UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE FARMACIA

Departamento de Edafología

TESIS DOCTORAL

Papel de los constituyentes edáficos en la disponibilidad de metales pesados en suelos calcáreos de uso agrícola del área mediterránea. Enfoque químico y biológico

Role of soil constituents in heavy metal availability in calcareous agricultural soils in the Mediterranean area. Chemical and bilogical approaches

Rôle des constituants du sol dans la disponibilité d'éléments trace métalliques das des sols calcaires agricoles de la région Méditerranéenne. Approche chimique et biologique

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Ana de Santiago Martín

Directoras

Antonio López Lafuente
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Under the direction of Doctors / Sous la direction des Docteurs

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Para aspirar al grado de Doctor

Madrid, 2013

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D. Antonio López Lafuente, Director del Departamento de Edafología de la Facultad de Farmacia de la Universidad Complutense de Madrid,

CERTIFICA,

Que el trabajo que constituye la presente memoria "Papel de los constituyentes edáficos en la disponibilidad de metales pesados en suelos calcáreos de uso agrícola del área Mediterránea. Enfoque químico y biológico", que presenta Dª Ana de Santiago Martín, para optar al grado de Doctor en Farmacia, ha sido realizada bajo la dirección del Dr. López Lafuente y de la Dra. González Huecas.

Y para que así conste, firmo la presente certificación en Madrid, 8 de enero de 2013.

Antonio López Lafuente

A mis abuelos

The top 20 cm of soil is all that stands between us and extinction

Agradecimientos

Esta Tesis no podría haberse llevado a cabo sin la colaboración de mucha gente, por lo que me gustaría agradecérselo.

A mis directores, por haber confiado en mí desde el principio y por haberme brindado la oportunidad de llevar a cabo esta Tesis. Quisiera agradecer, especialmente, el gran esfuerzo y el duro trabajo de los últimos meses, sin el cual no hubiésemos podido cumplir los plazos que nos habíamos propuesto.

Al personal tanto académico, técnico y administrativo, como becarios y precarios que están, o han estado, en el Departamento de Edafología. De estos años no sólo me llevo la experiencia docente y científica, sino que he tenido la suerte de estar rodeada de unos compañeros inmejorables que hacen del Departamento de Edafología un sitio donde es un placer trabajar.

A las personas con las que tuve el placer de compartir tan buenos momentos durante las estancias en el CIEMAT y en el INRA. Además de la experiencia científica, en las estancias he hecho grandes amigos que recordaré siempre.

Por último, a mi familia (gallega y bolañega), a mis amigos y a Juan, porque han colaborado en muchas etapas de mi Tesis (tamizando, "graficando", corrigiendo, traduciendo, adaptando la bibliografía…) y porque su cariño constante es el motor que me mueve día a día. Además, debo agradecerles la paciencia que han tenido conmigo este último año, pues no he podido dedicarles todo el tiempo que se merecen.

Muchas gracias,
Ana

** Esta Tesis ha sido financiada por la Universidad Complutense de Madrid y la Comunidad de Madrid, a través del Proyecto GR58/08 del Equipo de Investigación 950605, y por la Red CARESOIL (ref. P2009/AMB-1648).*

Índice Páginas

Resumen

Las características climáticas y el contenido en carbonato de los suelos agrícolas calcáreos del área mediterránea favorecen la acumulación y retención de metales pesados en los horizontes superficiales del suelo. Sin embargo, en este contexto, existe un riesgo potencial de movilización de los metales, incrementando la fracción de metal biodisponible para las plantas y los microorganismos del suelo. Constituyentes edáficos, como la materia orgánica, la arcilla y/o los óxidos de hierro, podrían jugar un papel clave minimizando este proceso. No obstante, un insuficiente contenido de alguno de estos componentes, como el habitualmente observado para la materia orgánica en los suelos agrícolas, podría ser un factor limitante. Aunque la información aportada por la bibliografía es relativamente amplia, este aspecto no está del todo resuelto.

Por todo ello, el objetivo principal de esta Tesis ha sido investigar el papel que ejercen los constituyentes del suelo, desde un enfoque tanto químico como biológico, en la disponibilidad de Cd, Cu, Pb y Zn en suelos agrícolas calcáreos del área Mediterránea. Para ello, se evaluaron los patrones de extractabilidad de los metales, la biodisponibilidad metálica en *Lactuca sativa* L. y la alteración de una serie de parámetros bioquímicos y microbiológicos frente a la contaminación metálica.

Las muestras seleccionadas fueron contaminadas con una mezcla de Cd, Cu, Pb y Zn a dos niveles (Tt1 y Tt2), dentro de los límites permitidos en la legislación Europea vigente. Posteriormente, las muestras fueron incubadas durante 12 meses con el fin de minimizar el efecto del tiempo de contacto. A diferentes intervalos de tiempo (1 día, 1, 3, 6 y 12 meses), se tomaron sub-muestras en las que se realizaron extracciones químicas simples de metales con el fin de estimar las fracciones metálicas móviles (sales neutras) y potencialmente móviles (agentes complejantes). Los datos analíticos, derivados de las extracciones químicas realizadas a los 12 meses de contacto, se complementaron con investigaciones mineralógicas y modelos de especiación mediante el programa Visual Minteq. La biodisponibilidad de los metales fue evaluada en hojas y raíces de dos variedades de *Lactuca sativa* L., romana e iceberg, cultivadas en sub-muestras de suelo tomadas a los 6 y 12 meses de tiempo de contacto. Finalmente, se determinaron una serie de parámetros bioquímicos

(actividades fosfatasa, ureasa, β-galactosidasa, arilsulfatasa y deshidrogenasa) y microbiológicos (concentración de ADN total, bacteriano y fúngico) en las muestras incubadas a los 12 meses.

Los elevados porcentajes de extracción de los metales con extractantes complejantes ponen de manifiesto que, a pesar de la elevada capacidad de sorción metálica de estos suelos, existe un importante riesgo de movilidad potencial. Sin embargo, aunque los resultados confieren a la fracción carbonatada un papel relevante en la minimización de estos procesos, se ha observado que dicha fracción no puede explicar, por sí sola, los patrones de extractabilidad de Cd, Cu, Pb y Zn en los suelos estudiados. En su lugar, la acción conjunta de la fracción carbonatada con otras fracciones del suelo, como la orgánica, los óxidos de hierro y la arcilla, resultó ser determinante en el control de estos patrones. En el caso de la extractabilidad de Cd, la materia orgánica y los óxidos de hierro mostraron estar involucrados, aunque el carbonato resultó ser el factor más relevante. Sin embargo, la fracción mineral fina y el contenido en materia orgánica recalcitrante destacaron en el control de la extractabilidad de Cu. La elevada retención de Pb, como consecuencia de la formación de $4PbCO_3 \cdot 3PbO$ (corroborado por difracción de rayos X), impidió concretar qué componentes del suelo intervienen en los procesos de desorción. La distribución de tamaño de partícula fue el único factor que explicó los procesos tanto de sorción como de extracción de Zn.

La alta fitotoxicidad y acumulación de metales observada en hojas y raíces, de ambas variedades de *Lactuca sativa* L., mostró que la fracción carbonatada no fue del todo eficaz en impedir la absorción metálica. En este sentido, se observó un resultado equivalente al obtenido con las extracciones químicas, ya que el carbonato, la materia orgánica recalcitrante, los óxidos de hierro y la arena gruesa fueron los principales factores explicativos de los patrones de biodisponibilidad de los metales tanto para las hojas como para las raíces. Cabe destacar el papel dual observado en el caso de la materia orgánica en función de su composición. Así, la fracción lábil limitó la absorción metálica por las raíces, en el nivel Tt2 para el Pb y en los dos niveles para el Cu, aunque favoreció la translocación de Cu y Pb en ambos niveles. Los resultados también indicaron que los procesos de adsorción de los metales a las raíces, así como la absorción y la translocación pudieron estar controlados en gran medida por

procesos competitivos entre los metales. Los patrones de biodisponibilidad de los metales en las hojas, fueron simulados satisfactoriamente por sales neutras y por el método LMWOA (ácidos orgánicos de bajo peso molecular). Sin embargo, merece especial atención la idoneidad observada de varios métodos de extracción relacionados con fracciones potencialmente móviles de los metales, como los extractantes complejantes.

Todas las actividades enzimáticas estuvieron altamente afectadas por la contaminación metálica. No obstante, se observó que la inhibición de estas actividades era menor en aquellos suelos con mayores proporciones de fracción de mineral fina, materia orgánica, óxidos de hierro cristalino y cationes divalentes en la solución del suelo. Este patrón fue similar al obtenido en la cuantificación de la concentración de ADN bacteriano. Cabe resaltar que la disminución de la concentración de ADN fúngico en los suelos contaminados no fue tan marcada, conllevando a una alteración de la relación de ADN fúngico/bacteriano.

En resumen, el alto porcentaje de extracción metálica, la elevada fitotoxicidad y bioacumulación metálica, así como la fuerte alteración de los parámetros bioquímicos y microbiológicos, ponen de manifiesto la vulnerabilidad de estos suelos. Los resultados obtenidos, por tanto, muestran que los suelos objeto de estudio se verían altamente afectados ante una creciente acumulación metálica en áreas agrícolas periurbanas. Teniendo en cuenta este escenario y que una mayor proporción de las fracciones orgánicas e inorgánicas (carbonatada, mineral fina y óxido de hierro) en los suelos podría minimizar este impacto, sería aconsejable que los límites individuales propuestos por la legislación europea actual fueran establecidos de acuerdo con las características edáficas y/o revisados cuando la contaminación se produce por una mezcla metálica. Desde esta perspectiva, se plantea una posible línea de investigación a desarrollar en futuros trabajos, con el fin de mejorar las bases para proponer estándares adecuados de calidad de acuerdo con las estrategias europeas para la protección de los suelos.

Abstract

The climate characteristics and carbonate content of calcareous agricultural soils in the Mediterranean area favour the accumulation and retention of heavy metals in the surface horizons of the soil. This leads to a potential risk of mobilisation of the metals, thereby increasing the fraction of bioavailable metal for plants and soil microorganisms. Soil constituents such as organic matter, clay and/or iron oxide may play a key role in minimising this process. However, an insufficient content in some of these components –such as is commonly observed in the case of organic matter in agricultural soils– may be a limiting factor. Although the bibliography provides relatively extensive information, this aspect has not yet been completely resolved.

Therefore, the main objective of this thesis was to investigate the role played by soil constituents from both a chemical and biological perspective in Cd, Cu, Pb and Zn availability in calcareous agricultural soils in the Mediterranean area. This was done by evaluating the extractability patterns of the metals, metal bioavailability in *Lactuca sativa* L. and the alterations occurring in a series of biochemical and microbiological parameters in the presence of metal contamination.

The samples selected were contaminated with a mixture of Cd, Cu, Pb and Zn at two levels (Tt1 and Tt2) within the limits permitted under current European legislation. Subsequently, the samples were incubated for 12 months in order to minimise the effect of contact time. At different time intervals (1 day, 1, 3, 6 and 12 months), sub-samples were taken and simple chemical extractions of metals were performed in order to estimate the mobile metal fractions (neutral salts) and potentially mobile fractions (complexing agents). The analytical data derived from the chemical extractions obtained after 12 months of contact were supplemented with mineralogical research and speciation models using the Visual Minteq program. Metal bioavailability was assessed in leaves and roots of two varieties of *Lactuca sativa* L. –romaine and iceberg– cultured in soil sub-samples taken after 6 and 12 months of contact time. Finally, a series of biochemical parameters (phosphatase, urease, β-galactosidase, arylsulfatase and dehydrogenase activities) and microbiological parameters (total DNA, bacterial and fungal concentration) were determined in the incubated samples after 12 months.

The higher percentages of metal extraction with the complexing extractants highlight the fact that, in spite of the high metal sorption capacity of these soils, there is a significant risk of potential mobility. However, although the results point to the importance of the carbonated fraction in minimising these processes, it was noted that this fraction alone cannot account for the extractability patterns of Cd, Cu, Pb and Zn in the soils in the study. Instead, the action of the carbonated fraction combined with other soil fractions such as those of carbonate, organic matter, iron oxide and clay, was clearly a determining factor in controlling these patterns. Organic matter and iron oxide also proved to have an influence on Cd extractability, although carbonate was the most important factor. However the fine mineral fraction and the recalcitrant organic matter content had a notable effect on the extractability of Cu. The high retention of Pb as a consequence of the formation of $4PbCO_3 \cdot 3PbO$ (corroborated by X-ray diffraction) made it impossible to determine with any certainty which soil components are involved in the desorption processes. Particle size distribution was the only factor that explained the processes of both sorption and Zn extraction.

The high phytotoxicity and metal accumulation observed in the leaves and roots of both varieties of *Lactuca sativa* L. showed that the carbonated fraction was not completely effective in preventing metal absorption. An equivalent result was observed to the one obtained with chemical extractions, as carbonate, recalcitrant organic matter, iron oxide and coarse sand were the main factors explaining metal bioavailability patterns in both leaves and roots. It is worth noting the dual role observed in the case of the organic matter according to their composition. Thus, the labile organic fraction limited metal absorption by the roots, at the Tt2 level for Pb and at both levels for Cu, although favoured Cu and Pb translocation at both levels. The results also indicated that the processes of adsorption of metals to roots, absorption and translocation may be largely controlled by competitive processes between the metals. The metal bioavailability patterns in lettuce leaves were successfully simulated by neutral salts and by the LMWOA method (low molecular weight organic acids). It is particularly worth noting the usefulness of various extraction methods in regard to potentially mobile metal fractions such as complexing extractants.

All enzymatic activity is highly affected by metal contamination. However, the inhibition of these activities proved to be lower in soils having greater proportions of fine mineral fraction, organic matter, crystalline iron oxides and divalent cations in the soil solution. This pattern was similar to that obtained in the quantification of the concentration of bacterial DNA. It should be highlighted that the decrease in the concentration of fungal DNA in contaminated soils was not as notable, and involved an alteration in the relationship between fungal/bacterial DNA.

In summary, the high percentage of metal extraction, the elevated phytotoxicity and metal bioaccumulation and the significant alterations in the biochemical and microbiological parameters highlight the vulnerability of these soils. The results thus demonstrate that the soils in this study would be greatly affected in the case of an increased accumulation of metals in periurban agricultural areas. Taking into account this scenario and that a greater proportion of organic and inorganic fractions (carbonate, fine mineral and Fe-oxide) in soils could minimize this impact, it would be recommended that the individual limits proposed by the current European legislation were established according to the characteristics of the soils, and/or revised when the contamination is produced by a combination of metals. This approach suggests a possible line to be developed in future research works in order to improve the basis for the proposal of adequate quality standards within the European strategies for the protection of soils.

Résumé

Les caractéristiques du climat et la teneur en carbonate dans les sols calcaires agricoles de la région Méditerranéenne favorisent l'accumulation et la rétention des éléments traces métalliques (ETM) dans les horizons de surface du sol. Néanmoins, dans ce contexte, il existe un risque potentiel de mobilisation des ETM, ce qui peut augmenter la fraction des ETM biodisponibles pour les plantes et les microorganismes du sol. Constituants du sol, tels que la matière organique, l'argile et/ou les oxydes de fer, pourraient jouer un rôle clé en minimisant ce processus. Cependant, une teneur insuffisante en l'un de ces composants, comme cela est habituellement observé pour la matière organique dans les sols agricoles peut être un facteur limitant. Bien que les informations fournies par la littérature soient relativement importantes, cette question n'est pas entièrement résolue.

Pour cela, l'objectif principal de cette Thèse a été d'étudier le rôle joué par les constituants du sol, par une approche chimique et biologique, en relation à la disponibilité de Cd, Cu, Pb et Zn dans des sols calcaires agricoles de la région Méditerranéenne. Pour ce faire, nous avons évalué les patrons d'extractibilité des ETM, la biodisponibilité métallique en *Lactuca sativa* L. et l'altération d'une série de paramètres biochimiques et microbiologiques face à la contamination métallique.

Les échantillons sélectionnés ont été contaminés par un mélange de Cd, Cu, Pb et Zn à deux niveaux (Tt1 et Tt2), dans les limites permises par la législation européenne en vigueur. Les échantillons ont été en suite incubés pendant 12 mois afin de minimiser l'effet du temps de contact. Des sous-échantillons ont été prélevés à différents intervalles de temps (1 jour, 1, 3, 6 et 12 mois) et des extractions chimiques simples des ETM ont été effectuées afin d'estimer les fractions métalliques mobiles (sels neutres) et potentiellement mobiles (agents complexants). Les données analytiques, provenant des extractions chimiques effectuées à 12 mois de contact, ont été complétées avec des investigations minéralogiques et modèles de spéciation à la l'aide du logiciel Visual Minteq. La biodisponibilité des ETM a été évaluée dans les feuilles et les racines des deux variétés de *Lactuca sativa* L., romaine et iceberg, cultivées dans des sous-échantillons de sol prélevés à 6 et 12 mois de temps de contact. Finalement, nous avons déterminé une série des paramètres biochimiques

(activités phosphatase, uréase, β-galactosidase, arylsulfatase et déshydrogénase) et microbiologiques (concentration d'ADN total, bactérienne et fongique) dans les échantillons incubés à 12 mois.

Les forts pourcentages d'extraction des ETM avec des extractants complexants montrent que, malgré la haute capacité de sorption métallique de ces sols, il existe un risque important de mobilité potentielle. Cependant, bien que les résultats confèrent à la fraction carbonatée un rôle relevant en minimisant ces processus, il a été constaté que cette fraction ne peut pas expliquer pour elle même les patrons d'extractibilité de Cd, Cu, Pb et Zn dans les sols étudiés. Au lieu de cela, l'action conjointe de la fraction carbonate avec d'autres fractions du sol, tels que l'organique, les oxydes de fer et l'argile, a été décisive dans le control de ces patrons. Dans le cas de l'extractibilité du Cd, la matière organique et les oxydes de fer se sont révélés être impliqués, bien que le carbonate a prouvé être le facteur le plus important. Cependant, la fraction minérale fine et la teneur en matière organique récalcitrante a été mis en évidence dans le contrôle de l'extractibilité du Cu. La forte rétention de Pb, due à la formation de $4PbCO_3 \cdot 3PbO$ (confirmée par diffraction des rayons X), nous a empêché de spécifier quels sont les composants du sol impliqués dans le processus de désorption. La distribution de taille des particules a été le seul facteur qui a expliqué aussi bien les processus de sorption que d'extraction du Zn.

La forte phytotoxicité et concentration des ETM observée dans les feuilles et les racines des deux variétés de *Lactuca sativa* L. a montré que la fraction carbonate n'est pas été entièrement efficace dans la prévention de l'absorption métallique. À cet égard, nous avons observé un résultat équivalent à celui obtenu avec des extractions chimiques, puisque le carbonate, la matière organique récalcitrant, les oxydes de fer et la sable grossier ont été les principaux facteurs explicatifs des patrons de biodisponibilité des ETM par les feuilles et les racines. On peut signaler le double rôle observé dans le cas de la matière organique en fonction de sa composition. Ainsi, la fraction labile métallique a réduit l'absorption métallique par les racines, au niveau Tt2 pour le Pb et aux niveaux Tt1 et Tt2 pour le Cu, bien que translocation du Cu et du Pb aie été favorisée dans les deux niveaux. Les résultats ont indiqué également que l'adsorption des métaux sur les racines, ainsi que l'absorption et la translocation pourraient être contrôlées en grande partie par des processus compétitifs parmi les

ETM. Les patrons de biodisponibilité des ETM dans les feuilles ont été simulés de manière appropriée par les sels neutres et par la méthode LMWOA (acides organiques à faible poids moléculaire). Cependant, il mérite une attention particulière l'adéquation des différentes méthodes d'extractions liées aux fractions potentiellement mobiles des ETM, tels que les agents d'extraction complexants.

Toutes les activités enzymatiques ont été fortement perturbées par la contamination métallique. Toutefois, il a été observé que l'inhibition de ces activités était plus faible chez les sols avec une plus haute proportion de fraction minérale fine, matière organique, oxydes cristallins de fer et cations divalents dans la solution du sol. Ce patron est similaire à celui obtenu avec la quantification de la concentration de l'ADN bactérien. Il est à noter que la diminution de la concentration de l'ADN fongique dans les sols contaminés n'était pas tellement important, entraînant une altération du rapport de l'ADN fongique/bactérien.

