 2003). This is in line with the study of Vazquez et al. (2006), who found an increase in soil pH upon white lupin cultivation in an acidic soil. An increase in soil pH generally reduces As sorption, especially in soils rich in metal oxides (Smith et al., 1999). This could also explain the large increase in soluble As found in the rhizosphere in Fe+lime at day 48, when porewater pH was 1.2 units higher in rhizosphere than in bulk soil (Fig. 4.2.1). Indeed, the concentration of As in porewater was significantly correlated with porewater pH in this treatment ($r = 0.701$, $P < 0.01$), which suggests that, besides other mechanisms, the rhizosphere alkalinisation was an important factor affecting As solubilisation.

Results in As plant uptake seem to reflect the slight mobilisation of As observed in the rhizosphere/rooted soil. Despite Fe+lime and Fe+BC treatments led to a significant reduction in the soluble and exchangeable As fractions (Fig. 4.2.1 and 4.2.2), As accumulation in lupin was similar in all treatments (Table 4.2.2). Significantly lower ($P < 0.05$) concentration of As was found in Fe+BC shoots, but it could be related to a slight increase in shoots growth (Table 4.2.3) and therefore due to a dilution effect. Likewise, As root uptake efficiency was significantly lower ($P < 0.05$) in Fe+BC than in Control (Table 4.2.2).

The higher percentage of As(V) in roots of control plants, compared to Fe+lime and Fe+BC, was related to higher As concentration in porewater, which suggests that part of As desorbed from soil would remain on the roots surface, likely associated to iron oxides that precipitated on roots, similarly to that described by Fresno et al. (2016) in a hydroponic experiment.

***Copper mobility and plant uptake***

Results on plant Cu uptake were not consistent with results from soil analyses. While a reduction of soluble (Fig. 4.2.1) and $(NH_4)_2SO_4$-extractable Cu (Fig. 4.2.2) was observed upon addition of Fe+lime and Fe+BC, the treatments did not lead to a reduction in Cu plant uptake (Table 4.2.2).

Copper solubilisation could have occurred due to its complexation by organic anions exuded by roots, as observed by (Tao et al., 2004). However, here we did not see an increase in soluble Cu (Fig. 4.2.1), potentially because the plant Cu uptake rate exceeded the soil capacity to resupply Cu to the soil solution.

The remarkably wider depletion zone observed in the lupin rhizosphere, compared to the other elements assessed (Fig. 4.2.3), besides the significantly lower concentration ($P < 0.05$) of $(NH_4)_2SO_4$-extractable Cu in control rhizosphere soil (Fig. 4.2.2), suggest high Cu uptake efficiency of this plant. In any case, Cu concentration in shoots is far from the toxic limit of 20 $\mu g^{-1}$ DW established for most crop species (Marschner, 2012).

Copper concentration in the soil solution did not exceed 2 µmol $L^{-1}$, even in the control soil (Fig. 4.2.1), and it decreased (or remained very low) throughout the experiment in both bulk and rhizosphere soil in all treatments, so for the soil tested in this study the plant-induced mobilisation of Cu should not represent a problem in terms of toxicity to plants.

### 4.2.5. Conclusions

Despite As immobilisation provoked by the addition of Fe+lime and Fe+BC to the contaminated soil, we found higher soluble As concentration in the *Lupinus albus* rhizosphere soil than in the bulk soil.

Since chemical mapping of the lupin rhizosphere performed by DGT LA-ICP-MS showed an increase of labile Fe and, in a lower extent, of labile As in the vicinity of lupin roots, we hypothesise that As was released by the dissolution of iron oxides, which would be mediated by organic anions excreted by roots, likely as a consequence of low soluble P. Besides, rhizosphere alkalinisation seemed to have enhanced As desorption from soil surface.

Arsenic mobilisation in the rhizosphere was reflected by similar plant uptake between treatments, despite the immobilisation observed in the bulk soil. A similar effect was observed for Cu, suggesting also some plant-induced mobilisation of this element.

Even though root-induced As solubilisation was observed in all treatments, it was not reflected by an increase in As mobility in bulk soil in any treatment. The effect of white lupin on As mobility seems to be highly localised and should not pose a risk in terms of As leaching. However, special care should be taken in controlling As transfer to lupin shoots, especially in contaminated agricultural systems.