En résumé, le haut pourcentage d'extraction des ETM, la forte phytotoxicité et bioaccumulation métallique, ainsi que la perturbation des paramètres biochimiques et microbiologiques mettent en évidence la vulnérabilité de ces sols. Les résultats obtenus montrent donc que les sols d'étude seraient très affectés face à une croissante accumulation métallique dans des zones périurbaines agricoles. Compte tenu de ce scénario et qu'une proportion plus élevée des fractions organiques et inorganiques (carbonate, minérale fine et des oxydes de fer) pourrait minimiser cet impact, il serait conseillé que les limites individuelles proposées par la législation européenne actuelle étaient établis en conformité avec les caractéristiques du sol et/ou révisées en cas de contamination par un mélange des ETM. Au sein de cette perspective, nous proposons une possible ligne de recherche à développer dans des travaux futurs, afin d'améliorer les bases pour proposer des normes de qualité appropriées selon les stratégies européennes pour la protection des sols.

1. Introducción

Introducción

El suelo es un componente esencial de la biosfera ya que, además de ser un sumidero geoquímico para los contaminantes, también actúa como una barrera natural en el control del transporte de elementos químicos y de sustancias a la atmósfera, la hidrosfera y la biota. Sin embargo, la función más importante del suelo es su productividad, básica para la supervivencia de los seres humanos. Por ello, el mantenimiento de las funciones ecológicas y agrícolas del suelo es responsabilidad de la humanidad (Kabata-Pendias y Pendias, 2001). La degradación del suelo, inducida o agravada por la actividad humana, supone un serio problema en Europa. Las prácticas agrícolas y forestales inadecuadas, las actividades industriales, el turismo, la expansión urbana y la construcción pueden conllevar un impacto negativo para los suelos. Esto se traduce en una pérdida de fertilidad y biodiversidad, una menor capacidad de retención de agua, la alteración de los ciclos de los nutrientes y la reducción de la degradación de los contaminantes. Es decir, el suelo se hace más vulnerable, al verse afectada de forma negativa alguna de sus funciones, tales como: a) producción de biomasa, (b) filtrado, tampón, almacenamiento y transformación y (c) hábitat biológico y reserva genética (Batjes, 2000).

El número de emplazamientos potencialmente contaminados en la Unión Europea se estima en unos 3,5 millones, siendo la contaminación difusa una de las principales amenazas para el suelo (Comisión Europea, 2006). Teniendo en cuenta que la persistencia de los contaminantes en el suelo es mucho mayor que en otros compartimentos de la biosfera, la contaminación del suelo, especialmente por metales pesados, parece ser casi permanente (Kabata-Pendias y Pendias, 2001). Un ejemplo de ello lo encontramos en las áreas metropolitanas, donde el riesgo de contaminación se debe, fundamentalmente, al desarrollo industrial y urbano, lo que conduce a un aumento de "elementos urbanos" en los suelos –Ba, Cd, Co, Cu, Mg, Pb, Sb, Ti y Zn– (de Miguel *et al.*, 1997). Este hecho queda patente en suelos agrícolas periurbanos que se ven afectados por procesos de contaminación, o que pueden llegar a estarlo en el futuro a menos que se apliquen criterios de sostenibilidad (Steinitz *et al.*, 2011). Para paliar en lo posible este riesgo, la Comunidad Europea, en 1986, publica la primera reglamentación específica (Directiva 86/278/EEC), en la que se establecen los valores límite de concentración total de metales pesados en suelos, referida al uso de lodos de depuradora en suelos agrícolas (Tabla 1).

Tabla 1. Valores límite de concentración de metales pesados en suelos y en lodos destinados a su utilización en la agricultura (mg kg^{-1} de materia seca de suelos de pH 6-7).

Elemento	Valores límite en suelos	Valores límite en lodos
Zn	150-300	2500-4000
Cd	1-3	20-40
Cu	50-140	1000-1750
Ni	30-75	300-400
Pb	50-300	750-1200
Hg	1-1,5	16-25

La contaminación por metales pesados puede afectar a la calidad de los suelos agrícolas, conllevando una fitotoxicidad y transferencia de metales a la dieta humana a través de su absorción por las plantas de cultivo. La reducción de la biomasa de las plantas ante una contaminación metálica ha sido atribuida a la interacción que se produce entre los metales y los nutrientes, dando como resultado graves deficiencias de elementos esenciales y, por tanto, afectando a su productividad (Capelo *et al.*, 2012;

Monteiro *et al.*, 2009). Además, las plantas que crecen en suelos contaminados pueden absorber y acumular metales en los tejidos comestibles en grandes cantidades sin signos visibles, lo que supone un mayor riesgo de entrada de metales pesados en la cadena trófica (Kabata-Pendias y Pendias, 2001). Peris *et al.* (2007) observaron que el contenido de metales en las partes comestibles de vegetales, cultivados en suelos agrícolas del área mediterránea cercanos a zonas urbanas o industriales, era superior al nivel máximo establecido por la legislación europea actual para cultivos hortícolas (Reglamento nº466/2001).

En este sentido, el riesgo potencial de que se produzca una contaminación metálica, y de su acumulación en las plantas de cultivo, está directamente relacionado con la movilidad y disponibilidad de los metales en los suelos. La mayor o menor retención o movilidad depende de la acción combinada de varios factores, incluyendo la naturaleza del metal y su concentración en el suelo, factores ambientales, y constituyentes del suelo. Estos factores determinan el equilibrio de los metales entre la fase sólida y la solución del suelo, a través de procesos tales como precipitación-disolución, adsorción-desorción, complejación-disociación y oxidación-reducción. No obstante, aún cuando estos procesos no son igualmente importantes para cada elemento, todos ellos se ven afectados por el pH del suelo y los procesos biológicos (He *et al.*, 2005).

El mecanismo de retención de los iones metálicos en el suelo se suele denominar con el término *sorción* que, en general, implica la pérdida de un ion metálico de la solución acuosa, para quedar retenido en la fase sólida contigua; y consta de tres procesos importantes: adsorción, precipitación en superficie y fijación. La adsorción de los metales pesados se describe en la bibliografía científica en términos de dos mecanismos básicos: *adsorción específica*, que se caracteriza por reacciones más selectivas y menos reversibles, incluyendo la quimisorción en complejos de esfera interna, y *adsorción no específica* (o intercambio iónico), lo que implica complejos de esfera externa menos selectivos y más débiles. El mecanismo de adsorción específica se produce mediante el establecimiento de uniones fuertes e irreversibles, con la materia orgánica y los minerales de carga variable, mientras que en el de adsorción no específica, los cationes del agua de los poros son intercambiados por aquellos cercanos a la superficie a través de fenómenos de carácter electrostático.

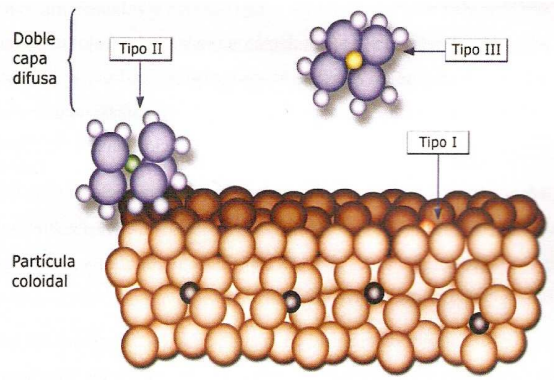

Fig. 1. Representación de los procesos de adsorción de iones en la superficie de una partícula coloidal. Tipo I se refiere a un complejo de esfera interna, Tipo II a un complejo de esfera externa y Tipo III a un ión disociado en la capa difusa (adaptación de Pérez-Esteban, 2011, de Sposito, 1989).

La *precipitación en superficie* se caracteriza por el crecimiento de una nueva fase sólida, que se repite en las tres dimensiones y forma una red 3-D, siendo dependiente del pH y de las cantidades relativas de los metales y los aniones presentes. Así, los metales pueden precipitar en los suelos como óxidos, hidróxidos, carbonatos, sulfatos o fosfatos. La *fijación* implica la difusión de una especie metálica en la fase sólida; al igual que en el caso de la precipitación en superficie, la fijación es tridimensional en la naturaleza. Los metales pesados, que están específicamente adsorbidos en los minerales de arcilla y en los óxidos metálicos, pueden difundir en las estructuras reticulares de estos minerales, fijándose en el espacio poroso de la estructura mineral (Bradl, 2004).

Un mecanismo de especial interés, en la retención de los iones metálicos, es el que tiene lugar en suelos desarrollados en el clima mediterráneo. En este clima, la escasa precipitación y la elevada evapotranspiración limitan la movilidad de los metales en los suelos, favoreciendo su acumulación. Además, la retención de los metales se ve incrementada en los suelos mediterráneos de naturaleza calcárea debido al contenido en carbonato (Chicharro Martín *et al.*, 1998; Lafuente *et al.*, 2008). La fracción carbonatada en los suelos afecta a la solubilidad de los metales, bien directamente, mediante interacciones superficiales, bien indirectamente, por el efecto del pH sobre los componentes del suelo. Por ello, se podría pensar *a priori* que estos

suelos de naturaleza carbonatada del área mediterránea son suelos no vulnerables ante un proceso de contaminación metálica (Conde *et al.*, 2007). Sin embargo, aunque esta alta capacidad de retención de los metales favorece su acumulación en los primeros centímetros del suelo, las fuerzas de retención son débiles, lo que implica una posible removilización metálica (Plassard *et al.*, 2000). La acción de los organismos del suelo, tales como las plantas o la fauna detritívora (Recatalá *et al.*, 2011; Ruiz *et al.*, 2009; Sayyad *et al.*, 2010; Udovic y Lestan, 2007), y/o los cambios en las condiciones del entorno, como una disminución del pH del suelo (Bolan *et al.*, 2003; Martínez y Motto, 2000; Plassard *et al.*, 2000), pueden favorecer este incremento en la concentración metálica móvil (Fig. 2).

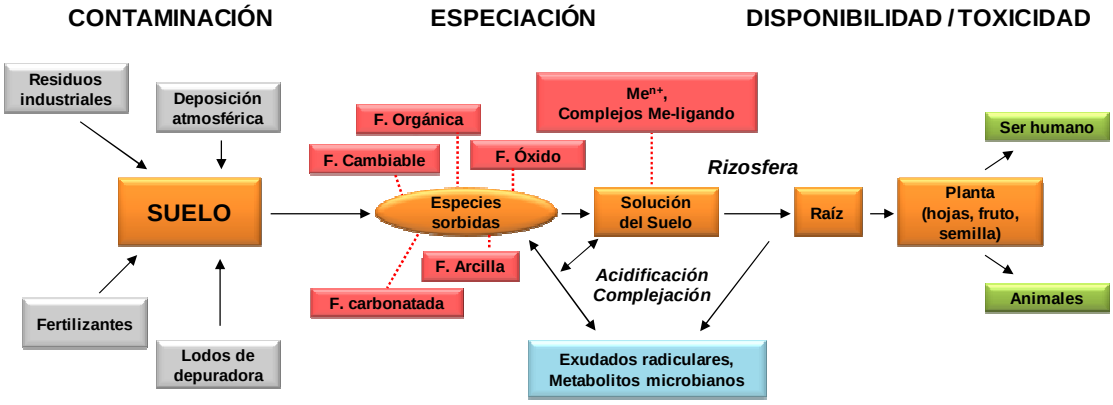

Fig. 2. Ciclo contaminación-especiación-disponibilidad/toxicidad de los metales pesados en agrosistemas terrestres (adaptación de Krishnamurti, 2000).

Por todo ello, la alta pero finita capacidad de los suelos calcáreos para almacenar metales potencialmente movilizables sugiere que se debe considerar a estos suelos como suelos muy vulnerables (Batjes, 2000). No obstante, esta posible removilización metálica puede ser minimizada por la acción de otros componentes del suelo, además de los carbonatos. En la bibliografía, se refleja el importante papel que juegan otros constituyentes y propiedades del suelo en la distribución y disponibilidad de los metales en suelos calcáreos, como la textura, la composición mineralógica, el contenido y tipo de materia orgánica, el pH del suelo y el contenido de óxidos de Fe, Al y Mn (Buekers *et al.*, 2007; Clemente y Bernal, 2006; Jalali y Khanlari, 2008; Jalali y

Moharrami, 2007; Micó *et al.*, 2006; Moreno *et al.*, 1992; Sánchez-Camazano *et al.*, 1998; Sipos *et al.*, 2008). En general, el pH del suelo es un buen indicador predictivo de la solubilidad de los metales, sin embargo, se ha visto que no lo es en cuanto a la toxicidad metálica.

La fracción orgánica del suelo es un factor clave en el control de estos procesos de removilización metálica. No obstante, la materia orgánica juega un papel dual ante un evento de contaminación (Clemente *et al.*, 2006; Zeng *et al.*, 2011). Por una parte, amortigua la removilización metálica, inmovilizando los metales a través de la formación de complejos estables metal-humus (Dabkowska-Naskret, 2003; Kalis *et al.*, 2006; Smith, 2009). Mientras que, por otra parte, favorece su movilización mediante la formación de complejos orgánicos móviles con los metales (Kaschl *et al.*, 2002; Wong *et al.*, 2007), aunque ésta podría estar inhibida en suelos calcáreos. Este papel dual se debe, en gran medida, a la composición de la fracción orgánica y a la naturaleza del metal (Cattani *et al.*, 2006; Inaba y Takenaka, 2005). Sin embargo, aunque varios autores han demostrado el papel ejercido por esta fracción en la disponibilidad de los metales en suelos calcáreos, la mayoría de los estudios se han llevado a cabo con suelos enmendados, y pocos son los trabajos realizados en suelos con un gradiente natural en el contenido y la composición de la materia orgánica. Por ello, consideramos esencial llevar a cabo estudios donde se profundice en esta dirección. El estudio del papel que ejerce el contenido y composición de las fracciones carbonatada, orgánica, óxido y arcilla en la disponibilidad y biodisponibilidad de metales pesados en suelos calcáreos es uno de los principales objetivos de esta Tesis, tal y como se reflejará en los Capítulos de Resultados 3, 4 y 5.

Ya que los suelos son sistemas dinámicos, cuando se plantean estudios encaminados tanto a la evaluación de la movilidad y disponibilidad de los metales, como a la determinación de riesgos potenciales, la cuantificación del contenido total de metales ha mostrado no ser una herramienta útil. Con el fin de comprender la interacción química de los metales pesados con otros componentes del suelo, tales como los minerales de arcilla o la materia orgánica, y/o estudiar su movilidad y disponibilidad, para las plantas, los animales, los microorganismos y los seres humanos, es necesario evaluar sus fracciones móviles o disponibles en el suelo (Ramos-Miras *et al.*, 2011; Ure, 1996). Para ello, habitualmente se emplean diferentes

métodos de extracción de metales, en función del objetivo perseguido, como son: contenido total, contenido pseudo-total, extracción simple, extracción secuencial y lixiviación en columna (Fig. 3).

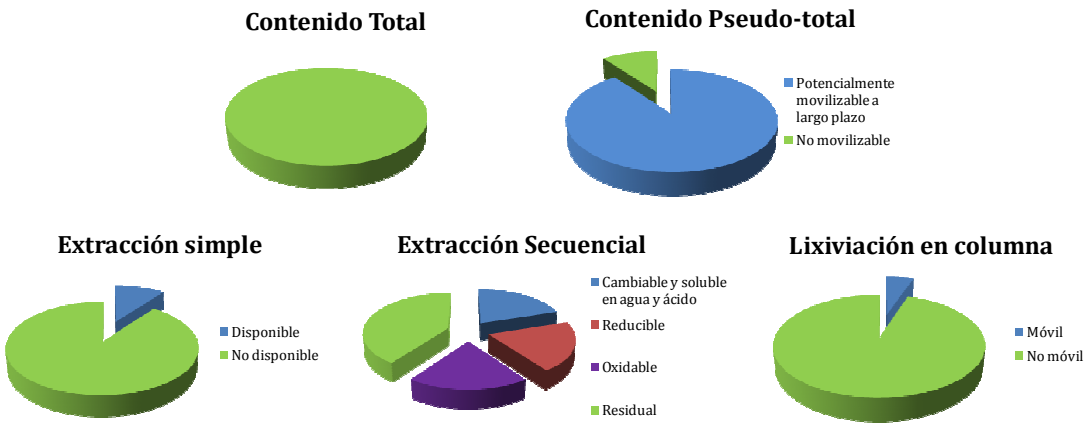

Fig. 3. Métodos de extracción química frecuentemente empleados en la determinación de metales en los suelos (adaptación de Rao *et al.*, 2008).

La medida de los contenidos *pseudo-totales* de los metales del suelo, mediante digestiones con ácidos fuertes, da una idea del contenido máximo potencialmente soluble o móvil de los metales, lo que estimaría el riesgo potencial máximo que podría ocurrir a largo plazo o en regímenes ambientales extremos (Rao *et al.*, 2008). No obstante, existe un equilibrio muy delicado entre la fracción pseudo-total de los metales, la *fracción móvil* y la *fracción potencialmente móvil* (Gupta *et al.*, 1996). El uso de métodos de extracción química de metales de diferentes fuerzas nos permite distinguir entre estas fracciones.

Con el objetivo de dilucidar la proporción de metal cambiable, móvil y/o disponible para los organismos se suelen emplear extracciones químicas simples en un paso, mientras que para estudiar las formas en las que los metales están presentes en el suelo, así como los sitios de unión, se utilizan más a menudo las extracciones secuenciales (Ure, 1996). Las *extracciones químicas simples en un paso* tienen como ventaja la simplicidad y facilidad de manipulación. Estos métodos se suelen emplear como estimadores de la *fracción biodisponible*, entendida ésta como la fracción metálica que

está disponible para un organismo receptor (Vig *et al.*, 2003). En este sentido, las sales neutras no tamponadas estimarían la fracción inmediatamente biodisponible y las sales tamponadas y los agentes complejantes la potencialmente biodisponible (Tabla 2).

Tabla 2. Métodos de extracción y características de las fracciones metálicas en el suelo (adaptación de Gupta *et al.*, 1996).

Fracciones en el suelo	Características de la fracción	Métodos de extracción comúnmente usados
Móvil	Activa (biodisponible y fácilmente lixiviable)	Soluciones salinas neutras no tamponadas ($NaNO_3$, $CaCl_2$, NH_4NO_3…)
Movilizable/ Potencialmente móvil	Potencialmente Activa + Activa (potencialmente biodisponible y potencialmente lixiviable)	Soluciones de agentes complejantes tamponados y no tamponados (NH_4Ac, EDTA, DTPA, HAc...)
Pseudo-total	No Activa + Potencialmente Activa (potencialmente movilizable)	Soluciones ácidas fuertes: agua regia, HNO_3, HCl
Inmóvil	No reactiva (pseudo-total – potencialmente movilizable)	

Ya que estos métodos no son específicos de una fase del suelo en particular, a menudo se emplean *extracciones secuenciales*, que se caracterizan por utilizar reactivos de naturaleza química y fuerza de extracción diferentes, en general, un ácido diluido, un agente reductor y un agente oxidante. Las distintas etapas se establecen en orden creciente de fuerza de extracción de los reactivos elegidos. Aún siendo numerosos los procedimientos formulados, en función de la naturaleza de los reactivos o el número de etapas, los más frecuentemente utilizados son los esquemas propuestos por Tessier (1979) y el BCR (Rauret *et al.*, 1999). A partir de estos procedimientos de extracción secuencial, el cálculo del *factor de movilidad* puede ser una herramienta útil en la evaluación de la movilidad de los metales en el suelo (Achiba *et al.*, 2009).

En cualquier caso, la combinación de los datos analíticos derivados de las extracciones químicas de los metales con modelos de especiación química de los extractos, así como con investigaciones mineralógicas, ha sido propuesta como una herramienta útil para dilucidar los componentes y procesos químicos del suelo, que afectan a la extractabilidad, movilidad y disponibilidad de los metales en los suelos

(Ettler *et al.*, 2007; Fabas *et al.*, 2011; Pérez-Esteban *et al.*, 2013; Sipos *et al.*, 2008; Vidal *et al.*, 2004). En los Apartados 3.1, 3.2 y 3.3 de esta Tesis, pertenecientes al primer Bloque de Resultados, se abordará el estudio de los patrones de extractabilidad de metales, en suelos calcáreos de uso agrícola del área mediterránea, en función de los constituyentes de los suelos.

No obstante, para validar un procedimiento dado, debe ser considerada la relación entre las formas disponibles de los metales en el suelo y su concentración en un organismo diana (fracción biodisponible) o la toxicidad producida. Con este fin, deben emplearse organismos con diferentes rutas de exposición y sensibilidades al mismo tiempo, tales como productores primarios y consumidores detritívoros. En este sentido, las plantas vasculares son extremadamente sensibles a la contaminación metálica. Por ello, los bioensayos con plantas vasculares han mostrado ser de gran utilidad y versatilidad en la evaluación de los efectos de tales contaminantes en el suelo. A pesar de la elevada variabilidad de la bioacumulación de los metales entre las diferentes especies vegetales, se han podido establecer valores de concentraciones metálicas en tejidos de hoja, de acuerdo a un rango considerado normal o bien a un rango tóxico (Tabla 3).

Tabla 3. Concentraciones de metales pesados (mg kg^{-1} peso seco) en tejidos de hoja generalizados para varias especies vegetales (adaptación de Kabata-Pendias y Pendias, 2001).

Elemento	Deficiente	Suficiente o normal	Excesiva o tóxica	Tolerable en cultivos
Zn	10-20	27-150	100-400	50-100
Cd	-	0,05-0,2	5-30	0,05-5
Cu	2-5	5-30	20-100	5-20
Ni	-	0,1-5	10-100	1-10
Pb	-	5-10	30-300	0,5-10
As	-	1-1,7	5-20	0,2
Hg	-	-	1-3	0,2
Cr	-	0,1-0,5	5-30	2

La cuantificación de la bioacumulación de metales y nutrientes en los tejidos de las plantas de cultivo, así como la medida de la inhibición de la biomasa de las plantas, de la germinación de las semillas, de la elongación radicular y de la necrosis radicular han mostrado ser herramientas útiles, sensibles y reproducibles en la determinación de

la fitotoxicidad causada por metales pesados y en la estimación del riesgo asociado a la entrada de metales en la cadena trófica (Alexander *et al.*, 2006; Escoto Valerio *et al.*, 2007; Monteiro *et al.*, 2009; Recatalá *et al.*, 2010, 2011). Una gran parte de estos trabajos se han llevado a cabo empleando distintas variedades de lechuga (*Lactuca sativa* L.), pues se ha visto que es un organismo sensible a la contaminación y que, a su vez, puede acumular elevadas concentraciones de metales (Chapman *et al.*, 2012; Lamb *et al.*, 2010). Además, la lechuga es una de las hortalizas más consumidas en el área mediterránea, siendo de especial preocupación, por tanto, la bioacumulación metálica en las partes comestibles.

La mayor o menor bioacumulación de metales en las plantas depende de una amplia variedad de factores: procesos de sorción-desorción de los metales en el suelo, especiación de los metales y naturaleza de los enlaces químicos establecidos con la fase sólida, competencia entre los metales, reacciones de los metales en la rizosfera y mecanismos de absorción y translocación de las plantas. La mayoría de estos factores dependen en gran medida del tipo de suelo y de sus características.

Como se ha mencionado anteriormente, en los suelos calcáreos de uso agrícola del área mediterránea, la fracción carbonatada juega un papel clave en la inmovilización metálica. Sin embargo, se ha visto que esta fracción podría no ser del todo eficaz, al detectar en estos suelos una proporción significativa de metales potencialmente biodisponibles para las plantas (Sayyad *et al.*, 2010). En este contexto, se hace necesario identificar qué constituyentes del suelo gobiernan los patrones de biodisponibilidad de los metales para las plantas, así como qué constituyentes minimizan la toxicidad metálica. Este aspecto será objeto de estudio en los Apartados 4.1 y 4.2 de esta Tesis, correspondientes al segundo Bloque de los Resultados.

Con el objetivo de simular la biodisponibilidad de los metales para las plantas, se han empleado diferentes métodos de extracción química de metales (Chaignon *et al.*, 2003; Feng *et al.*, 2005; Meers *et al.*, 2007). Sin embargo, la comunidad científica aún no ha logrado establecer un único método estandarizado, para la predicción de la biodisponibilidad de los diferentes metales, en cualquier tipo de suelo y para cualquier especie diana. Las sales neutras no tamponadas ($CaCl_2$, $MgCl_2$, $NaNO_3$, NH_4NO_3), que extraen los metales fácilmente intercambiables, se consideran métodos adecuados con fines ecotoxicológicos en la evaluación del riesgo de entrada de metales en la

cadena trófica (Meers *et al.*, 2007; Menzies *et al.*, 2007). No obstante, se ha visto que ejercen una débil competencia por los sitios de adsorción en la materia orgánica y en las superficies de los óxidos, por lo que no son representativas, en todas las ocasiones, de la fracción biodisponible para las plantas. En su lugar, métodos de extracción química, basados en el empleo de ácidos orgánicos de bajo peso molecular, podrían simular de forma más precisa la interacción que ocurre en el sistema raíz-suelo-microorganismos (Feng *et al.*, 2005). Otros agentes complejantes de mayor fuerza de extracción también han resultado ser apropiados en la simulación de los patrones de biodisponibilidad de los metales, como el DTPA (ácido dietilen triamino pentaacético), en suelos neutros o cercanos a la alcalinidad, o el EDTA (ácido etilen diamino tetraacético), en suelos ácidos. Sin embargo, se han obtenido un mayor número de correlaciones entre los metales extraídos con DTPA o EDTA y el contenido de metales en las raíces, que los extraídos con respecto al contenido en las hojas (Brun *et al.*, 2001).

La amplia variedad de resultados descritos en la bibliografía puede deberse a la multitud de factores implicados en la absorción de los metales, tales como la unión competitiva a la superficie de la raíz y la fuerte unión a la materia orgánica del suelo y a la superficie radicular (Black *et al.*, 2011). Además, la fracción biodisponible de los metales en las plantas no sólo está afectada por la fracción disponible de los metales en el suelo, sino también por los mecanismos de absorción de nutrientes, por la competitividad entre cationes, reacciones en la rizosfera, por el crecimiento de las plantas y por las estrategias intrínsecas de cada especie vegetal (Heemsbergen *et al.*, 2010; Kidd et al., 2009; Monteiro *et al.*, 2009; Ramos *et al.*, 2002; Zorrig *et al.*, 2010). La relación entre los patrones de disponibilidad de los metales y los de biodisponibilidad para las plantas de *Lactuca sativa* L. será tratada en los Apartados 4.1 y 4.2 de esta Tesis, correspondientes al segundo Bloque de los Resultados.

Los metales pesados presentan, además, efectos tóxicos hacia la biota del suelo, pudiendo disminuir el número y la actividad de los microorganismos (Giller *et al.*, 1998; Obbard, 2001). La alteración de la actividad enzimática del suelo se puede producir de forma directa o indirecta. De forma directa, por inactivación de las enzimas mediante la interacción de los metales con el centro activo, con los sustratos, con los complejos enzima-sustrato y/o con los productos enzimáticos. De forma

indirecta, por alteración de la comunidad microbiana que sintetiza enzimas (Kandeler *et al.*, 2000; Kunito *et al.*, 2001) o por reducción de la biomasa microbiana del suelo, dando como resultado la selección de organismos resistentes o tolerantes metabólicamente menos eficientes (Kizilkaya *et al.*, 2005; Kumpiene *et al.*, 2009). Ya que la actividad enzimática del suelo está implicada en el ciclo de los nutrientes principales, la cuantificación de este parámetro bioquímico ha resultado ser un buen bio-indicador del efecto derivado de una perturbación natural y/o antrópica (Renella *et al.*, 2003), incluso más sensible que las plantas o los animales (Hinojosa *et al.*, 2004). La medida de las diferentes actividades enzimáticas del suelo no sólo es altamente sensible al efecto de la contaminación metálica sino que, además, la metodología para su determinación es rápida, sencilla y de bajo coste (Mikanova, 2006).

En el área mediterránea, el clima favorece los procesos de descomposición de la fracción orgánica del suelo, resultando a menudo en un contenido muy bajo de materia orgánica. Ya que esta fracción desempeña un papel fundamental, tanto para la estabilización física como precursor para la síntesis enzimática, la actividad microbiana de estos suelos es, en general, muy baja (Bastida *et al.*, 2006). Esta situación podría limitar la respuesta de las poblaciones microbianas ante el estrés resultante de una contaminación metálica, poniendo de manifiesto la fragilidad de estos suelos (Baldantoni *et al.*, 2010; Moreno *et al.*, 2009). Por ello, ampliar el conocimiento sobre la implicación de los constituyentes del suelo, principalmente la materia orgánica, en la afectación de la actividad de las comunidades microbianas es un factor clave en los estudios de toxicidad por metales pesados en suelos.

Ya que la toxicidad de los metales en el suelo varía significativamente de acuerdo con sus características, la variabilidad natural en las propiedades de los suelos podría ser un impedimento decisivo para el uso de un único parámetro biológico (Nannipieri *et al.*, 1990). Además, se han observado reducciones en la actividad enzimática en suelos contaminados por metales pesados sin que se hayan producido cambios en la estructura de la comunidad microbiana (Kandeler *et al.*, 2000). Por ello, utilizar una combinación de varias propiedades biológicas con fines de diagnóstico podría proporcionar una mejor visión de la alteración de los procesos y el funcionamiento del suelo (Acosta-Martínez *et al.*, 2003). En este sentido, se emplean con frecuencia parámetros biológicos que estimen, no sólo la actividad o

funcionalidad, sino también el tamaño de las poblaciones microbianas, como son la medida del carbono de la biomasa microbiana, la tasa de respiración y/o la cuantificación del ADN bacteriano y fúngico mediante PCRs cuantitativas en tiempo real –qPCR– (reacción en cadena de la polimerasa ~ Polymerase Chain Reaction). La combinación de estos parámetros, además, puede ser otra herramienta útil en la interpretación de los resultados, pues la actividad enzimática extracelular es generalmente dependiente del estado del organismo (activo, inactivo o muerto), existiendo un desfase temporal entre la muerte celular y la disminución de las actividades enzimáticas (Brookes, 1995). En el Apartado 5.1 de esta Tesis, perteneciente al tercer Bloque de los Resultados, se abordará el estudio de la alteración de una combinación de parámetros biológicos ante la contaminación metálica y su relación con los constituyentes del suelo.

Bibliografía

Acosta-Martínez, V., Zobeck, T.M., Gill, T.E., Kennedy, A.C., 2003. Enzyme activities and microbial community structure in semiarid agricultural soils. Biol. Fertil. Soils 38, 216-227.

Alexander, P.D., Alloway, B.J., Dourado, A.M., 2006. Genotypic variations in the accumulation of Cd, Cu, Pb and Zn exhibited by six commonly grown vegetables. Environ. Pollut. 144, 736-745.

Baldantoni, D., Leone, A., Iovieno, P., Morra, L., Zaccardelli, M., Alfani, A., 2010. Total and available soil trace element concentrations in two Mediterranean agricultural systems treated with municipal waste compost or conventional mineral fertilizers. Chemosphere 80, 1006-1013.

Bastida, F., Moreno, J.L., Hernández, T., García, C., 2006. Microbiological degradation index of soils in a semiarid climate. Soil Biol. Biochem. 38, 3463-3473.

Batjes, N.H., 2000. Soil Vulnerability to Diffuse Pollution in Central and Eastern Europe SOVEUR Project (Version 1.0). FAO and ISRIC

Black, A., McLaren, R.G., Reichman, S.M., Speir, T.W., Condron, L.M., 2011. Evaluation of soil metal bioavailability estimates using two plant species (*L. perenne* and *T. aestivum*) grown in a range of agricultural soils treated with biosolids and metal salts. Environ. Pollut. 159, 1523-1535.

Bolan, N.S., Adriano, D.C., Curtin, D., 2003. Soil Acidification and Liming Interactions with Nutrient and Heavy Metal Transformation and Bioavailability. Adv. Agron. 78, 215-272.

Bradl, H.B., 2004. Adsorption of heavy metal ions of soils and soils constituents. J. Colloid Interface Sci. 277, 1-18.

Brookes, P.C., 1995. The use of microbial parameters in monitoring soil pollution by heavy metals. Biol. Fertil. Soils 19(4), 269-279.

Brun, L.A., Maillet, J., Hinsinger, P., Pépin, M., 2001. Evaluation of copper availability to plants in copper-contaminated vineyard soils. Environ. Pollut. 111, 293-302.

Buekers, J., Van Laer, L., Amery, F., Van Buggenhout, S., Maes, A., Smolders, E., 2007. Role of soil constituents in fixation of soluble Zn, Cu, Ni and Cd added to soils. Eur. J. Soil Sci. 58, 1514-1524.

Capelo, A., Santos, C., Loureiro, S., Pedrosa, M.A., 2012. Phytotoxicity of lead on *Lactuca sativa*: effects on growth, mineral nutrition, photosynthetic activity and oxidant metabolism. Fresen. Environ. Bull. 21, 450-459.

Cattani, I., Fragoulis, G., Boccelli, R., Capri, E., 2006. Copper bioavailability in the rhizosphere of maize (Zea mays L.) grown in two Italian soils. Chemosphere 64, 1972-1979.

Chaignon, V., Sanchez-Neira, I., Herrmann, P., Jaillard, B., Hinsinger, P., 2003. Copper bioavailability and extractability as related to chemical properties of contaminated soils from a vine-growing area. Environ. Pollut. 123, 229-238.

Chapman, E.E.V., Dave, G., Murimboh, J.D., 2012. Ecotoxicological risk assessment of undisturbed metal contaminated soil at two remote lighthouse sites. Ecotoxicol. Environ. Saf. 73, 961-969.

Clemente, R., Bernal, M.P., 2006. Fractionation of heavy metals and distribution of organic carbon in two contaminated soils amended with humic acids. Chemosphere 64, 1264-1273.

Clemente, R., Escolar, A., Bernal, M.P., 2006. Heavy metals fractionation and organic matter mineralization in contaminated calcareous soil amended with organic materials. Bioresour. Technol. 97, 1894-1901.

Conde, P., Martín Rubí, J.A., Ballesta, R.J., 2007. Chemical vulnerability of red soils in La Mancha (Central Spain). Sci. Total Environ. 378, 228-232.

Dabkowska-Naskret, H., 2003. The role of organic matter in association with zinc in selected arable soils from Kujawy Region, Poland. Org. Geochem. 34, 645-649.

de Miguel, E., Llamas, J.F., Chacon, E., Berg, T., Larssen, S., Røyset, O., Vadset, M., 1997. Origin and patterns of distribution of trace elements in street dust: unleaded petrol and urban lead. Atmos. Environ. 31(17), 2733-2740.

Directiva 86/278/EEC de 12 Junio de 1986 relativa a la protección del medio ambiente y, en particular, de los suelos, en la utilización de los lodos de depuradora en agricultura.

Escoto Valerio, M., Fernández García, J., Martín Peinado, F., 2007. Determination of phytotoxicity of soluble elements in soils, based on a bioassay with lettuce (*Lactuca sativa* L.). Sci. Total Environ. 378, 63-66.

Ettler, V., Mihaljevič, M., Šebek, O., Grygar, T., 2007. Assessment of single extractions for the determination of mobile forms of metals in highly polluted soils and sediments - Analytical and thermodynamic approaches. Anal. Chim. Acta 602(1), 131-140.

Favas, P.J.C., Pratas, J., Gomes, M.E.P., Cala, V., 2011. Selective chemical extraction of heavy metals in tailings and soils contaminated by mining activity: Environmental implications. J. Geochem. Explor. 111, 160-171.

Feng, M., Shan, X., Zhang, S. and Wen, B., 2005. A comparison of the rhizosphere-based method with DTPA, EDTA, $CaCl_2$, and $NaNO_3$ extraction methods for prediction of bioavailability of metals in soil to barley. Environ. Pollut. 137, 231-240.

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

Gupta, S.K., Vollmer, M.K., Krebs, R., 1996. The importance of mobile, mobilisable and pseudo total heavy metal fractions in soil for three-level risk assessment and risk management. Sci. Total Environ. 178, 11-20.

He, Z.L., Yang, X.E., Stoffella, P.J., 2005. Trace elements in agroecosystems and impacts on the environment. J. Trace Elem. Med. Biol. 19, 125-140.

Heemsbergen, D.A., McLaughlin, M.J., Whatmuff, M., Warne, M.St.J., Broos, K., Bell, M., Nash, D., Barry, G., Pritchard, D., Penney, N., 2010. Bioavailability of zinc and copper in biosolids compared to their soluble salts. Environ. Pollut. 158, 1907-1915.

Hinojosa, M.B., Carreira, J.A., García-Ruíz, R., Dick, R.P., 2004. Soil moisture pre-treatment effects on enzyme activities as indicators of heavy metal-contaminated and reclaimed soils. Soil Biol. Biochem. 36, 1559-1568.

Inaba, S., Takenaka, C., 2005. Effects of dissolved organic matter on toxicity and bioavailability of copper for lettuce sprouts. Environ. Internat. 31, 603- 608.

Jalali, M., Khanlari, Z. V., 2008. Effect of aging process on the fractionation of heavy metals in some calcareous soils of Iran. Geoderma 143, 26-40.

Jalali, M., Moharrami, S., 2007. Competitive adsorption of trace elements in calcareous soils of western Iran. Geoderma 140, 156-163.

Kabata-Pendias, A., Pendias, H., 2001. Trace Elem. Soils. Plants., third ed. Boca Ratón, New York.

Kalis, E.J.J., Temminghoff, E.J.M., Weng, L., van Riemsdijk, W.H., 2006. Effects of humic acid and competing cations on metal uptake by *Lolium perenne*. Environ. Toxicol. Chem. 25(3), 702-711.

Kandeler, E.D., Tscherco, D., Bruce, K.D., Stemmer, M., Hobs, P.J., Bardget, R.D., Amelung, W., 2000. Structure and function of the soil microbial community in microhabits of a heavy metal polluted soil. Biol. Fertil. Soils 32, 390-400.

Kaschl, A., Römheld, V., Chen, Y., 2002. The influence of soluble organic matter from municipal solid waste compost on trace metal leaching in calcareous soils. Sci. Total Environ. 291, 45-57.

Kidd, P., Barceló, J., Bernal, M.P., Navari-Izzo, F., Poschenrieder, C., Shilev, S., Clemente, R., Monterroso, C., 2009. Trace element behaviour at the root–soil interface: Implications in phytoremediation. Environ. Exp. Bot. 67, 243-259.

Kızılkaya, R., Aşkın, T., Bayraklı, B., Sağlam, M., 2004. Microbiological characteristics of soils contaminated with heavy metals. Eur. J. Soil Biol. 40, 95-102.

Kumpiene, J., Guerri, G., Landi, L., Pietramellara, G., Nannipieri, P., Renella, G., 2009. Microbial biomass, respiration and enzyme activities after in situ aided phytostabilization of a Pb- and Cu- contaminated soil. Ecotoxicol. Environ. Saf. 72, 115-119.

Kunito, T., Saeki, K., Goto, S., Hayashi, H., Oyaizu, H., Matsumoto, S., 2001. Copper and zinc fractions affecting microorganisms in long-term sludge-amended soils. Bioresour. Technol. 79, 135-146.

Lafuente, A.L., González, C., Quintana, J.R., Vázquez, A., Romero, A., 2008. Mobility of heavy metals in poorly developed carbonate soils in the Mediterranean region. Geoderma 145, 238-244.

Lamb, D.T., Ming, H., Megharaj, M., Naidu, R., 2010. Heavy metal (Cu, Zn, Cd and Pb) partitioning and bioaccessibility in uncontaminated and long-term contaminated soils. J. Hazard. Mater. 171, 1150-1158.