# Chapter 5

# General Discussion

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# General Discussion

Although As naturally exists in soils and groundwaters, anthropogenic activities such as mining or smelting processes have increased its concentration, contaminating environmental compartments at a global scale. Arsenic is classified by the WHO as a class 1 carcinogenic and identified as one of the ten chemicals of major public health concern. Copper is an essential micronutrient for plants, but at high concentration, it can result toxic for organisms.

Phytostabilisation technologies aim at stabilising contaminants in the soil and often combine plants and amendments to decrease trace elements mobility, improve soil properties and support plant growth. In As-contaminated soils, iron oxides have been found to stabilise trace elements, but barely improved soil properties. Meanwhile, organic amendments often improved soil fertility, support plant growth and immobilised metals but enhanced As mobility. Therefore, the remediation of multicontaminated soils is a challenge when contaminants have contrasting chemistries.

In this Thesis, we evaluated the combination of an iron amendment (iron sulfate) with several organic materials to remediate an As- and Cu-contaminated soil. For that, we established two investigation lines. i) Firstly, we evaluated the effect of several soil treatments on As and Cu geochemistry, analysing their mobility and distribution in the soil. The improvement of soil functions was also evaluated using several parameters such as soil nutrients and the establishment of a plant cover (Chapter 3). ii) Secondly, the influence of iron-based amendments on As dynamics in the root-soil solution-soil interface (rhizosphere) was investigated (Chapter 4).

### *Effect of soil amendments on As and Cu mobility*

In the pot experiments, all treatments combining iron sulfate and organic materials significantly reduced As mobility. These treatments resulted in a decrease in the extractable (Figs. 3.1.1 and 3.2.2), leachable (Table 3.1.2) and soluble (Fig. 3.2.1) As fractions.

Fractionation of As performed in the iron-treated soils revealed an increase of As associated to amorphous iron (hydr)oxides with respect to the control soils, from 52%

to >80% (F5, Fig. 3.1.2). This suggests that addition of iron sulfate resulted in the formation of additional amorphous iron (hydr)oxides, which greatly affected As mobility in the treated soils.

Despite As mobility was mainly governed by iron oxides, there were other factors also modulating it and they should also be considered when immobilising As in the soil.

The influence of pH on As mobility was evident when Fe+lime was neither able to reduce the extractable nor the leachable As fractions, linked to the highest soil pH in this treatment (chapter 3.1). This was confirmed by the significant positive correlation between soil pH and extractable As, which showed the strong influence of soil pH on As mobility, especially in soils rich in oxide minerals (Fitz and Wenzel, 2002; M Sadiq, 1997). This also explained the greatest reduction in both soluble and extractable As fractions provoked by treatment Fe+BC, the one with the lowest pH.

After these observations, the application rate of $CaCO_3$ was adjusted in treatments Fe+lime, Fe+OMWC and Fe+BC (in this case holm oak biochar) in order to reduce differences in soil pH provoked by the treatments and minimise the effect of pH on As mobility (chapter 3.2). Despite this, we still found differences among treatments, but generally all of them reduced the concentration of extractable and soluble As in spite of the higher soil pH in the treated soils (Figs. 3.2.1 and 3.2.2). In view of this, when soil pH was adjusted to a neutral range (5.5-7), amorphous iron oxides efficiently controlled As mobility, mitigating the effects of soil pH.

Nevertheless, in this work we found other factors affecting soluble and extractable As. In view of the linear regression equations 3.2.1 and 3.2.2, competition between arsenate and phosphate for soil sorption sites might result in an increase in extractable As, while porewater pH seemed to be the most influential factor on soluble As among treated soils. Although competition between DOC and arsenate for soil sorption sites has been reported (Bauer and Blodau, 2006; Sharma et al., 2011; Arco-Lázaro et al., 2016), in view on that observed in Chapter 3.2., DOC did not seem to affect As solubility.