Chicharro Martín, A., Cala Ribero, V., Larrea Marín, M.T., 1998. Contamination by heavy metals in soils in the neighbourhood of a scrapyard of discarded vehicles. Sci. Total Environ. 212, 145-152.

Martínez, C.E., Motto, H.L., 2000. Solubility of lead, zinc and copper added to mineral soils. Environ. Pollut. 107, 153-158.

Meers, E., du Laing, G., Unamuno, V., Ruttens, A., Vangronsveld, J., Tack, F.M.G., Verloo, M.G., 2007. Comparison of cadmium extractability from soils by commonly used single extraction protocols. Geoderma 141, 247-259.

Menzies, N.W., Donn, M.J., Kopittke, P.M., 2007. Evaluation of extractants for estimation of the phytoavailable trace metals in soils. Environ. Pollut. 145, 121-130.

Mikanova, O., 2006. Effects of heavy metals on some soil biological parameters. J. Geochem. Explor. 88, 220-223.

Micó, C., Recatalá, L., Peris, M., Sánchez, J., 2006. Assessing heavy metal sources in agricultural soils of an European Mediterranean area by multivariate analysis. Chemosphere 65, 863-872.

Monteiro, M.S., Santos, C., Soares, A.M.V.M., Mann, R.M., 2009. Assessment of biomarkers of cadmium stress in lettuce. Ecotoxicol. Environ. Saf. 72, 811-818.

Moreno, J.L., Bastida, F., Ros, M., Hernández, T., García, C., 2009. Soil organic carbon buffers heavy metal contamination on semiarid soils: Effects of different metal threshold levels on soil microbial activity. Eur. J. Soil Biol. 45, 220-228.

Moreno, A.M., Pérez, L., González, J., 1992. Relaciones entre contenidos totales de Zn, Pb, Cu y Cd en suelos y plantas. Suelo y Planta 2(4), 757-771.

Nannipieri, P., Greco, S., Ceccanti, B., 1990. Ecological significance of the biological activity in soil. In: Bollag, J-M., Stotzky, G. (Eds.). Soil Biochem., vol. 6. Marcel Dekker, New York, pp. 293-355.

Obbard, J.P., 2001. Ecotoxicological assessment of heavy metals in sewage sludge amended soils. Appl. Geochem. 16, 1405-1411.

Pérez-Esteban, J., 2011. Biodisponibilidad de metales pesados en suelos mineros contaminados enmendados con materiales orgánicos. Tesis Doctoral. Universidad Politécnica de Madrid. Madrid.

Pérez-Esteban, J., Escolástico, C., Moliner, A., Masaguer, A., 2013. Chemical speciation and mobilization of copper and zinc in naturally contaminated mine soils with citric and tartaric acids. Chemosphere 90, 276-283.

Peris, M., Micó, C., Recatalá, L., Sánchez, R., Sánchez, J., 2007. Heavy metal contents in horticultural crops of a representative area of the European Mediterranean region. Sci. Total Environ. 378, 42-48.

Plassard, F., Winiarski, T., Petit-Ramel, M., 2000. Retention and distribution of three heavy metals in a carbonated soil: comparison between batch and unsaturated column studies. J. Contam. Hydrol. 42, 99-111.

Ramos, I., Esteban, E., Lucena, J.J., Gárate, A., 2002. Cadmium uptake and subcellular distribution in plants of *Lactuca* sp. Cd-Mn interaction. Plant. Sci. 162, 761-767.

Ramos-Miras, J.J., Roca-Perez, L., Guzmán-Palomino, M., Boluda, R., Gil, C., 2011. Background levels and baseline values of available heavy metals in Mediterranean greenhouse soils (Spain). J. Geochem. Explor. 110, 186-192.

Rao, C.R.M., Sahuquillo, A., Lopez Sanchez, J.F., 2008. A Review of the Different Methods Applied in Environmental Geochemistry For Single and Sequential Extraction of Trace Elements in Soils and Related Materials. Water Air Soil Pollut. 189, 291-333.

Rauret, G., Lopez-Sanchez, J.F., Sahuquillo, A., Rubio, R., Davidson, C., Ure, A., Queqauviller, Ph., 1999. Improvement of the BCR three-step sequential extraction procedure prior to the certification of new sediment and soil reference materials. J. Environ. Monit. 1, 57-61.

Recatalá, L., Sacristán, D., Arbelo, C., Sánchez, J., 2011. Can a Single and Unique Cu Soil Quality Standard be Valid for Different Mediterranean Agricultural Soils under an Accumulator Crop? Water, Air, Soil Pollut. 223(4), 1503-1517.

Recatalá, L., Sánchez, J., Arbelo, C., Sacristán, D., 2010. Testing the validity of a Cd soil quality standard in representative Mediterranean agricultural soils under an accumulator crop. Sci. Total Environ. 409, 9-18.

Reglamento (CE) nº 466/2001 de 8 de marzo de 2001 por el que se fija el contenido máximo de determinados contaminantes en los productos alimenticios.

Renella, G., Ortigoza, A.L.R., Landi, L., Nannipieri, P., 2003. Additive effects of copper and zinc on cadmium toxicity on phosphatase activities and ATP content of soil as estimated by the ecological dose (ED50). Soil Biol. Biochem. 35, 1203-1210.

Ruiz, E., Rodríguez, L., Alonso-Azcárate, J., 2009. Effects of earthworms on metal uptake of heavy metals from polluted mine soils by different crop plants. Chemosphere 75, 1035-1041.

Sánchez-Camazano, M., Sanchez-Martin, M. J., Lorenzo, L. F., 1998. Significance of soil properties for content and distribution of cadmium and lead in natural calcareous soils. Sci. Total Environ. 218, 217-226.

Sayyad, G., Afyuni, M., Mousavi, S.F., Abbaspour, K.C., Richards, B.K., Schulin, R., 2010. Transport of Cd, Cu, Pb and Zn in a calcareous soil under wheat and safflower cultivation - A column study. Geoderma 154, 311-320.

Sipos, P., Németh, T., Kovács Kis, V., Mohai, I., 2008. Sorption of copper, zinc and lead on soil mineral phases. Chemosphere 73, 461-469.

Smith, S.R., 2009. A critical review of the bioavailability and impacts of heavy metals in municipal solid waste composts compared to sewage sludge. Environ. Int. 35, 142-156.

Steinitz, C., del Pozo, C., Vargas-Moreno, J.C., Canfield, T., 2011. Futuros alternativos para los paisajes dinámicos del Corredor del Henares. Fundación Paisaje 2011.

Tessier, A., Campbell, P.G.C., Bisson, M., 1979. Sequential extraction procedure for the speciation of particulate trace metals. Anal. Chem. 51, 844-851.

Udovic, M., Lestan, D., 2007. The effect of earthworms on the fractionation and bioavailability of heavy metals before and after soil remediation. Environ. Pollut. 148, 663-668.

Ure, A.M., 1996. Single extraction schemes for soil analysis and related applications. Sci. Total Environ. 78, 3-10.

Vidal, J., Pérez-Sirvent, C., Martínez-Sánchez, M.J., Navarro, M.C., 2004. Origin and behaviour of heavy metals in agricultural Calcaric Fluvisols in semiarid conditions. Geoderma 121, 257-270.

Vig, K., Megharaj, M., Sethunathan, N., Naidu, R., 2003. Bioavailability and toxicity of cadmium to microorganisms and their activities in soil: a review. Adv. Environ. Res. 8, 121-135.

Wong, J.W.C., Li, K.L., Zhou, L.X., Selvam, A., 2007. The sorption of Cd and Zn by different soils in the presence of dissolved organic matter from sludge. Geoderma 137, 310-317.

Zeng, F., Ali, S., Zhang, H., Ouyang, Y., Qiu, B., Wu, F., Zhang, G., 2011. The influence of pH and organic matter content in paddy soil on heavy metal availability and their uptake by rice plants. Environ. Pollut. 159, 84-91.

Zorrig, W., Rouached, A., Shahzad, Z., Abdelly, C., Davidian, J., Berthomieu, P., 2010. Identification of three relationships linking cadmium accumulation to cadmium tolerance and zinc and citrate accumulation in lettuce. J. Plant Physiol. 167, 1239-1247.

2. Hipótesis y Objetivos

Hipótesis y Objetivos

Las características intrínsecas de los suelos calcáreos de uso agrícola del área mediterránea favorecen la acumulación y retención de metales pesados en los horizontes superficiales. En este contexto, los constituyentes edáficos adquieren un papel decisivo, pues le confieren al suelo una función ambiental de gran importancia, actuando como sumidero o como fuente de contaminantes. Sin embargo, el papel que ejercen, tanto el contenido, como la composición, de las diferentes fracciones del suelo aún no está bien definido. Basándonos en esta premisa, la presente Tesis Doctoral ha tenido como finalidad profundizar en el conocimiento del papel que ejercen los constituyentes del suelo en la disponibilidad de metales pesados en suelos calcáreos de uso agrícola del área mediterránea. Con este propósito, se seleccionaron muestras de suelo que, aún no estando contaminadas, presentan un riesgo potencial de contaminación debido a su ubicación y uso, a las que se le añadió una mezcla de Cd, Cu, Pb y Zn a dos niveles, con el fin de simular un futuro escenario de contaminación metálica.

A partir de este objetivo general, abordado desde un enfoque tanto químico como biológico, se establecieron los siguientes objetivos específicos, articulados en tres grandes bloques:

Bloque 1. Estudio de los patrones de extractabilidad de metales

- Analizar la influencia del nivel de contaminación y de los constituyentes del suelo en las tendencias temporales de la extractabilidad metálica bajo un proceso de incubación en condiciones de laboratorio.
- Evaluar la eficiencia de los métodos de extracción seleccionados, como estimadores de la movilidad inmediata y potencial de los metales.
- Identificar las fracciones del suelo que explican los patrones de extractabilidad de los metales.

Bloque 2. Estudio de los patrones de biodisponibilidad de metales en *Lactuca sativa* L.

- Evaluar la posible fitotoxicidad causada en hojas y raíces de dos variedades de *Lactuca sativa* L., romana e iceberg.
- Determinar la transferencia metálica en el sistema suelo-planta, así como la translocación a los tejidos comestibles.
- Identificar las fracciones del suelo que explican los patrones de biodisponibilidad de los metales.
- Estudiar las posibles relaciones existentes entre los patrones de biodisponibilidad de los metales en las plantas y los de disponibilidad en el suelo, estimados con métodos químicos de extracción.

Bloque 3. Estudio de la perturbación de las propiedades microbiológicas del suelo

- Evaluar la inhibición de las actividades enzimáticas del suelo implicadas en los principales ciclos biogeoquímicos de nutrientes.
- Detectar la posible perturbación de la biomasa fúngica y bacteriana.
- Identificar las fracciones del suelo que determinan la persistencia de las actividades enzimáticas y la supervivencia de las poblaciones microbianas.

Hypothesis and Objectives

The intrinsic characteristics of calcareous agricultural soils in the Mediterranean area favour the accumulation and retention of heavy metals in the surface horizons. In this context, the soil constituents acquire a decisive role, as they confer a major environmental function to soils, acting as either sink or source of contaminants. However, the role played by both content and composition of the different soil fractions is not properly defined. Based on this premise, this PhD. Thesis has been aimed at deepen the knowledge of the role played by soil constituents in metal availability in calcareous agricultural soils in the Mediterranean area. For this purpose, we selected soil samples which, while not contaminated, present a potential risk of contamination due to their location and use. A mixture of Cd, Cu, Pb and Zn at two levels was added to the aforementioned samples, in order to simulate a future scenario metal contamination.

From this general objective, addressed from both chemical and biological approaches, the following specific objectives were established, being articulated in three sections:

Section 1. Study of metal extractability patterns

- To analyze the influence of both contamination level and soil constituents in temporal trends of metal extractability over an incubation experiment under laboratory conditions.
- To assess the extraction efficiency of the selected methods as estimators of immediate and potential mobility of metals.
- To identify soil fractions explaining metal extractability patterns.

Section 2. Study of metal bioavailability patterns in *Lactuca sativa* L.

- To evaluate the potential phytotoxicity caused in leaves and roots in both romaine and iceberg varieties of *Lactuca sativa* L.
- To determine the metal transfer in the soil-plant system and the metal translocation to edible tissues.
- To identify soil fractions explaining metal bioavailability patterns.
- To study possible relationships between metal bioavailability patterns in plants and metal availability in soils, estimated by chemical extraction methods.

Section 3. Study of soil microbial disturbance

- To assess the inhibition of several enzyme activities involved in major soil biogeochemical cycles of nutrients.
- To detect a potential disturbance in the bacterial and fungal biomasses.
- To identify soil fractions determining the persistence of enzyme activities and the survival of the microbial populations.

Hypothèse et Objectifs

Les caractéristiques intrinsèques des sols calcaires agricoles de la région Méditerranéenne favorisent l'accumulation et la rétention des éléments traces métalliques (ETM) dans les horizons de surface du sol. Dans ce contexte, les constituants du sol acquièrent un rôle décisif, puisqu'ils confèrent au sol une fonction écologique d'une grande importance, en tant que source ou puits de contaminants. Cependant, le rôle joué par le contenu et la composition des différentes fractions du sol n'est pas bien défini. Sur cette prémisse, cette Thèse de Doctorat a eu pour but de mieux comprendre le rôle joué par les constituants du sol dans la disponibilité des ETM dans les sols calcaires agricoles de la région Méditerranéenne. À cette fin, nous avons sélectionné des échantillons de sol, bien que n'étant pas contaminés, qui présentent un risque potentiel de contamination due à son emplacement et usage, auxquelles a été ajouté un mélange de Cd, Cu, Pb et Zn à deux niveaux, afin de simuler un scénario futur de contamination par des ETM.

De cet objectif général, abordé dans une perspective à la fois chimique et biologique, nous avons établi les objectifs spécifiques suivants, formulés en trois sections:

Section 1. Étude des patrons d'extractibilité des ETM

- Analyser l'influence du niveau de contamination et des constituants du sol dans les tendances temporelles d'extractibilité des ETM au cours d'un essai d'incubation sous conditions de laboratoire.
- Évaluer l'efficacité de certaines méthodes d'extraction, comme estimateurs de la mobilité immédiate et potentielle des ETM.
- Identifier les fractions du sol explicatives des patrons d'extractibilité des ETM.

Section 2. Étude des patrons de biodisponibilité des ETM dans *Lactuca sativa* L.

- Évaluer le risque de phytotoxicité, à niveau des feuilles et des racines, dans deux variétés de *Lactuca sativa* L., romaine et iceberg.
- Déterminer le transfert des ETM dans le système sol-plante et la translocation dans les tissus comestibles.
- Identifier les fractions du sol explicatives des patrons de biodisponibilité des ETM.
- Étudier la relation entre les patrons de biodisponibilité des ETM dans les plantes et la disponibilité dans le sol, estimés par extractions chimiques.

Section 3. Étude de la perturbation des propriétés microbiennes du sol

- Évaluer l'inhibition des activités enzymatiques impliquées dans les principaux cycles biogéochimiques des éléments nutritifs du sol.
- Détecter les possibles perturbations de la biomasse bactérienne et fongique.
- Identifier les fractions du sol qui déterminent la persistance des activités enzymatiques et la survie des populations microbiennes.

3. Estudio de los patrones de extractabilidad de metales

Apartado 3.1

Soil properties affecting metal extractability patterns in periurban calcareous agricultural soils in the Mediterranean area

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International Journal of Environmental Research, in press

Resumen

El riesgo potencial de acumulación de metales pesados en áreas agrícolas periurbanas es un asunto de gran preocupación. Las características del clima Mediterráneo y el contenido en fracción carbonatada de los suelos agrícolas, muy abundantes en esta área, favorecen la acumulación de los metales en los horizontes superficiales. Sin embargo, existe también un riesgo de movilidad potencial metálica en estos suelos. Por ello, el objetivo del trabajo se centró en el estudio de las propiedades edáficas que, en estas condiciones, afectan a la movilidad de los metales. Los suelos fueron contaminados con una mezcla de Cd, Cu, Pb y Zn e incubados hasta 12 meses de tiempo de contacto, pasado este tiempo, se evaluaron, en las muestras de suelo, los patrones de extractabilidad metálica utilizando métodos de extracción química en un solo paso ($NaNO_3$, una mezcla de ácidos orgánicos de bajo peso molecular –LMWOA– y dietilen triamino pentaacético –DTPA–).

La concentración de Cd extraído fue un 50 % menor en suelos con mayores contenidos en carbonato. La concentración de Cu extraído con LMWOA fue mayor en suelos con menor contenido en materia orgánica (MO), en el día 1 del experimento de incubación, y en suelos con menor contenido tanto en carbonato como en fracción recalcitrante de la MO, a los 12 meses. Las fracciones minerales finas determinaron las menores concentraciones de Cu extraído con DTPA. La mayor retención de Pb se produjo en suelos con los mayores contenidos en arcilla y carbonato. Sin embargo, no se pudo establecer qué componente edáfico reguló los patrones de extractabilidad de Pb. El patrón de extractabilidad Zn se relacionó con la distribución de tamaño de partícula, siendo su extractabilidad más elevada en suelos con una baja proporción de fracciones minerales finas. En resumen, el carbonato, la granulometría y la materia orgánica fueron los factores más relevantes en el control la movilidad potencial de los metales en estos suelos. Los altos valores de extracción metálica con DTPA evidenciaron que existe un riesgo potencial de movilidad de metales en los suelos de estudio.

Abstract

The potential risk of metal accumulation in periurban agricultural areas is a matter for concern. The climate characteristics and carbonate content of calcareous agricultural Mediterranean soils typical in these areas favour metal accumulation at the surface level; however there is also a risk of potential metal mobility. Our study focuses on the soil properties affecting metal mobility in these soils. Metal extractability patterns were assessed in soils after they were spiked with a mixture of Cd, Cu, Pb, and Zn and incubated up to 12 months, using one-step extraction methods ($NaNO_3$, a mixture of low molecular weight organic acids –LMWOA and diethylene triamine pentaacetic acid –DTPA–). The concentration of extractable Cd was 50% lower in the soil with the highest carbonate content. LMWOA-extractable Cu was highest in soils with the lowest organic matter (OM) content at day 1 of the incubation experiment and in soils with the lowest carbonate and recalcitrant OM contents at 12 months. Fine mineral fractions determined the lowest DTPA-extractable Cu. The highest Pb retention was in soils with the highest carbonate and clay contents. However, we were unable to establish any soil component affecting Pb extractability patterns. The Zn extractability pattern was related to particle-size distribution, which was highest in soils with a low proportion of fine mineral fractions. To summarise, carbonate, particle-size distribution and OM are relevant to potential metal mobility in these soils. The high DTPA-extractable metal values are evidence of a potential risk of metal mobility in the soils in the study.

Key-words

Soil pollution, carbonate, metal mobility, one-step extractions, incubation experiment

Introduction

In recent decades, increasing industrialization and urbanization has led to a rise in the accumulation of trace metals in periurban agricultural areas (Roca-Perez *et al.*, 2010). The risk of metal contamination of groundwater and its accumulation in food crops is directly related to the potential mobility of metals in soils. The mobility, and availability metals in soils depends on the combined action of various factors, including the nature of the metal and its concentration in the soil, environmental factors, and soil components (Vega *et al.*, 2010). These factors determine the soil solution-solid phase equilibrium of metals through the mechanisms of sorption-desorption and dissolution-precipitation.