In order to observe the effect of iron and organic amendments in a longer term, the previous experiments were upscaled (chapter 3.3.); we used lysimeters (macrocosms of 240 $dm^3$, approximately 120 kg of soil) instead of pots and investigated the remediation process over almost 2 years without controlling the climatic conditions. In this work we assessed the same combinations of amendments, but differing in the

application rates of the organic materials, and we included green waste compost (GWC) as soil organic amendment to combine with iron sulfate. The effect of the treatments in this experiment did not fully match results obtained in the pot experiments (chapters 3.1 and 3.2), highlighting the importance of validating results at more realistic and closer to the field conditions.

Although As mobility changed along the macrocosm experiment and was differently affected by the treatments, the results were not similar to the pot experiments because the treatments did not reduce As mobility with respect to the control soil. Since no correlation between soil pH and extractable As was found at any sampling time, it is likely that the higher application of the organic amendments could have affected As mobility to some extent.

A remarkable result in this experiment was the increase in the concentration of extractable As between 14 and 20 months in all soils, including the control. This could be related to the transformation of iron (hydr)oxides to more crystalline phases, and hence the reduction in As adsorption capacity, or to the effect of ageing on As distribution in the original waste material, rather than due to specific treatment effects. In addition, it is possible that in this experiment DOM had a stronger influence on As mobility than in the previous experiments, since after 20 months extractable As was slightly higher in Fe+PS, Fe+BC and Fe+OMWC than in Fe+lime and the control, and was positively correlated with DOC concentration in the porewater.

We also investigated changes in the porewater chemistry between 15 and 20 months after the addition of the treatments. Soluble As was significantly higher only in Fe+PS, likely due to a concomitant effect of the increase in pH and DOC in this treatment, whereas the rest of the treatments did not significantly affect As solubility.

Noteworthy from this experiment, despite the intense application of organic matter and subsequent increase in DOC (Fig. 3.3.4), As was not significantly mobilised after the coapplication with $FeSO_4$, contrastingly to previous experiments, where strong As mobilisation was observed with single applications of compost or biochar (Clemente et al., 2010; Beesley et al., 2014; Moreno-Jiménez et al., 2013; Beesley et al., 2013; Manzano et al., 2016).

In several studies, Cu dynamics in soils has been shown to be significantly affected by soil pH and organic matter, being the former the most influential factor (Kumpiene et al., 2007; Soler-Rovira et al., 2010; Beesley et al., 2010; Forján et al., 2016). Based on the results obtained in chapter 3, we can support this statement.

Although we observed that the addition of Fe+OMWC and Fe+BC increased the Cu fraction associated to organic matter (Fig. 3.1.2), the effect on the most labile fraction ($CaCl_2$-extractable) was mainly related to changes in soil pH provoked by the treatments.

When soil pH was further controlled (due to the adjustment of lime application rates, chapter 3.2), all treatments effectively reduced the most labile Cu soil fractions (soluble and extractable). Soil pH was again the most influential factor affecting Cu mobility, although the formation of soluble Cu-DOM complexes was clear in treatment Fe+OMWC (Fig. 3.2.1, Table 3.2.5).

Similar results were obtained in the macrocosm experiment (chapter 3.3), where all treatments resulted in a significant and long-lasting reduction of both extractable and soluble Cu fractions. Its mobility seemed to be again mainly governed by effects of the treatments on soil pH, and maybe other physico-chemical properties (such as CEC), as in this case no correlation was found between DOC and labile Cu concentrations in the treated soils.

Since Cu is an essential element for plants, its available concentration in contaminated soils should be reduced but kept in a concentration high enough to assure plant nutrition. Based on the lack of Cu deficiency or toxicity symptoms in plants of any experiment and given that Cu concentration in shoots was in all cases within the sufficiency range (Mills et al., 1996; Marschner, 2012 ), we do not expect a negative effect of the treatments in terms of Cu nutrition in the tested soil.

In summary, chapter 3 showed that the combination of iron sulfate with organic materials is a suitable tool to reduce As and Cu mobility; the iron sulfate application prevented from any As mobilisation and the increase in soil pH provoked by the treatments reduced Cu mobility. However, the application rates tested in small scales and short-term experiments should not be extrapolated to the field. Larger scale and longer-term experiments should be additionally carried out in order to investigate the effects of soil heterogeneity, ageing and environmental factors before establishing a remediation strategy *in situ*.