In the Mediterranean climate, low rainfall and high evapotranspiration limit metal mobility in soils, and metal retention is increased in calcareous Mediterranean soils owing to their carbonate content. It has been suggested that these characteristics imply that these soils are not vulnerable to chemical degradation (Conde *et al.*, 2007). However, their high metal retention capacity enhances metal accumulation in the first few centimetres of the soil, where plants can increase their concentrations of soluble metal, thereby leading to significant leaching and particularly to the uptake of metals (Sayyad *et al.*, 2010). Martínez and Motto (2000) observed that metal solubility is more susceptible to decreases in pH in calcareous soils than in non-calcareous soils, making them more vulnerable. Although carbonates play a decisive role in sorption-desorption processes, some studies have highlighted the fact that other soil constituents could play a key role in metal distribution and availability in calcareous soils (Buekers *et al.*, 2007; Jalali and Khanlari, 2008; Micó *et al.*, 2006; Sánchez-Camazano *et al.*, 1998; Sipos *et al.*, 2008). However, the information in the literature is far from homogeneous. In previous studies we observed a high metal retention capacity (> 90 %) in calcareous agricultural soils in the Mediterranean area and we reported that metal mobility is conditioned by the mineralogical characteristics (Lafuente *et al.*, 2008). The complexity of the interaction between metals and the constituents of calcareous soils, combined with the vulnerability of these soils which allows metals to move from one environmental echelon to another, highlights the

importance of the study of the soil properties affecting the mobility –and thus the availability– of metals in these soils.

It is generally accepted that to assess the mobility and availability of metal in soils it is not sufficient merely to analyse the total metal content in soils, nor is this a useful tool for determining potential risks. One-step extraction methods are widely used in studies of the potential mobility and availability of metals: chelating agents to assess metal phytoaccessibility and ecotoxicity, neutral salts for toxicity-related measurement of metal availability (Conder *et al.*, 2001; Gupta and Aten, 1993), and recently low molecular weight organic acids to simulate the rhizosphere ambient (Feng *et al.*, 2005). These procedures are useful as they may provide comparative information on the potential mobility of metals, and help to evaluate the relative availability of various metals in multi-element contaminated soils.

In the present work, we placed particular emphasis on the soil constituents and properties affecting metal availability in calcareous soils, since the role of soil constituents in metal desorption processes is not well defined for these soils. We therefore selected various periurban calcareous agricultural soils representative of the Mediterranean area with a gradient in their carbonate content, and whose remaining soil components differed. In order to obtain information on metal availability in soils with these characteristics, samples were spiked with a mixture of metals and incubated for up to 12 months. Metals were extracted with one-step extraction methods ($NaNO_3$, LMWOA and DTPA) at different time intervals.

Materials and Methods

Study area, soil characteristics and sampling

The study area is located in Alcalá de Henares (Spain) at an altitude of 588 m. It is part of a periurban axis that combines agricultural activity and the main residential and industrial uses in the Madrid region. This scenario is typical of European metropolitan areas, and contains agricultural soils which are affected and likely to become more so in the future unless sustainability criteria are applied (Steinitz *et al.*, 2011). The soil samples come from different plots in the "El Encín" Agricultural Research Station, located on Henares River on quaternary sediments

(IGME, 1990). These alluvial sediments have led to an ancient calcaric Fluvisol (Moreno Merino, 1998) which presents Anthric characteristics today (FAO, 2006) mainly as a result of agricultural use. The average annual temperature is slightly over 13 ºC; average annual rainfall is 401 mm $year^{-1}$; and potential evaporation is about 760 mm $year^{-1}$. The site is typical of a Mediterranean pluviseasonal-oceanic bioclimate on an upper meso-Mediterranean low dry bioclimatic belt (Rivas-Martínez, 1987).

We selected six agricultural soil samples with different carbonate contents and amounts of organic matter. Samples included a mixture of 30-40 kg of the soil surface horizons (0-30 cm), which were not been differentiated due to agricultural activity. Soil samples were air-dried and passed through a 2 mm sieve.

Experimental design

For the experimental design, we selected unpolluted agricultural soil samples that are at potential risk of contamination due to their location. Based on the premise that the addition of a mixture of heavy metals in the soil samples would mimic the situation which would occur in a multi-element contaminated soil, the unpolluted soil samples were spiked with a multi-element metal salt solution. Cd, Cu, Pb, and Zn were selected owing to their different speciation, mobility and extractability in soils. Ten kg of each soil sample were subjected to an incubation experiment. Each soil sample was placed in an individual plastic container (40 cm wide x 59 cm long x 21 cm high) and spiked with nitrate salts from heavy metals in an aqueous solution at the rate of 3 mg kg^{-1} of Cd, 140 mg kg^{-1} of Cu, 300 mg kg^{-1} of Pb, and 300 mg kg^{-1} of Zn, corresponding to the limit values proposed by current European legislation (Directive 86/278/EEC).

The soil samples and the metallic solution were mixed and left to equilibrate for a period of 12 months at room temperature without cover or drainage. During this equilibration period, the soils were air-dried, mixed and rewetted with distilled water in cycles of about 2 weeks, in order to favour metal redistribution into the soil matrix and to minimize the disruption of the metal speciation patterns (Zheng and Zhang, 2011). When a soluble metal salt is added to soil, it enters the soil pore water and initially increases the concentration of soluble metal ions, as a function of contact time; metal ions are redistributed from weakly-binding sites to more strongly-binding

sites (McLaughlin, 2001). These processes play a key role in determining the extractability and availability of metals in soils and the soil toxicity status (Zapusek and Lestan, 2009). Three duplicate subsamples were randomly removed from each spiked soil sample at different time intervals (1 day, 1, 3, 6 and 12 months) for one-step extraction methods of metals ($NaNO_3$, LMWOA and DTPA-methods). Metal chemical extractions were also performed in unpolluted soils for use as a reference. In order to evaluate the soil sorption capacity, a sorption test was conducted prior to the incubation experiment.

Analytical methods

All chemicals were obtained from Merck (Germany). All glassware used was pre-washed with an aqueous solution of HNO_3 1:1000 for 24 h and rinsed with distilled water.

Soil physicochemical parameters

According to ISRIC-methods (2002), the following parameters were determined: soil pH in a 1:2.5 soil to water ratio, equivalent $CaCO_3$ (ECC) by the acid neutralization method, total organic C by Walkley and Black wet oxidation procedure, particle-size distribution by the Robinson's pipette method, cation exchange capacity (CEC) by the ammonium acetate method and crystalline and amorphous Fe and Mn oxide contents by dithionite-citrate extraction, followed by acid oxalate extraction. We used the two-step acid hydrolysis procedure with H_2SO_4 to determine recalcitrant pool of organic matter (Rovira and Vallejo, 2000). Total N was determined by elemental analysis (LECO CNS 2000I analyzer). Total Ca, Mg, Cd, Cu, Pb, and Zn contents of the soil samples were determined after wet digestion with a mixture of nitro-perchloric-hydrofluoric acids under high-pressure conditions (SISS, 1985). Fe, Mn, Ca, Mg, Cd, Cu, Pb, and Zn concentration in the corresponding extracts was quantified by atomic absorption spectroscopy –AAS– (Analytikjena NovAA 300). All samples were extracted and analyzed in duplicate.

Soil mineralogical analyses

Mineral composition of soil samples (fine earth) was examined by X-ray diffraction (XRD) using an EQ 0434520 31 02 (X'Pert MPD) diffractometer with Cu Kα radiation operated at 45 kV and 40 mA. All XRD patterns were recorded with a dwell time of 1s and 0.04º2θ step. Soils were examined on randomly-orientated powders. Abundance of soil minerals was semi-quantitatively (Bish, 1994).

One-step extraction methods of metals

$NaNO_3$-extractable metals were determined by shaking 16 g of soil with 40 ml of 0.1 M $NaNO_3$ for 2 h (Gupta and Aten, 1993). LMWOA-extractable metals were determined by shaking 5 g of soil with 50 ml of a combined solution of low molecular weight organic acids (acetic, lactic, citric, malic and formic acids with a molar ratio of 4:2:1:1:1) for 16 h (Feng *et al.*, 2005). DTPA-extractable metals were determined by shaking 20 g of soil with 40 ml of 0.005 M DTPA + 0.01 M $CaCl_2$ + 0.01 M triethanolamine (TEA) for 2 h (Lindsay and Norwell, 1978).

Sorption capacity

For the sorption capacity test, the metallic solution and each soil sample (1:2 w:v) were mechanically shaken for 24 h in centrifuge tubes. The supernatant of each extraction was subsequently centrifuged at 3500 rpm for 15 min and then filtered. Cd, Cu, Pb and Zn concentration in the supernatant was quantified by AAS. Distribution coefficients (K_d) of metals were calculated from the sorption data obtained (Lafuente *et al.*, 2008) using the equation: $K_d = (M_{sorbed})/(M_{solution})$; where M_{sorbed} is the amount of sorbed metal per unit weight of soil (mg kg^{-1}), and $M_{solution}$ is the amount of metal in solution per unit volume of liquid (mg L^{-1}).

Results and Discussion

Soil characteristics

The main results of the soil physicochemical analyses are shown in Table 1. All unpolluted soils showed pH values above 8. The ECC content ranged from moderate (M1, M2, and M3 soils) to low (L1 and L2 soils) and very low (VL1 soil). The total organic C (TOC) ranged from very low to high content and recalcitrant pool (RP) was about 67 %. The textural classes of soils ranged from sandy-loam to sandy-clay loam. Fine sand was the most frequent size fraction. Crystalline Fe oxide content was low. Crystalline Mn and amorphous Fe-Mn oxide content was very low (data not shown). The total Cu, Pb, and Zn content were similar to those obtained by other authors for agricultural soils in the Mediterranean area (Jiménez Ballesta *et al.*, 2010; Micó *et al.*, 2006; Peris *et al.*, 2007) and in no case exceeded the levels established by the European Union (Directive 86/278/EEC). The total Cd content was lower than the quantification limit.

Table 1. Selected physicochemical parameters of the unpolluted soil samples.

Soil sample	pH	ECC	OM	CS	FS	Silt	Clay	cry-Fe	CEC	Total Ca	Total Mg	Total Cu	Total Pb	Total Zn
		g kg^{-1}							$cmol_c$ kg^{-1}	g kg^{-1}		mg kg^{-1}		
M1	8.4	190	10.2	111	603	126	161	8.1	7.1	94.2	10.5	8.0	14.0	54.7
M2	8.1	148	20.3	114	569	124	193	8.5	8.7	93.5	10.7	12.1	55.8	62.7
M3	8.1	125	30.6	23	592	215	170	11.7	10.6	41.3	10.5	10.0	23.8	63.3
L1	8.2	106	31.6	159	590	78	172	13.4	6.9	40.1	7.0	12.0	25.5	52.9
L2	8.1	100	13.4	112	462	167	259	10.3	13.3	33.8	9.4	8.5	21.5	55.1
VL1	8.1	32	21.0	45	459	168	328	11.4	19.1	10.0	6.2	10.1	24.0	62.1

ECC = equivalent $CaCO_3$; OM =organic matter; CS = coarse sand; FS = fine sand; cry-Fe = crystalline Fe oxides; CEC = cation exchange capacity

In the metal-spiked soil samples, pH values decreased by 0.2-0.6 units as a result of the metallic solution added (pH 3.5). We also noted that there was a 2.8-fold decrease of RP content in L2 soil, 1.7 in VL1 soil, and 1.3 in L1 soil. These soils corresponded to the soil samples with a lower ECC content, which suggests that soils with low ECC levels show lower OM stabilization (Grünewald *et al.*, 2006). The

decrease in RP may be related to the toxicity produced by the mixture of metals added. In a previous experiment we observed an increase in the fungal:bacterial ratio in some metal-spiked soils (de Santiago *et al.*, 2011), leading to a disruption of key soil processes such as the fungal decomposition of recalcitrant OM (de Boer *et al.*, 2005).

Examination of the fine earth fractions by X-ray diffraction (Table 2) indicated high quartz and low kaolinite contents. Phyllosilicates 2:1 were the main mineral in this fraction with peaks at 0.99 and 0.33 nm and in varying proportions. The presence of plagioclase feldspars was confirmed by the reflections at 0.32 and 0.31 nm. Calcite (0.303, 0.209 and 0.187 nm) was the predominant mineral in the carbonate group. Dolomite appeared only in M1 and M2 soil samples (0.288 nm and 0.180 nm).

Table 2. Composition and relative abundance of fine earth in the unpolluted soil samples.

Soil Sample	Phyllosilicates 2:1	Kaolinite	Feldspars	Quartz	Calcite	Dolomite
M1	++	tr	++	++++	+++	++
M2	+	tr	+	++++	+++	+
M3	++	+	++	++++	++	tr
L1	++	tr	++	++++	++	tr
L2	+	tr	++	++++	++	nd
VL1	++	tr	++	++++	tr	nd

Number of +´s is proportional to abundance: (++++) most abundant, (+) least abundant, (tr) trace, and (nd) not detected.

Metal extractability patterns

The metal sorption capacity was above 98 % for Cd and 99 % for Cu, Pb, and Zn in all six soils studied (data not shown). Distribution coefficients (K_d) are shown in Fig. 1. High K_d values indicate that the metal was retained in the solid phase through adsorption, precipitation and complexing reactions, whereas low K_d values show that high concentrations of metals remain in solution (Anderson and Christensen, 1988). Pb was retained in the highest quantities: as shown, the calculated K_d ranged from 5 to $7 \cdot 10^3$. Cu and Zn presented intermediate values of K_d while Cd had the lowest K_d value. These results concur with the lowest percentage of extractable Pb and the highest percentage of extractable Cd obtained with all the extraction methods tested.

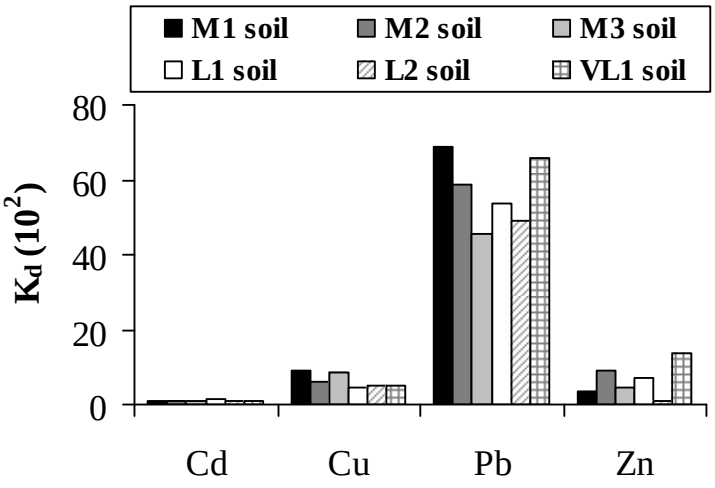

Fig. 1. Distribution coefficient (K_d) calculated in the metal-spiked soil samples at 24 h of contact time.

The metal concentration values obtained in single chemical extractions in the unpolluted and metal-spiked soils incubated for 1 day and 12 months are shown in Table 3. Other values were not included for reasons of space. The metal concentration values in the metal-spiked soils as a percentage of total metal are shown in Fig. 2.

In general, the extraction efficiency at the end of the incubation experiment for all tested soils and metals applied as a multi-element metal salt solution followed the extraction sequence: DTPA (36-80 %) > LMWOA (1-11 %) > $NaNO_3$ (0-3 %), as specified by Feng *et al.* (2005). In some cases, the metal concentrations extracted were too low to allow a consistent interpretation of the data ($NaNO_3$-extractable Cu, Pb and Zn and LMWOA-Pb). Moreover, the $NaNO_3$ method was incapable of discriminating among different soil types for metal extractability. However, the influence of soil properties on metal extractability could be observed with LMWOA and more clearly with the DTPA method from the start of the experiment, and more markedly so at the end. This behaviour is reflected in the trend plots shown in Figure 2.

Table 3. Extractable Cd, Cu, Pb, and Zn concentrations (mg kg^{-1}) in the unpolluted and in the metal-spiked soil samples at day 1 and at 12 months of contact time.

Metal	Extraction method	Contact time	Soil sample					
			M1	M2	M3	L1	L2	VL1
Cd	$NaNO_3$	Unpolluted	LQL	LQL	LQL	LQL	LQL	LQL
		1 day	0.43	0.42	0.43	0.42	0.43	0.43
		12 months	0.09	0.08	0.08	0.09	0.09	0.08
	LMWOA	Unpolluted	LQL	LQL	LQL	LQL	LQL	LQL
		1 day	0.63	0.73	0.66	0.69	0.71	0.74
		12 months	0.17	0.28	0.22	0.30	0.20	0.32
	DTPA	Unpolluted	LQL	LQL	LQL	LQL	LQL	LQL
		1 day	2.06	1.97	2.53	2.65	2.32	1.97
		12 months	1.07	1.67	1.65	2.20	1.70	1.93
Cu	$NaNO_3$	Unpolluted	0.59	0.42	0.43	0.58	0.58	0.55
		1 day	1.65	1.79	4.04	2.75	2.34	1.74
		12 months	0.43	0.63	0.53	1.06	0.43	0.29
	LMWOA	Unpolluted	0.19	0.18	0.18	0.91	0.32	0.21
		1 day	25.27	20.20	6.87	9.33	19.30	15.16
		12 months	3.06	2.35	2.68	6.27	3.22	9.30
	DTPA	Unpolluted	1.32	1.47	1.70	0.93	0.85	1.49
		1 day	90.91	71.08	86.31	81.64	83.50	65.97
		12 months	111.70	112.70	100.90	121.00	104.90	92.64
Pb	$NaNO_3$	Unpolluted	LQL	LQL	LQL	LQL	LQL	LQL
		1 day	0.24	0.22	0.25	0.27	0.28	0.26
		12 months	0.49	0.48	0.48	0.45	0.52	0.52
	LMWOA	Unpolluted	0.98	0.56	0.68	0.54	1.08	0.93
		1 day	1.09	0.62	0.72	0.74	1.15	1.25
		12 months	2.65	2.44	2.19	2.74	2.45	2.42
	DTPA	Unpolluted	2.24	7.12	5.38	3.63	3.52	6.27
		1 day	186.76	161.64	197.09	193.09	184.96	162.88
		12 months	167.20	171.20	145.50	166.20	157.70	174.80
Zn	$NaNO_3$	Unpolluted	LQL	LQL	LQL	LQL	LQL	LQL
		1 day	4.91	3.54	2.04	3.02	4.98	2.65
		12 months	0.24	0.21	0.10	0.64	0.20	0.07
	LMWOA	Unpolluted	LQL	LQL	LQL	LQL	LQL	LQL
		1 day	3.19	2.74	2.64	4.99	3.79	3.01
		12 months	20.76	22.97	5.98	35.38	11.94	8.77
	DTPA	Unpolluted	1.37	1.84	2.22	2.36	1.88	2.53
		1 day	181.34	148.85	188.73	193.12	175.16	125.39
		12 months	198.90	210.00	193.20	244.60	213.20	164.20

LQL = lower quantification limit.

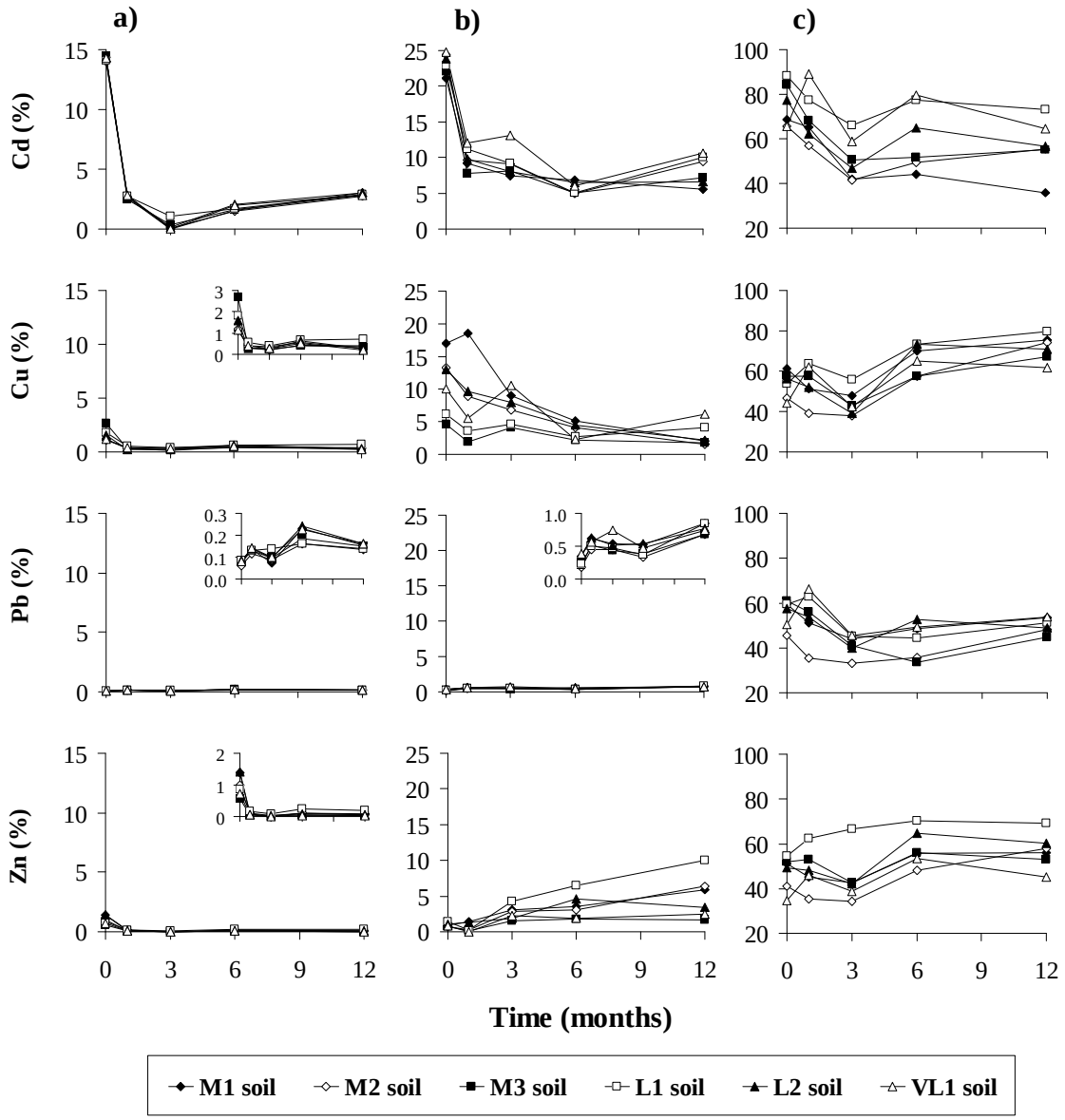

Fig. 2. Relative metal extractability (%) with $NaNO_3$ (a), LMWOA (b) and DTPA (c) methods in the metal-spiked soil samples as a function of the incubation time.

$NaNO_3$-extractable metal percentages showed a general downward trend throughout the incubation experiment, mainly during the first month of contact time. The percentage values of LMWOA-extractable Cd and Cu decreased throughout the incubation experiment, while LMWOA-extractable Zn showed a slight upward trend. The temporal trends with the DTPA-method were more irregular. These differing temporal trends thus revealed the strong affectation of soil constituents on metal

extractability with these extraction methods, as discussed below for each metal studied.

Cadmium extractability patterns

As expected, the values for $NaNO_3$-extractable Cd concentration presented a downward trend during the first month of the incubation experiment (Fig. 2) and similar percentage values of Cd –about 14 % at day 1 and about 3 % at 12 months– were extracted from different soils. At 12 months, the relative concentration of extractable Cd in M1 soil (with the highest ECC content) was half that found in L1 and VL1 soils (with a lower ECC content). This pattern was more evident in the case of DTPA than with the LMWOA method. This highlighted the fact that the ECC content plays a key role in Cd sorption-desorption processes in calcareous soils at the Cd concentration used in this study (Buekers *et al.*, 2007; Sánchez-Camazano *et al.*, 1998).

However, although this behaviour is clearly shown in the soils with very different ECC contents, no clear pattern could be established between the carbonate gradient in the soils tested and the percentage of extractable Cd. Hence other soil properties and/or constituents such as CEC and OM (Krishnamurti and Naidu, 2003) may affect Cd extractability as the carbonate gradient decreases. In previous studies on these types of soil, we reported that Cd retention was affected by mineralogy (Lafuente *et al.*, 2008). However, in the present research, we observed that although the M1 soil had the highest content of calcite and dolomite (trace in L1 soil and not detected in VL1 soil), the mineralogical study did not offer any additional information on the extractability pattern.

From these results it can be concluded that although the ECC content appears to play a key role, this constituent alone proved insufficient for us to establish a pattern of Cd extractability when it is added as a multi-element metal salt solution.

Copper extractability patterns

The study of soil properties affecting Cu extractability was more complex. In the case of the $NaNO_3$-extraction method, it should be noted that similar Cu concentrations were extracted in unpolluted soils and in metal-spiked soils at 12

months (Table 3 and Fig. 2). This may suggest that Cu redistributes with contact time, reducing its concentration in exchangeable positions (Sayen *et al.*, 2009). However, the low extraction percentage was not sufficient to reveal a Cu extractability pattern with this method.

LMWOA-extractable Cu decreased throughout the incubation experiment, showing a different pattern of extractability at the beginning and at the end. At day 1, the soils with the highest OM contents (M3 and L1 soils) showed the lowest % of extractable Cu (about 5 %), while soils with the lowest OM content (M1 soil) showed the highest extractability values (about 17 %). However, after 12 months, soils with a low ECC content (VL1 and L1 soils), regardless of their OM content, showed the highest Cu extractability, which coincides with the pattern observed in unpolluted soils. This observation could indicate *a priori* that the ECC content affects Cu extractability with LMWOA. Nevertheless, the alteration of the organic fractions noted in metal-spiked soils at the end of the incubation experiment in soils with less ECC content could explain this behaviour. This fact suggests that Cu-OM complexes may be established at the start and then degrade over the course of the incubation experiment (Martínez *et al.*, 2003) and facilitating its subsequent extraction with LMWOA. This suggests that OM and carbonate may compete for Cu retention in these calcareous soils (Besnard *et al.*, 2001). In view of these results, we cannot determine whether higher Cu-extractability with LMWOA is affected by either a lower ECC content or by further degradation of recalcitrant OM in soils with a lower ECC content, or possibly both.

In the case of DTPA, soils with the highest silt and clay contents (VL1, M3 and L2 soils) presented the lowest Cu extractability after 12 months. There was no pattern related to OM content. However, Jalali and Khanlari (2008) highlighted the influence of the silt fraction on the amount of Cu bound to the organic fraction in calcareous soils. In these soils, clay-humic complexes dominate organometallic complexes, and the former are more stable. If this type of complex had been established, the DTPA method, due to its greater extracting power, might have been able to release the Cu retained in these complexes. Studies of soil particle-size fractionation have shown that Cu was mainly concentrated in the clay-sized fraction < 2 μm, and in the coarse particulate organic matter > 50 μm (Besnard *et al.*, 2001). This

was attributed to the high reactivity of the mineral constituents and organo-mineral associations present in the < 2 μm fraction, and would explain the relationships found between fine mineral fractions and OM, and Cu extractability with DTPA. As noted by Jalali and Khanlari (2008), further research is required in this direction.

Although Cu extractability was affected by ECC, silt and OM, the OM appears to be the most influential factor. This reveals that in the soils studied with a high Cu sorption capacity the sorption-desorption processes could be affected by the OM, despite their low OM content (Buekers *et al.*, 2007; Micó *et al.*, 2006), and highlights the vulnerability of these soils, since an increase in the mineralization of OM (very common in the Mediterranean climate) could affect the storage capacity, mobility and availability of metals in soils, leading to a significant risk of environmental contamination (Hernandez-Soriano and Jimenez-Lopez, 2012; Martínez *et al.*, 2003). It is worth noting that we extracted about 60-80 % of Cu with DTPA. This high extraction percentage reveals the risk of the potential mobility and availability of Cu, even in soils such as these, where the Cu sorption capacity is high.

Lead extractability patterns

The chemistry of Pb in soils is mainly affected by specific adsorption, precipitation of stable compounds, and formation of relatively stable complexes with OM. As shown by the calculated K_d values, the highest retention of Pb occurred in M1 and VL1 soils, with the highest ECC and clay contents respectively, thus showing the influence of these constituents on Pb retention after 24 h of contact time (Fig. 1). However, at 12 months, DTPA-extractable Pb concentrations were very similar for all soils, making it impossible to discern which soil properties affect Pb extractability with this method (Fig. 2). Jalali and Khanlari (2008) observed that with longer contact times than the first 3 h-1 day, Pb was distributed from exchangeable forms to more strongly-binding sites (reducible and oxidizable fractions). The sorption-desorption processes in these fractions are strongly regulated by soil pH. However, the pH range of the soils in the study is so narrow that we are unable to distinguish which of the constituent parts of these fractions affect Pb extractability with DTPA. Therefore, the DTPA method was impractical for fulfilling the aims of this paper. Nevertheless, it is

worth noting the high percentage of DTPA-extractable Pb obtained in the metal-spiked soils (about 50 %).

Zinc extractability patterns

The concentrations of Zn extracted with the LMWOA and DTPA methods showed similar extractability patterns, with an upward trend over the course of the incubation experiment (Fig. 2). All soils showed similar LMWOA-extractable Zn concentration values at the beginning of the study and became differentiated during the incubation experiment. After 12 months, the different Zn extractability pattern corresponded to the soil silt content, and the highest extractable Zn values were obtained in soils with the lowest proportions of silt, following the soil sequence: L1>M2>M1>L2>VL1>M3. In this regard, Jalali and Khanlari (2008) noted that a lower proportion of Zn in the exchangeable fraction of calcareous soils was observed in soil samples with the highest silt content.

In the case of the DTPA method, the highest extraction percentage corresponded to soils with the highest coarse sand content, following the soil sequence: L1>L2=M2=M1>M3>VL1. In the unpolluted soils (Table 3), more Zn was extracted with DTPA in the soil with the lowest silt content (L1 soil). Although the distribution of forms of Zn is dose-dependent (Xiang *et al.*, 1995), in our case both unpolluted soils and metal-spiked soils appeared to show the same extractability pattern. For the Zn-K_d value, the highest sorption was obtained in VL1 soil, which had the highest CEC values and clay content. The latter is dominated by 2:1 silicates (Table 2), which have a greater fixing capacity for this metal. Nevertheless, due to the low percentage of $NaNO_3$-extractable Zn, the main mechanism for binding to these constituents appears not to be adsorption, but immobilization in the octahedral layers of phyllosilicates (Jacquat *et al.*, 2009; Sipos *et al.*, 2008).

In view of these results, we conclude that particle-size distribution can be the most important factor affecting both the sorption and extractability of Zn with all the selected extraction procedures in these calcareous agricultural soils, under experimental conditions. As in the case of the other metals, Zn extractability with DTPA was high (45-70 %), which may indicate a risk of phytotoxicity, despite the high sorption capacity of these soils.

Conclusions

The information obtained shows that the carbonate content alone cannot explain the extractability patterns of Cd, Cu, Pb, and Zn in the soils studied, under the experimental conditions used. Thus although carbonate was observed to play a key role in regulating Cd extractability, carbonate content was insufficient to establish a Cd-extractability pattern. Cu-extractability was affected by the content of carbonate, silt and organic matter, with the latter as the most influential constituent. Although carbonate and clay content was seen to govern Pb retention, it could not be established which soil components affect Pb-extractability patterns. Particle-size distribution was found to be a significant factor affecting both sorption and extraction processes of Zn.

It has become apparent that the combination of several soil properties and constituents, such as particle-size distribution and organic matter, plays a key role in metal extractability patterns in calcareous soils. These results point to the conclusion that, despite the low organic matter content, long-term transformations of organic matter and organo-mineral associations could alter metal storage capacity and, consequently, metal availability. From the high Cd, Cu, Pb, and Zn concentrations extracted with the DTPA method we can infer a potential risk of metal mobility and availability in the periurban calcareous agricultural soils of the Mediterranean area studied, with a high metal retention capacity (> 98 %). Since our results provide evidence that these soils could not withstand the environmental impact from metal accumulation in periurban areas, we believe that they should be considered vulnerable in the long term. Nevertheless, further research work is necessary.

Acknowledgements

This work was supported by the Universidad Complutense de Madrid (UCM) and the Madrid Autonomous Region through Grant GR58/08, Research Team 950605 and Network CARESOIL, Ref. P2009/AMB-1648. We especially wish to thank Folkert van Oort and Isabelle Lamy (Pessac, INRA) for their helpful advice in the development of the present work and Ms. Prudence Brooke-Turner for the revision of the English version of the manuscript.

References

Anderson, P.R., Christensen, T.H., 1988. Distribution coefficients of Cd, Co, Ni, and Zn in soils. J. Soil Sci. 39, 15-22.

Besnard, E., Chenu, C., Robert, M., 2001. Influence of organic amendments on copper distribution among particle-size and density fractions in Champagne vineyard soils. Environ. Pollut. 112, 329-337.

Bish, D.L., 1994. Quantitative x-ray diffraction analysis of soils. In J.E. Amonette, & L.W. Zelazny (Eds.), Quantitative methods in soil mineralogy, pp. 267-295. SSSA Misc. Publ. SSSA, Madison, WI.

Buekers, J., Van Laer, L., Amery, F., Van Buggenhout, S., Maes, A., Smolders, E., 2007. Role of soil constituents in fixation of soluble Zn, Cu, Ni and Cd added to soils. Eur. J. Soil Sci. 58, 1514-1524.

Conde, P., Martín Rubí, J.A., Ballesta, R.J., 2007. Chemical vulnerability of red soils in La Mancha (Central Spain). Sci. Total Environ. 378, 228-232.

Conder, J.M., Lanno, R.P., Basta, N.T., 2001. Assessment of metal availability in smelter soil using earthworms and chemical extractions. J. Environ. Qual. 30, 1231-1237.

de Boer, W., Folman, L.B., Summerbell, R.C., Boddy, L., 2005. Living in a fungal world: impact of fungi on soil bacterial niche development. FEMS Microbiol. Rev. 29(4), 795-811.

de Santiago, A., Cheviron, N., Quintana, J.R., González, C., Lafuente, A.L., Mougin, C., 2011. Heavy metal availability and their relationships with soil microbial characteristics in agricultural carbonated soils. Book of Abstracts of 6th International Symposium of Interactions of Soil Minerals with Organic Components and Microorganisms, Montpellier.

Directive 86/278/EEC of 12 June 1986 on the protection of the environment, and in particular of the soil, when sewage sludge is used in agriculture.

FAO, 2006. World reference base for soil resources, 2006. A framework for international classification, correlation and communication. FAO, Roma.

Feng, M., Shan, X., Zhang, S., Wen, B., 2005. A comparison of the rhizosphere-based method with DTPA, EDTA, $CaCl_2$, and $NaNO_3$ extraction methods for

prediction of bioavailability of metals in soil to barley. Environ. Pollut. 137, 231-240.

Grünewald, G., Kaiser, K., Jahn, R., Guggenberger, G., 2006. Organic matter stabilization in young calcareous soils as revealed by density fractionation and analysis of lignin-derived constituents. Org. Geochem. 37, 1573-1589.

Gupta, S.K., Aten, C., 1993. Comparison and evaluation of extraction media and their suitability in a simple model to predict the biological relevance of heavy metal concentrations in contaminated soils. Int. J. Environ. Anal. Chem. 51, 25-46.

Hernandez-Soriano, M.C., Jimenez-Lopez, J.C., 2012. Effects of soil water content and organic matter addition on the speciation and bioavailability of heavy metals. Sci. Total Environ. 423, 55-61.

IGME (Instituto Geológico y Minero de España), 1990. Mapa geológico de España nº 535. Escala 1:50.000 (Algete), Madrid.

ISRIC (International Soil Reference and Information Center), 2002. Procedures for Soil Analysis, 3th ed. International Soil Reference and Information Center, Wageningen.

Jacquat, O., Voegelin, A., Kretzschmar, R., 2009. Local coordination of Zn in hydroxy-interlayered minerals and implications for Zn retention in soils. Geochim. Cosmochim. Acta 73, 348-363.

Jalali, M., Khanlari, Z.V., 2008. Effect of aging process on the fractionation of heavy metals in some calcareous soils of Iran. Geoderma 143, 26-40.

Jiménez Ballesta, R., Conde Bueno, P., Martín Rubí, J.A., García Giménez, R., 2010. Pedo-geochemical baseline content levels and soil quality reference values of trace elements in soils from the Mediterranean (Castilla La Mancha, Spain). Eur. J. Geosci. 2(4), 441-454.

Krishnamurti, G.S.R., Naidu, R., 2003. Solid-solution equilibria of cadmium in soils. Geoderma 113, 17-30.

Lafuente, A.L., González, C., Quintana, J.R., Vázquez, A., Romero, A., 2008. Mobility of heavy metals in poorly developed carbonate soils in the Mediterranean region. Geoderma 145, 238-244.

Lindsay, W.L., Norvell, W.A., 1978. Development of a DTPA soil test for zinc, iron, manganese and copper. Soil Sci. Soc. Am. J. 42, 421-428.

Martínez, C.E., Jacobson, A.R., McBride, M.B., 2003. Aging and temperature effects on DOC and elemental release from a metal contaminated soil. Environ. Pollut. 122, 135-143.

Martínez, C.E., Motto, H.L., 2000. Solubility of lead, zinc and copper added to mineral soils. Environ. Pollut. 107, 153-158.

McLaughlin, M.J., 2001. Aging of metals in soils changes bioavailability. Fact Sheet Environ. Risk Assess. 4, 1-6.

Micó, C., Recatalá, L., Peris, M., Sánchez, J., 2006. Assessing heavy metal sources in agricultural soils of an European Mediterranean area by multivariate analysis. Chemosphere 65, 863-872.

Moreno Merino, L., 1998. Estudio de la influencia del suelo sobre la composición de las aguas subterráneas a través de la solución del suelo. Modelo en Fluvisoles calcáricos. PhD thesis, Universidad Complutense de Madrid.

Peris, M., Micó, C., Recatalá, L., Sánchez, R., Sánchez, J., 2007. Heavy metal contents in horticultural crops of a representative area of the European Mediterranean region. Sci. Total Environ. 378, 42-48.

Rivas-Martínez, S., 1987. Memoria del mapa de series de vegetación de España. 1:400.000. Madrid: ICONA.

Roca-Perez, L., Gil, C., Cervera, M.L., Gonzálvez, A., Ramos-Miras, J., Pons, V., Bech, J., Boluda R., 2010. Selenium and heavy metals content in some Mediterranean soils. J. Geochem. Explor. 107, 110-116.

Rovira, P., Vallejo, V.R., 2000. Evaluating thermal and acid hydrolysis methods as indicators of soil organic matter quality. Commun. Soil Sci. Plant Anal. 31, 81-100.

Sánchez-Camazano, M., Sanchez-Martin, M.J., Lorenzo, L.F., 1998. Significance of soil properties for content and distribution of cadmium and lead in natural calcareous soils. Sci. Total Environ. 218, 217-226.

Sayen, S., Guillon, E., 2009. Aging effect on the copper sorption on a vineyard soil: Column studies and SEM–EDS analysis. J. Colloid Interface Sci. 331, 47-54.

Sayyad, G., Afyuni, M., Mousavi, S.F., Abbaspour, K.C., Richards, B.K., Schulin, R., 2010. Transport of Cd, Cu, Pb and Zn in a calcareous soil under wheat and safflower cultivation - A column study. Geoderma 154, 311-320.

Sipos, P., Németh, T., Kovács Kis, V., Mohai, I., 2008. Sorption of copper, zinc and lead on soil mineral phases. Chemosphere 73, 461-469.

SISS (Società Italiana della Scienza del Suolo), 1985. Metodi Normalizzati di Analisi del Suolo. Edagricole Ed., Bologna.

Steinitz, C., del Pozo, C., Vargas-Moreno, J.C., Canfield, T., 2011. Futuros alternativos para los paisajes dinámicos del Corredor del Henares. Fundación Paisaje.

Vega, F.A., Andrade, M.L., Covelo, E.F., 2010. Influence of soil properties on the sorption and retention of cadmium, copper and lead, separately and together, by 20 soil horizons: Comparison of linear regression and tree regression analyses. J. Hazard. Mater. 174, 522-533.

Xiang, H.F., Tang, H.A., Ying, Q.H., 1995. Transformation and distribution of forms of zinc in acid, neutral and calcareous soils of China. Geoderma 66, 121-135.