***Improvement of soil functions: establishment of a plant cover***

Since the presence of plants can minimise the dispersion of contaminants, establishment and development of a healthy vegetation cover was a main goal of the remediation strategy evaluated. For that, an improvement of soil functions is essential.

The economical sustainability of phytostabilisation strategies can be achieved by selecting plants that provide added value. Co-cropping or rotation of tolerant species with $N_2$-fixing legumes, such as white lupin, can provide atmospheric $N_2$-fixation inputs, which may represent a benefit in terms of soil fertility and growth of other species (Wong, 2003; Reichman, 2007; Fumagalli et al., 2014). Winter rye (*Secale cereale* L.) straw and grains can be used to produce biogas and ethanol and, due to its high resistance and tolerance to a wide range of climatic conditions, this species can be potentially grown throughout the majority of Europe (Petersson et al., 2007; Tuck et al., 2006).

The results on nutrient availability in a short-term (chapters 3.1 and 3.2) revealed that Fe+lime and Fe+PS had little effect on soil nutrients, as their application did neither result in an enhancement of extractable K, Mg and P nor in total organic carbon and total nitrogen (TOC and TN) (Tables 3.1.3 and 3.2.2 and Figure 3.2.2).

Fe+OMWC supplied nutrients, as shown by the increased concentrations of exchangeable K, Mg, available P and TOC and TN contents (Tables 3.1.3 and 3.2.2 and Figure 3.2.2), and also slightly increased soil pH (Fig. 3.1.1 and 3.2.2), what generally improves soil fertility and plant nutrition. This treatment also led to an enhancement of *A. elatius* and white lupin growth.

The effect of Fe+BC on soil nutrients depended on the feedstock used: olive tree pruning-BC or holm oak-BC. Although both increased TOC and TN contents, holm oak-BC had generally greater effect on K, Mg and P availability. Relative to plant growth, we found a decrease in *A. elatius* biomass production when using olive tree pruning-BC, likely related to the decrease in soil pH, while holm oak-BC improved white lupin roots and shoots growth.

Since effects of soil treatments on soil functions may be overestimated in short-term experiments carried out in microcosms, we also evaluated the effect of combining iron sulfate and organic amendments almost two years after their application (chapter 3.3). The greatest effects were provoked by the Fe+OMWC and Fe+BC (holm oak) once again, and also with Fe+GWC. These treatments enhanced macronutrients availability and supplied organic carbon and nitrogen (Table 3.3.7), causing the

highest rye biomass production (Fig. 3.3.5). Treatment Fe+BC provided the best environment for plant growth, as its addition resulted not only in the highest shoots dry weight but also in greater and more homogeneous plant cover (Fig. 3.3.6).

Our results supports previous research (Pardo et al., 2014; Gil-Loaiza et al., 2016; Touceda-González et al., 2016), which found general improvement of soil physico-chemical properties and plant growth upon addition of organic amendments to contaminated soils.

The improvement of soil health was also evaluated by several enzymatic activities and by toxicity bioassays. As shown in chapter 3.1, the toxicity bioassays performed, *i.e. Lactuca sativa* germination success and toxicity of soil leachates towards the marine bacteria *Vibrio fischeri* (Table 3.1.4), did not match the chemical evaluation, suggesting that other factors can affect these organisms rather than the presence of pollutants.

As shown in Chapter 3.3, dehydrogenase activity was enhanced by most of the treatments with respect to the control soil, showing a general improvement of soil health (Fig. 3.3.7). The fact that this enzyme activity was lower in Fe+BC-treated soil than in the other treatments after two years of the remediation strategy implementation suggests that BC provided more stable C source than PS and both composts, which can mean an advantage in terms of C sequestration in soils.

The effects on acid phosphatase and β-glucosidase activities are more difficult to link to an improvement of soil quality, as other factors such as soil pH and the availability of nutrients may affect these soil enzymes.