Zapusek, U., Lestan, D., 2009. Fractionation, mobility and bio-accessibility of Cu, Zn, Cd, Pb and Ni in aged artificial soil mixtures. Geoderma 154, 164-169.

Zheng, S., Zhang, M., 2011. Effect of moisture regime on the redistribution of heavy metals in paddy soil. J. Environ. Sci. 23(3), 434-443.

Apartado 3.2

Temporal trends of metal extractability in calcareous soils affected by soil properties and metal contamination levels

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Manuscrito en preparación

Resumen

El papel desempeñado por los constituyentes edáficos en el control de las tendencias temporales de la movilidad de los metales pesados en suelos calcáreos contaminados del área mediterránea merece una atención especial, debido tanto a las propiedades particulares de estos suelos como a las características climáticas. La reacciones que se producen durante el tiempo de contacto entre el suelo y el metal, o efecto "aging", modifican la movilidad de los metales, siendo factores determinantes, en los resultados obtenidos, el nivel de contaminación empleado y el tipo de suelo estudiado. Las investigaciones centradas en la movilidad inmediata y potencial de los metales son de particular interés para la gestión de suelos contaminados.

El objetivo del presente trabajo fue investigar el papel que tanto la dosis metálica aplicada, como los constituyentes del suelo, ejercen en las tendencias temporales de la movilidad inmediata y potencial de los metales en suelos que presentan un gradiente natural de carbonato, y difieren en el resto de sus constituyentes. Las muestras de suelo fueron contaminadas con una mezcla de Cd, Cu, Pb y Zn, a dos niveles y, posteriormente, dejadas en contacto durante un período de incubación de 12 meses. Durante este periodo, se extrajeron submuestras de suelo a diferentes intervalos de tiempo (1 día, 1, 3, 6 y 12 meses). En ellas, se analizó, con métodos de extracción química de un solo paso, la movilidad metálica inmediata ($NaNO_3$) y la movilidad metálica potencial (ácido dietilen triamino pentaacético –DTPA–).

En los dos niveles de contaminación ensayados, los valores de concentración de Cd, Cu y Zn extraídos con $NaNO_3$ alcanzaron el equilibrio dentro del período de incubación. La tendencia temporal de la movilidad metálica inmediata estuvo determinada por la fracción carbonatada, para el Cd y el Cu, y por la fracción más fina del carbonato, para el Zn. En el caso de la movilidad potencial de los metales, se observó que las concentraciones de los metales extraídos con DPTA no alcanzaron el equilibrio dentro del periodo de incubación. En este caso, la acción combinada de las fracciones carbonatada, orgánica, óxido de Fe y arcilla, fue determinante para definir las diversas tendencias temporales observadas para cada metal en ambos niveles.

Abstract

The role played by soil constituents in governing temporal trends of metal mobility in contaminated calcareous Mediterranean soils deserves special attention due to the particular soil and climate characteristics. Reactions occurring during the aging modify the metal mobility over time, and the rate of metal application and type of soil can be decisive in the outcome of aging reactions in soils. Investigations of the immediate and potential metal mobility are of particular interest in the management of contaminated soils. The aim of the present work was to investigate the role that both metal dose and soil constituents play in temporal trends of (potential) metal mobility in soils offering a natural gradient of carbonate and whose remaining soil components differed. Soil samples were spiked with a mixture of Cd, Cu, Pb and Zn at two different levels and then left aging for a period of 12 months incubation after spiking. During this period, metals were extracted at different time intervals (1 day, 1, 3, 6 and 12 months) with one-step extraction methods to estimate immediate metal mobility ($NaNO_3$) and potential metal mobility (diethylene triamine pentaacetic acid –DTPA–).

At both levels of contamination, $NaNO_3$-extractable Cd, Cu and Zn concentration values reached equilibrium within the period of incubation. Temporal trend of immediate metal mobility was governed by carbonate fraction for Cd and Cu and by the finest carbonate fraction for Zn. In the case of potential metal mobility, DPTA-extractable metal concentrations did not reach equilibrium within the incubation time. In this case, the combined action of carbonate, organic, Fe-oxide and clay fractions were decisive to define the different temporal trends observed for each metal at both levels.

Keywords

Calcareous soils, incubation experiment, metal mobility, soil properties, temporal trends

Introduction

In any study aimed at accurately predicting the behaviour of metals in soils, it is important to know the metal sorption-desorption processes in the preliminary stages of contamination, and also to investigate the dynamics of metals over time (McLaughlin, 2001). Metal sorption processes in soils are characterized by an initial stage of relatively rapid sorption, followed by a secondary stage that may continue over weeks, months or even years (Smolders and Degryse, 2007). These processes, known as "natural attenuation" or "aging", are caused by metal diffusion to internal sorption sites by migration, adsorption, surface precipitation or redox processes (Pueyo *et al.*, 2008). Reactions occurring during aging modify metal mobility over time, and should consequently be considered for risk assessment purposes (Sayen *et al.*, 2009).

It has been reported that the outcome of aging reactions in soils where metal loadings are higher are similar to those in metal deficient soils, although the processes may differ due to the higher metal concentrations involved, i.e. precipitation reactions may become more important at high metal loadings and at longer contact times, months or years (McLaughlin, 2001). In this context, factors such as the rate of metal application and the type of soil can be decisive (Han and Banin, 1999). In calcareous soils, due to the presence of $CaCO_3$ compounds and to soil pH values, aging processes can be expected to be more pronounced than in soils with a low pH (Lock and Janssen, 2003). Nevertheless, in recent works conducted in metal-spiked calcareous soils after 12 months of contact time, we observed a high potential mobility of metals despite the elevated metal sorption capacity of these soils (de Santiago-Martín *et al.*, in press). Furthermore, we also reported that the carbonate fraction alone could not explain the metal mobility patterns, indicating that other soil fractions may play a key role in governing the temporal trends of metal mobility.

The aim of the present work was to investigate the role that both metal dose and soil constituents play in the temporal trends of (potential) metal mobility in soils with a natural carbonate gradient and whose remaining soil components differed. With this purpose, soil samples were spiked with a mixture of Cd, Cu, Pb and Zn at two different levels, and allowed to age for 12 months under incubation before the

metals were extracted with one-step extraction methods at different time intervals. Differences in temporal trends among metals, dose levels and soils were discussed.

Materials and Methods

Soil sampling

The soil samples come from different plots in an Agricultural Research Station (Alcalá de Henares, Madrid, Spain), at an altitude of 588 m, on quaternary sediments (IGME, 1990). Soils classified as calcaric Fluvisol (Moreno Merino, 1998) present Anthric characteristics today (FAO, 2006). The average annual temperature is 13 ºC, average annual rainfall is 401 mm $year^{-1}$ and potential evaporation is about 760 mm $year^{-1}$.

A set of six soil samples with different carbonate content –moderate (M1 and M2), low (L1 and L2) and very low (VL2 and VL3)– was selected based on information from previous studies (de Santiago-Martin *et al.*, in press; Lafuente *et al.*, 2008). Several properties of the soil samples are shown in Table 1. Total metal content values were similar to those obtained by other authors for agricultural soils in the Mediterranean area (Jiménez Ballesta *et al.*, 2010; Micó *et al.*, 2006; Peris *et al.*, 2007), and in no case exceeded the levels set by the European Union (Directive 86/278/EEC). Sampling was done at randomly selected points. To avoid potential bias, 30-40 kg were taken from each sampling point (0-30 cm) and homogenized. Soil samples were air-dried and passed through a 2 mm sieve.

Table 1. Physicochemical parameters of un-spiked soil samples.

Soil sample	pH	Carbonate fraction		Organic fraction				Oxide fraction	Particle-size distribution			
		ECC	AL	TOC	OM	RP	C/N	Cry-Fe	CS	FS	Silt	Clay
		g kg^{-1}		g kg^{-1}		% TOC		g kg^{-1}	g kg^{-1}			
M1	8.4	190	77	6	10	69	8	8	111	603	126	161
M2	8.1	148	53	12	20	48	12	9	114	569	124	193
L1	8.2	106	21	18	32	72	11	13	159	590	78	172
L2	8.1	100	42	8	13	75	10	10	112	462	167	259
VL2	8.2	27	15	12	21	74	11	12	99	386	172	344
VL3	8.2	9	6	8	14	52	8	7	245	560	70	124

ECC = equivalent $CaCO_3$; AL = active lime; TOC = total organic C; OM =organic matter; RP = recalcitrant pool; TOC = total organic C; CS = coarse sand; FS = fine sand.

Metal-spiking and soil aging

The selected unpolluted soil samples were spiked with a multi-element salt solution of metals to mimic the situation which would occur in multi-element contaminated soils. Cd, Cu, Pb, and Zn were selected owing to their different speciation, mobility and extractability in soils. Each soil sample was subjected to an incubation experiment. Two containers (40 cm wide x 59 cm long x 21 cm high), weighing 10 kg each, were set for each sample, and nitrate salts of heavy metals in aqueous solution were spiked at two different levels (Tt1 and Tt2). The concentrations used were based on the heavy metal concentration provided by current European legislation on the protection of soils (Directive 86/278/EEC): the Tt1 level corresponds to the limit values of heavy metal concentration in soils (3 mg kg^{-1} Cd + 140 mg kg^{-1} Cu + 300 mg kg^{-1} Pb + 300 mg kg^{-1} Zn) and the Tt2 level corresponds to the semi-maximum concentrations permitted in sewage sludge (20 mg kg^{-1} Cd + 875 mg kg^{-1} Cu + 600 mg kg^{-1} Pb + 2000 mg kg^{-1} Zn).

The soil sample and the metal solution were mixed and left to equilibrate for a period of 12 months at room temperature without cover or drainage. During this equilibration period, the soils were air-dried, mixed and rewetted with distilled water in cycles of about 2 weeks, in order to favour metal redistribution into the soil matrix (McLaughlin, 2001). Duplicate subsamples were randomly removed from each metal-spiked soil sample at different time intervals (1 day, 1, 3, 6 and 12 months) using one-step metal extraction methods which estimate the easily exchangeable and mobile fraction (0.1 M $NaNO_3$) and the potentially mobile fractions (0.005 M DTPA) of metals (Gupta *et al.*, 1996).

Analytical methods

All chemicals were obtained from Merck (Germany) and Panreac (Spain). All glassware used was pre-washed with an aqueous solution of HNO_3 1:1000 for 24 h and rinsed with distilled water.

Soil physicochemical analyses

According to ISRIC methods (2002), the following parameters were determined in un-spiked soil samples: soil pH in a 1:2.5 soil to water ratio; equivalent

$CaCO_3$ –ECC– according to the acid neutralization method; total organic C by the Walkley and Black wet oxidation procedure; particle-size distribution by Robinson's pipette method; crystalline and amorphous Fe oxide contents by dithionite-citrate extraction; in addition to acid oxalate extraction. The active equivalent $CaCO_3$ or "active lime" –AL– was determined with NH_4-oxalate as described by Drouineau (1942). To characterize the nature of the organic carbon, we used the two-step acid hydrolysis procedure with H_2SO_4 to determine labile I and II and recalcitrant pools of organic C –LPI, LPII and RP– (Rovira and Vallejo, 2000). Total Cd, Cu, Pb, and Zn contents of the soil samples were determined after wet digestion with a mixture of nitro-perchloric-hydrofluoric acids under high-pressure conditions (SISS, 1985). Metal concentration in the extracts was quantified by flame atomic absorption spectroscopy –AAS– (Analytikjena NovAA 300). All samples were extracted and analysed in duplicate.

One-step extraction methods of metals

To determine extractable metals in metal-spiked soil samples, we used the following procedures: $NaNO_3$-extractable metals were determined by shaking 16 g of soil with 40 ml of 0.1 M $NaNO_3$ for 2 h (Gupta and Aten, 1993); DTPA-extractable metals were determined by shaking 20 g of soil with 40 ml of 0.005 M diethylenetriaminepentaacetic acid (DTPA) + 0.01 M $CaCl_2$ + 0.01 M triethanolamine (TEA) for 2 h (Lindsay and Norwell, 1978). The supernatant of each extraction was centrifuged at 3500 rpm for 15 min and then filtered. Dilutions were made with the corresponding extraction solution. Cadmium, Cu, Pb and Zn concentration in the extracts was quantified by AAS.

Results and Discussion

Temporal trends of metal extractability with $NaNO_3$

The temporal trends of the $NaNO_3$-extractable metal concentration values were substantially affected by the nature of the metal and the concentration of the mixture added (Fig. 1). A similar temporal trend was observed for Cd, Cu and Zn extractability, mainly at the Tt1 level, and a different trend for Pb.

At the Tt1 level, $NaNO_3$-extractable Cd, Cu and Zn concentration values reached equilibrium within the first month of contact time, and remained constant in all soil samples over the subsequent 11 months, implying a rapid redistribution of the metal in the different soil fractions (McLaughlin, 2001). The influence of some soil constituents and particularly of carbonate –a key constituent differentiating our soil samples– on metal extractability with incubation time was revealed mainly at the highest contamination level (Tt2). Carbonate and fine mineral fractions appeared to play an important role in Cd, Cu and Zn extractability. Thus, $NaNO_3$-extractable Cd and Cu concentration values at the Tt2 level reached equilibrium at different contact times depending on the ECC content (Jalali and Khanlari, 2008).

In soil samples with higher ECC content, extractable Cd and Cu values sharply decreased, and equilibrium was reached during the first month; thereafter concentrations of both levels tended to equalize despite the fact that Cd and Cu concentrations ~ 6 times higher were added at the Tt2 level than at the Tt1 level. This pattern was more evident in the case of Cu, suggesting a higher Cu sorption capacity in calcareous soils than for Cd or Zn (Jalali and Moharrami, 2007). Nevertheless, as the ECC content decreases, a longer contact time was required to reach equilibrium – up to 6 months for Cu and > 12 months for Cd– and differences in concentrations between the two levels were more evident.

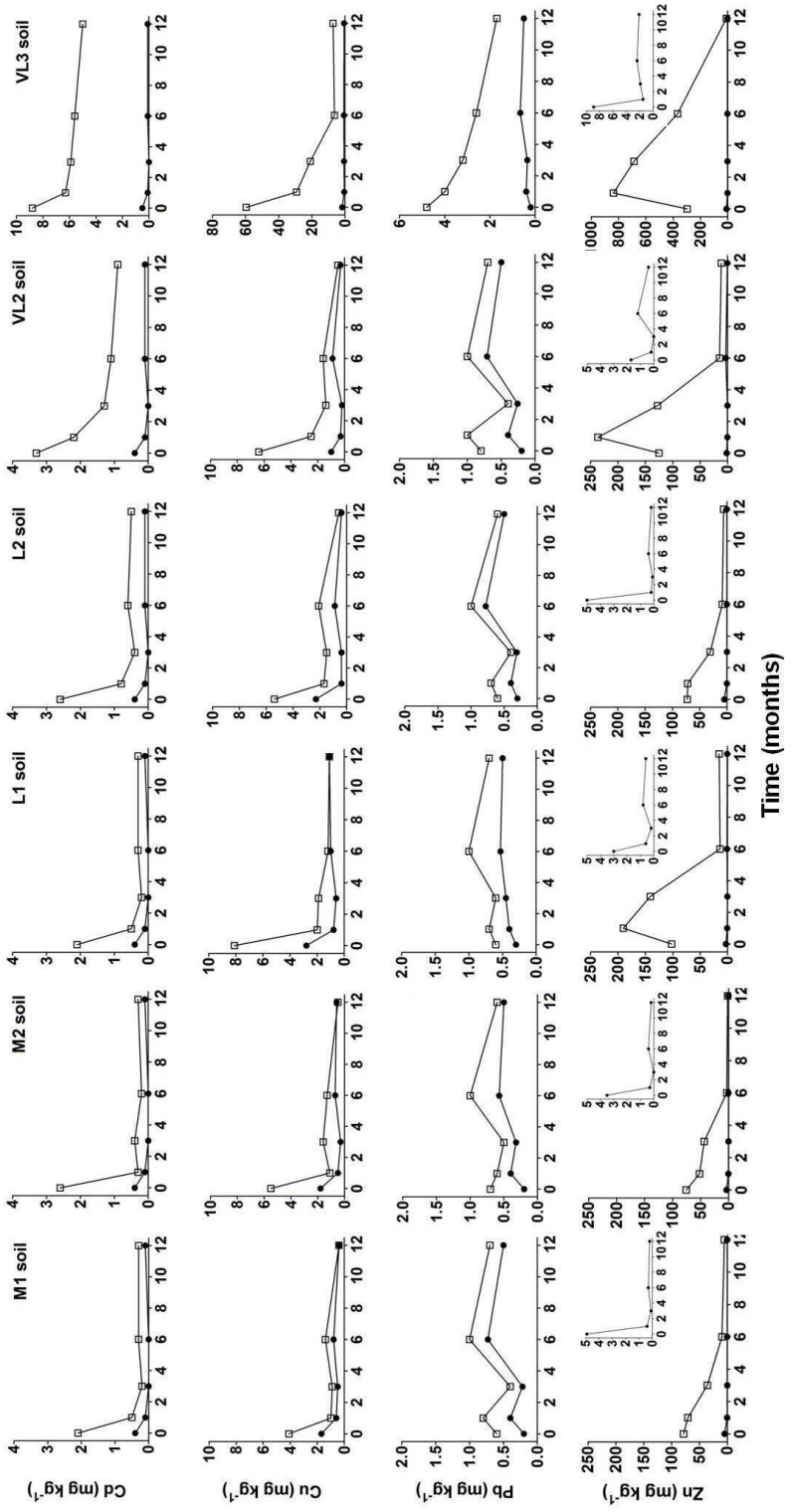

Fig 1. Temporal trends of Cd, Cu, Pb and Zn extractability with $NaNO_3$ in metal-spiked soil samples at the Tt1 (●) and at the Tt2 levels (□).

In the case of Zn extractability at the Tt2 level, equilibrium was reached within the incubation time (> 6 months), except in the VL3 sample, and Zn concentrations at this level were approaching those of the Tt1 level. However, two clear temporal trends could be distinguished during the first month of incubation, highlighting the key role played by soil constituents in Zn redistribution. Thus in M1, M2 and L2 soil samples, a gradual decline in Zn extractability was observed from the beginning of the experiment. Conversely, in L1, VL2 and VL3 soil samples, extractable Zn values increased during the first month of contact time and then subsequently decreased sharply. A rapid Zn redistribution in the carbonate fraction could explain the temporal trend observed in the case of samples with higher ECC content (M1 and M2). In soil samples with very low ECC content (VL2 and VL3), the competitive processes exerted by other metals (Cd and Cu) or major cations in soil solution with a higher affinity to the carbonate fraction could account for the initial increase in extractable Zn concentration values. The longer time required for extractable Cd and Cu concentrations to reach equilibrium in these soil samples would support this hypothesis.

As previously reported, the simultaneous presence of metals can increase the amount of metals released in the exchangeable fraction (Morera *et al.*, 2001). In our case, this behaviour was more evident as the ECC content decreased. Nevertheless, the opposite temporal trends were detected in the case of L1 and L2 soil samples, despite the fact that the ECC content was the same in these samples, ~ 100 g kg^{-1}, showing that the ECC content alone cannot explain these temporal trends. Instead, active lime (AL) was the soil constituent that defined these trends, assigning a dominant role to the finest carbonate fraction in governing the temporal trend of $NaNO_3$-extractable Zn concentration in the shorter term. During the following 11 months, Zn may be redistributed to other soil mineral fractions such as the phyllosilicate fraction, explaining the subsequent decrease in Zn extractability (Jacquat *et al.*, 2009). In previous works we reported that the fine mineral fraction was the main factor in explaining Zn extractability patterns in these soils at 12 months of contact time (de Santiago-Martín *et al.*, in press). It should be noted that in the VL3 soil sample, extractable Zn values did not reach equilibrium within 12 months of

incubation, probably due to the fact that this sample has the peculiarity of presenting poor ECC, AL and clay contents.

In the case of Pb, despite the slight increase in its extractable concentration with contact time at both the Tt1 and Tt2 levels, the Pb concentrations extracted were too low to assess the temporal trend, except in the case of VL3 soil at the Tt2 level. The low Pb concentration extracted was attributed to the high Pb retention in these soils, probably through the formation of lead oxide carbonate, as stated in previous works (data not published). This could also explain the pattern observed in the VL3 soil sample, which can be attributed to its very low proportion of carbonate fraction.