***Influence of the rhizosphere on As mobility and its uptake by Lupinus albus L.***

Mitigation of potentially toxic elements plant uptake and their transfer to the food chain is one of the major concern issues when aided phytostabilisation is implemented. Since root activities may alter the dynamics of trace elements in the rhizosphere, the interaction of iron-based amendments and As in white lupin roots and rhizosphere was also investigated in this thesis.

The formation of an iron plaque on the surface of lupin roots was induced by adding a high concentration of iron sulfate to the growth medium (chapter 4.1). Our aim was to evaluate the effects of this plaque on As uptake and its distribution within

the plant. The results demonstrated that iron plaque can be formed on the roots of white lupin under aerobic conditions. This plaque was able to sequester As on the roots surface, thus mitigating its absorption and its transfer to the shoots (Table 4.1.2). Our hypothesis is that iron plaque can be also formed in the rhizosphere of plants growing in oxic soils, which would mean that As stabilisation due to formation of iron oxides would not only occur in the bulk soil, but also in the soil-plant interface.

In a rhizobag experiment (chapter 4.2), we found As mobilisation in the lupin rhizosphere despite soil amendments resulted in a significant decrease in soluble and extractable As fractions in the bulk soil (Figs 4.2.1 and 4.2.2). This mobilisation was even greater in the iron-treated soils than in the control. Arsenic mobilisation was likely provoked by exudation of organic compounds as a response to low soluble P. Chemical mapping of the lupin rhizosphere revealed Fe and As co-solubilisation in the root vicinity (Fig. 4.2.3), which suggests that root exudates triggered the dissolution of iron oxides and therefore As release, likely enhanced by slight rhizosphere alkalinisation, especially in Fe+lime. Due to this, As uptake by white lupin was similar in the control and the treated soils.

These results show that iron enrichment in the rhizosphere not always mitigates As uptake, as root activities may increase As phytoavailability. Therefore, the selection of plants and amendments should be done taking into account rhizosphere processes.

The proportion of As(V) in roots was higher when As concentration increased in soil porewater, suggesting that part of the As released from iron oxides could precipitate on roots surface, likely associated to iron. Although this effect did not seem to reduce As transfer to the shoots, in contrast to that observed in chapter 4.1, these results suggest that manipulating iron in the rhizosphere can lead to iron plaque formation and help to mitigate As transfer to shoots.

# Capítulo 6. Conclusiones

# *Chapter 6. Conclusions*

# Conclusiones

De los resultados obtenidos en la presente Tesis Doctoral se pueden extraer las siguientes conclusiones:

- La aplicación de sulfato de hierro y materiales orgánicos al suelo da lugar a la formación de óxidos amorfos de hierro, que son los que principalmente controlan la movilidad del As. La aplicación de materia orgánica no interfiere en la capacidad de los óxidos de hierro para adsorber As y son otros factores, especialmente el pH, los que tienen una cierta influencia. Los factores más influyentes en la dinámica del Cu en los suelos tratados son el pH y la materia orgánica.

- Al aumentar la escala de trabajo, se observa una reducción de la efectividad de los óxidos de hierro para controlar la movilidad del As y el aporte de materia orgánica cobra más importancia en la dinámica del As en el suelo.

- La combinación de sulfato de hierro y compost o biochar mejora las funciones del suelo, aumentando la disponibilidad de nutrientes y estimulando el crecimiento vegetal. La enmienda orgánica más prometedora es el biochar (de encina), que da lugar a una cubierta vegetal más homogénea y a una mayor producción de biomasa (centeno).

- Los resultados obtenidos resaltan la necesidad de realizar estudios a mayor escala y más largo plazo antes de implementar una estrategia de recuperación *in situ.*

- La formación de una placa de hierro sobre las raíces de *Lupinus albus* L. (altramuz) da lugar a una reducción en la absorción y la transferencia de As a la parte aérea de la planta.

- Pese a la estabilización de As provocada por la enmiendas de hierro, se observó una ligera movilización de éste en la rizosfera de altramuz, ligada a la dinámica de otros elementos. Esta movilización dio lugar a una acumulación de As en la planta similar a la del suelo control. Estos resultados enfatizan la necesidad de estudiar procesos rizosféricos cuando se evalúe una estrategia de fitoestabilización.

**La combinación de sulfato de hierro y enmiendas orgánicas se presenta como una alternativa adecuada para la recuperación de suelos contaminados con As y Cu mediante fitoestabilización asistida, ya que reduce o mantiene estable la movilidad de estos elementos y da lugar a una mejora global de las funciones del suelo. Además, esta estrategia presenta la ventaja adicional del reciclaje en el suelo de residuos orgánicos, aplicados como enmiendas en forma de compost y biochar.**

# Conclusions

From the results obtained in this PhD Thesis, the following conclusions can be drawn:

- The addition of iron sulfate and organic materials to soil leads to the formation of amorphous iron oxides, which mainly control As mobility. Organic matter addition did not interfere with the capacity of iron oxides to sorb As and other factors, especially soil pH, have certain influence. The most influential factors on Cu dynamics in the treated soils are pH and organic matter.

- In larger scale, iron oxides are found to be less effective at controlling As mobility and organic matter addition seems to have relatively more influence on As dynamics in soil.

- The combination of iron sulfate and compost or biochar improves soil functions, increases nutrients availability and stimulates plant growth. Biochar (holm oak) is the organic material that shows the most promising results, as it creates the most homogeneous plant cover and better enhances biomass production (rye).

- The results obtained highlight the importance of performing larger-scale and larger-term studies before *in situ* implementation of a soil remediation strategy.

- Iron plaque formed on white lupin roots provokes a decrease in As uptake and translocation to the shoots.

- In spite of the As stabilisation provoked by the amendments, slight As mobilisation was found in the rhizosphere of white lupin, which seems to be linked to other elements dynamics. This mobilisation led to similar As uptake in the control and the treated soils. These results emphasise the necessity of investigating rhizosphere processes when evaluating phytostabilisation strategies.

**Combining iron sulfate and organic materials is shown as a suitable alternative for aided phytostabilisation of As- and Cu-contaminated soils, as it reduces or stabilises the mobility of these trace elements. Besides, this strategy presents the advantage of recycling organic wastes by using them as amendments such as compost and biochar.**

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# ANEXO

# ANNEX

# ANEXO / ANNEX


**Figure A-1**. Lupinus albus plants grown for 48 days in the control and in the soils treated with Fe+lime, Fe+PS, Fe+OMWC and Fe+GWC (Chapter 3.2).

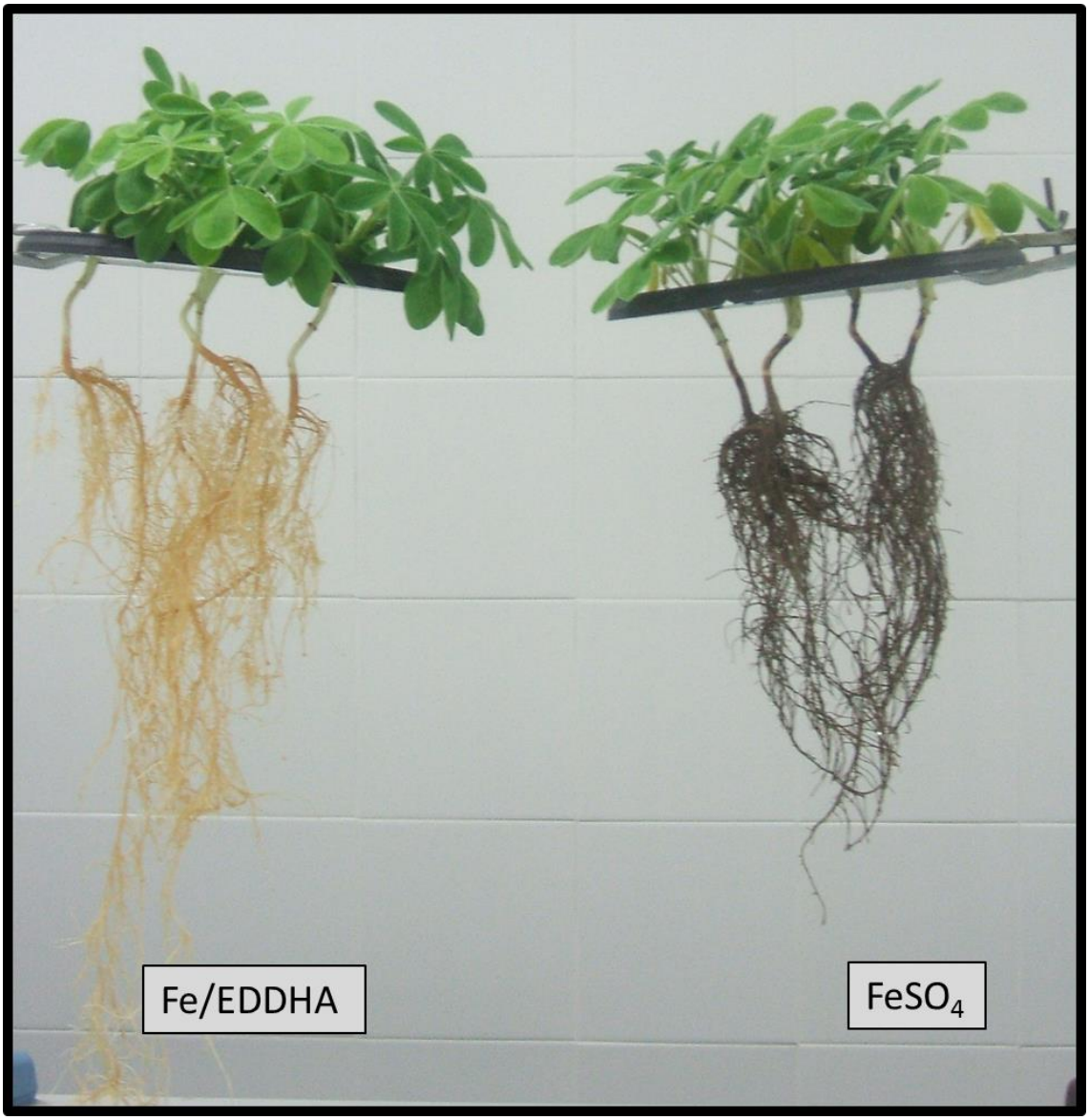


**Figure A-2.** White lupin plants grown in the Control (without As) with 3 mg $L^{-1}$ of Fe supplied as Fe/EDDHA (left) or 30 mg $L^{-1}$ of Fe as $FeSO_4$ (right). A surficial brownish coat was visible on the roots of lupin plants treated with $FeSO_4$.

**Figure A-3**. Details of the rhizobag system and porewater collection described in Chapter 4.2.

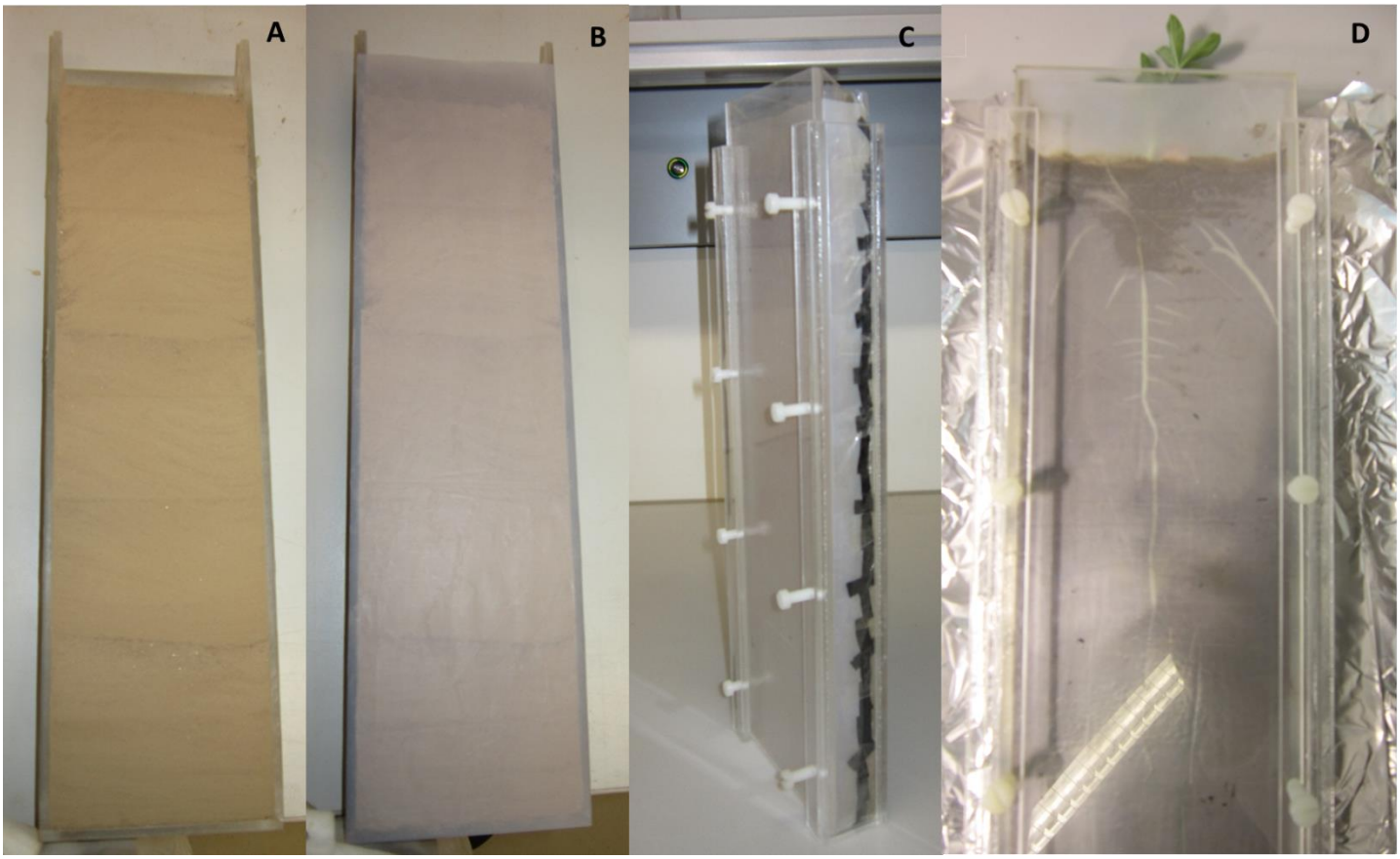


**Figure A-4.** Rhizotrons preparation: (**A**) the rhizotrons are carefully filled in order to obtain a very fine and flat soil surface; (**B**) soil surface is covered with a 10 µm thick polycarbonate filter membrane; (**C**) the membrane is covered with a plastic piece to keep soil humidity and protect the membrane and the removable plate is fixed with plastic screws; (**D**) white lupin plant grown in a rhizotron for 15 days.

**Figure A-5**. Detail of gel deployment on a white lupin root. Clamps are used to make pressure on the front plate of the rhizotron to assure contact between the gel and the soil surface (the nucleopore membrane is between the soil and the gel).

# Publicaciones derivadas de esta Tesis / Publications

T. Fresno, J.M. Peñalosa, J. Santner, M. Puschenreiter, T. Prohaska, E. Moreno-Jiménez. 2016. Iron plaque formed under aerobic conditions efficiently immobilizes arsenic in Lupinus albus L. roots. Environmental Pollution 216: 215-222.

T. Fresno, E. Moreno-Jiménez, J.M. Peñalosa. 2016. Assessing the combination of iron sulfate and organic materials as amendment for an arsenic and copper contaminated soil. A chemical and ecotoxicological approach. Chemosphere. 165: 539-546.

T. Fresno, J.M. Peñalosa, J. Santner, M. Puschenreiter, E. Moreno-Jiménez, Effect of *Lupinus albus* L. root activities on As and Cu mobility after addition of iron-based soil amendments. Ready for submitting.

Los artículos derivados de los capítulos 3.2 y 3.3 están en fase de elaboración.

Manuscripts resulting from chapters 3.2 and 3.3 are in preparation.

*Caminante son tus huellas el camino y nada más; [...]*

*[...] Caminante no hay camino sino estelas en la mar.*

Antonio Machado