Temporal trends of metal extractability with DTPA

The temporal trends of DTPA-extractable metal concentration values varied widely among metals and for each contamination level (Fig. 2). At the Tt1 level, the extractable Cd concentration decreased 17-33% at 12 months, without reaching equilibrium. The lowest Cd extractability at both 1 day and 12 months of contact time was recorded for M1, M2 and L2 soil samples, probably due to their higher proportion of carbonate fraction, and specifically of AL (Rajaie *et al.*, 2006). In the case of DTPA-extractable Cu and Zn, their concentration values did not reach equilibrium within the contact time of the incubation experiment, and showed a 15-36% increase for Cu and a 6-26% increase for Zn at 12 months.

These wide ranges among samples highlighted the key role that soil constituents play in affecting metal extractability with this method (Reyhanitabar and Gilkes, 2010). Thus, the highest increases were observed in soil samples with the lowest proportion of both RP and fine mineral fractions, coinciding with the greatest decreases in extractable Cd concentrations. Both the increase in Cu and Zn concentrations and the decrease in Cd could be due to Cu and Zn redistribution from the carbonate fraction to the soil fractions for which these metals have high affinity – such as the organic fraction, mainly in the case of Cu, and the fine mineral fraction, predominantly for Zn– probably through organo-mineral associations (Besnard *et al.*, 2001; Dabkoska-Naskret, 2003). In this scenario, soluble Cd species could be more easily diffused in the crystal defects and pores of the lime, occupying the sites left free

by Cu and Zn, and resulting in stable bonds (Buekers *et al.,* 2007), leading to decreased Cd extractability with DTPA.

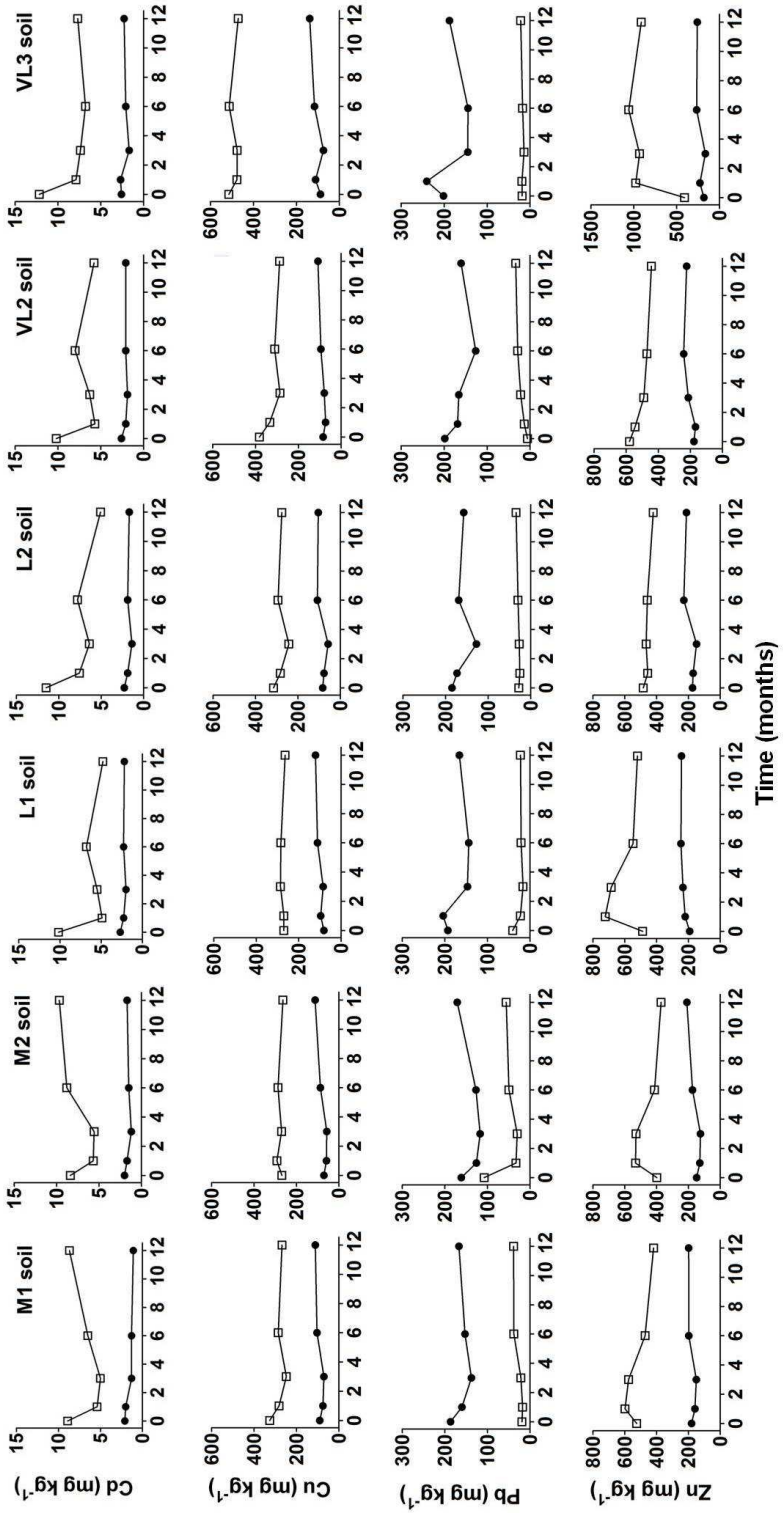

Fig 2. Temporal trends of Cd, Cu, Pb and Zn extractability with DTPA in metal-spiked soil samples at the Tt1 (●) and at the Tt2 levels (□).

At the Tt2 level, DTPA-extractable Cd concentration values in samples with low and very low ECC content (L1, L2, VL2 and VL3) decreased during the first month, followed by a tendency toward equilibrium. Surprisingly, Cd extractability in soil samples with a higher carbonate proportion (M1 and M2) increased over time without reaching equilibrium, showing that other soil fractions were also affecting the temporal trends of DTPA-extractable Cd. The lower crystalline Fe-oxide content of these latter samples (M1 and M2) could be the factor responsible for this behaviour, since reactive oxide phases may play a significant role in decreasing Cd solubility after adsorption or coprecipitation (Martínez and McBride, 2001). In the case of DTPA-extractable Cu at the Tt2 level, lower concentrations were extracted in M1 and M2 samples at 1 day of contact time. However, at 12 months, similar amounts of Cu were quantified in all samples, except in the particular case of the VL3 soil sample. Although Cu extractability remained almost constant throughout the contact time of the experiment, it is worth noting that a gradual decrease occurred during the first 3 months in soil samples with a higher RP content (M1, L2 and VL2).

The concentration of DTPA-extractable Zn at the Tt2 level was highly dependent on the fine mineral fraction, as indicated for $NaNO_3$ extractions. In fact, in soil samples with a higher clay content (L2 and VL2), Zn concentration values decreased slightly throughout the incubation time without reaching equilibrium. At 12 months, the highest Zn extractability was found in soil samples with a lower silt content (L1 and VL3), as previously reported (de Santiago-Martín *et al.*, in press). Considering that Zn redistribution with contact time to the fine mineral fraction is via specific adsorption, DTPA may exert a weak capacity to extract Zn from the adsorption sites of mineral surfaces. Nevertheless, in soil samples with a lower fine mineral proportion, Zn extractability was affected by the combined action of several soil fractions where Zn was weakly bound; however, due to the non-specificity of the DTPA method we were unable to distinguish them. In any case, these results indicate that in coarser textured soil samples a large percentage of Zn is present as a potentially mobile form, without attaining equilibrium, throughout most of the duration of our experiment.

With regard to DTPA-extractable Pb at the Tt1 level, slight variations in Pb concentrations (~ 3 % net decrease) were observed over the course of the incubation

experiment. This suggested a rapid Pb redistribution in the soil matrix during the first hours of contact time (Jalali and Khanlari, 2008). At the Tt2 level, the results were surprising, since Pb extractability was much lower than at the Tt1 level. The formation of lead oxide carbonate, as stated above, could explain its lower extractability when Pb is added at higher concentrations. Thus in these calcareous soils, lower concentrations of Pb added as soluble salts may involve greater potential mobility, entailing an increased environmental risk.

Conclusions

Results showed that the carbonate fraction was the main factor governing temporal trends of metal mobility, estimated using the $NaNO_3$-method. This fraction thus determined the temporal trend of Cd and Cu extractability with this method, whatever the contamination level, which led to concentrations of both levels tending to equalize at 12 months. Although the level of contamination was a determining factor in the case of temporal trends of $NaNO_3$-extractable Zn, the finest carbonate fraction was observed to play an important role. The low Pb concentrations extracted, attributed to precipitation processes, did not allow us to study the temporal trends of this metal.

In the case of metal potential mobility, estimated using the DTPA method, extractable metal concentrations did not attain equilibrium within the incubation time. The combined action of carbonate, organic, Fe-oxide and clay fractions determined the different temporal trends observed for each metal at both levels. Since this has provided evidence for the possible occurrence of competitive processes governed by the differing affinities of these metals to different soil fractions, further research in this direction would be of great interest.

Acknowledgements

This work was supported by the Universidad Complutense in Madrid and the Madrid Autonomous Region through Grant GR58/08, Research Team 950605 and Network CARESOIL, Ref. P2009/AMB-1648. We especially wish to thank Ms Pru Brooke-Turner for the revision of the English version of the manuscript.

References

Besnard, E., Chenu, C., Robert, M., 2001. Influence of organic amendments on copper distribution among particle-size and density fractions in Champagne vineyard soils. Environ. Pollut. 112, 329-337.

Buekers, J., Van Laer, L., Amery, F., Van Buggenhout, S., Maes, A., Smolders, E., 2007. Role of soil constituents in fixation of soluble Zn, Cu, Ni and Cd added to soils. Eur. J. Soil Sci. 58, 1514-1524.

Dabkowska-Naskret, H., 2003. The role of organic matter in association with zinc in selected arable soils from Kujawy Region, Poland. Org. Geochem. 34, 645-649.

de Santiago-Martín, A., Valverde-Asenjo, I., Quintana, J.R., González-Huecas, C., Lafuente, A.L., in press. Soil properties affecting metal extractability patterns in periurban calcareous agricultural soils in the Mediterranean area. Int. J. Environ. Res.

Directive 86/278/EEC of 12 June 1986 on the protection of the environment, and in particular of the soil, when sewage sludge is used in agriculture.

Drouineau, G., 1942. Dosage rapide du calcaire actif de sols. Ann. Agron. 12,441-450.

FAO, 2006. World reference base for soil resources, 2006. A framework for international classification, correlation and communication. FAO, Roma.

Gupta, S.K., Aten, C., 1993. Comparison and evaluation of extraction media and their suitability in a simple model to predict the biological relevance of heavy metal concentrations in contaminated soils. Int. J. Environ. Anal. Chem. 51, 25-46.

Gupta, S.K., Vollmer, M.K., Krebs, R., 1996. The importance of mobile, mobilisable and pseudo total heavy metal fractions in soil for three-level risk assessment and risk management. Sci. Total Environ. 178, 11-20.

Han F.X., Banin A., 1999. Long-term transformation and redistribution of potentially toxic heavy metals in arid-zone soils: II. Incubation at the field capacity moisture content. Water, Air, Soil Pollut. 114(3), 221-250.

IGME (Instituto Geológico y Minero de España), 1990. Mapa geológico de España nº 535. Escala 1:50.000 (Algete), Madrid.

ISRIC (International Soil Reference and Information Center), 2002. Procedures for Soil Analysis, 3th ed. International Soil Reference and Information Center, Wageningen.

Jacquat, O., Voegelin, A., Kretzschmar, R., 2009. Local coordination of Zn in hydroxy-interlayered minerals and implications for Zn retention in soils. Geochim. Cosmochim. Acta 73, 348-363.

Jalali, M., Moharrami, S., 2007. Competitive adsorption of trace elements in calcareous soils of western Iran. Geoderma 140, 156-163.

Jalali, M., Khanlari, Z.V., 2008. Effect of aging process on the fractionation of heavy metals in some calcareous soils of Iran. Geoderma 143, 26-40.

Jiménez Ballesta, R., Conde Bueno, P., Martín Rubí, J. A., García Giménez, R., 2010. Pedo-geochemical baseline content levels and soil quality reference values of trace elements in soils from the Mediterranean (Castilla La Mancha, Spain). Eur. J. Geosci. 2(4), 441-454.

Lafuente, A.L., González, C., Quintana, J.R., Vázquez, A., Romero, A., 2008. Mobility of heavy metals in poorly developed carbonate soils in the Mediterranean region. Geoderma 145, 238-244.

Lindsay, W.L., Norvell, W.A., 1978. Development of a DTPA soil test for zinc, iron, manganese and copper. Soil Sci. Soc. Am. J. 42, 421-428.

Lock, K., Janssen, C.R., 2003. Influence of ageing on zinc bioavailability in soils. Environ. Pollut. 126, 371-374.

Martínez C.E., McBride M.B., 2001. Cd, Cu, Pb, and Zn coprecipitates in Fe oxide formed at different pH: Aging effects on metal solubility and extractability by citrate. Environ. Toxicol. Chem. 20(1), 122-126.

McLaughlin, M. J., 2001. Aging of metals in soils changes bioavailability. Fact Sheet Environ. Risk Assess. 4, 1-6.

Micó, C., Recatalá, L., Peris, M., Sánchez, J., 2006. Assessing heavy metal sources in agricultural soils of an European Mediterranean area by multivariate analysis. Chemosphere 65, 863-872.

Moreno Merino, L., 1998. Estudio de la influencia del suelo sobre la composición de las aguas subterráneas a través de la solución del suelo. Modelo en Fluvisoles calcáricos. PhD thesis, Universidad Complutense de Madrid.

Morera, M.T., Echevarría, J.C., Mazkiarán, C., Garrido, J.J., 2001. Isotherms and sequential extraction procedures for evaluating sorption and distribution of heavy metals in soils. Environ. Pollut. 113, 135-144.

Peris, M., Micó, C., Recatalá, L., Sánchez, R., Sánchez, J., 2007. Heavy metal contents in horticultural crops of a representative area of the European Mediterranean region. Sci. Total Environ. 378, 42-48.

Rovira, P., Vallejo, V.R., 2000. Evaluating thermal and acid hydrolysis methods as indicators of soil organic matter quality. Commun. Soil Sci. Plant Anal. 31, 81-100.

Sayen, S., Mallet, J., Guillon, E., 2009. Aging effect on the copper sorption on a vineyard soil: Column studies and SEM–EDS analysis. J. Colloid Interface Sci. 331, 47-54.

SISS (Società Italiana della Scienza del Suolo) (Edagricole Ed.). 1985. Metodi Normalizzati di Analisi del Suolo. Bologna.

Smolders, E., Degryse, F., 2007. Fixation of cadmium and zinc in soils: implications for risk assessment. In: Hamon, R., McLaughlin, M., Lombi, E. (Eds.), Natural Attenuation of Trace Element Availability in Soils. SETAC Press, Florida, pp. 157-171.

Apartado 3.3

Metal extractability patterns to evaluate (potentially) mobile fractions in periurban calcareous agricultural soils in the Mediterranean area. Analytical and mineralogical approaches

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Resumen

Un conjunto de suelos calcáreos agrícolas periurbanos del área mediterránea fueron contaminados con una mezcla de Cd, Cu, Pb y Zn, a dos niveles de contaminación, dentro de los valores límites propuestos por la legislación Europea actual, y se incubaron durante 12 meses. Posteriormente, submuestras de los suelos se sometieron a diversos métodos de extracción química de un solo paso para estimar las fracciones metálicas móviles (sales neutras) y potencialmente móviles (complejantes y ácidos). Con los resultados obtenidos, se analizaron los patrones de extractabilidad metálica en función de las características del suelo. Los datos analíticos se complementaron con investigaciones mineralógicas y con modelos de especiación usando el programa Visual Minteq.

La formación de complejos metálicos solubles en los extractos –estimado por el programa Visual Minteq– conllevó a que la mayor eficiencia de extracción se produjese con extractantes complejantes. Los patrones de extractabilidad de los metales estuvieron relacionados con el contenido y composición de las fracciones carbonatada, orgánica, óxido de Fe y arcilla. Las fracciones metálicas potencialmente móviles estuvieron reguladas, principalmente, por la fracción coloidal del suelo (materia orgánica recalcitrante, caliza activa y minerales de la arcilla). En el caso del Pb, prácticamente no se obtuvieron correlaciones con los constituyentes del suelo, lo que fue atribuido a la alta retención de este metal, debido a la formación de $4PbCO_3 \cdot 3PbO$ (corroborado por Difracción de Rayos X). En definitiva, la elevada proporción de metales extraídos con agentes complejantes destacó la alta pero finita capacidad para almacenar metales potencialmente movilizables y la posible vulnerabilidad de estos suelos ante el impacto ambiental derivado de una acumulación metálica.

Abstract

A set of periurban calcareous agricultural Mediterranean soils was spiked with a mixture of Cd, Cu, Pb and Zn at two levels within the limit values proposed by current European legislation, incubated for up to 12 months, and subjected to various one-step extraction procedures to estimate mobile (neutral salts) and potentially mobile metal fractions (complexing and acidic extraction methods). The results obtained were used to study metal extractability patterns according to the soil characteristics. The analytical data were coupled with mineralogical investigations and speciation modeling using the Visual Minteq model.

The formation of soluble metal-complexes in the complexing extracts –predicted by the Visual Minteq calculations– led to the highest extraction efficiency with complexing extractants. Metal extractability patterns were related to both content and composition of carbonate, organic, Fe-oxide and clay fractions. Potentially mobile metal fractions were mainly affected by the soil colloidal fraction (recalcitrant organic matter, active lime and clay minerals). In the case of Pb, scarce correlations between chemical extractions and soil constituents were obtained which was attributed to high Pb retention due to the formation of $4PbCO_3 \cdot 3PbO$ (corroborated by X-Ray diffraction). In summary, the high metal proportion extracted with complexing agents highlighted the high but finite capacity to store potentially mobilizable metals and the possible vulnerability of these soils against environmental impact from metal accumulation.

Keywords

Metal mobility, calcareous soil, one-step extractions, speciation modeling, mineralogical composition

Introduction

Soil degradation is a serious problem in Europe, mainly driven or exacerbated by human activity. The number of potentially contaminated sites in the European Union-25 is estimated at approximately 3.5 million, with soil pollution from diffuse sources being recognized as one of the major soil threats by the European Union Soil Thematic Strategy (European Commission, 2006). This is especially true in metropolitan areas subjected to risks from pollution due to a strong industrial development and spreading urbanization, leading to an increase of "Urban Elements" in soils –Ba, Cd, Co, Cu, Mg, Pb, Sb, Ti and Zn– (de Miguel *et al.*, 1997). This scenario is typical of periurban agricultural soils which are affected and likely to become more so in the future unless sustainability criteria are applied (Iram *et al.*, 2012; Meng *et al.*, 2008; Peris *et al.*, 2007; Steinitz *et al.*, 2011). Based on these premises, there is a potential risk of metal contamination of groundwater and of accumulation in food crops which is directly related to the mobility of metals in soils.

The mobility of metals can be greatly decreased in calcareous Mediterranean soils where low rainfall and high evapotranspiration lead to metal accumulation in the first few centimetres of the soil. This metal accumulation capacity, enhanced by the high metal retention provided by carbonates, can however be affected by other capacity-controlling properties such as texture, content and type of organic matter, soil pH-redox conditions and the content of oxides of Fe, Al and Mn (Hesterberg *et al.*, 1992). Metals accumulated and retained could be potentially mobilizable by organism activity (Sayyad *et al.*, 2010) and/or by changes in the conditions in the environment (Bolan *et al.*, 2003). The high but finite capacity to store potentially mobilizable metals suggests that these soils should be considered to be the most vulnerable (Batjes, 2000). This high vulnerability which allows metals to move from one environmental echelon to another, combined with the complexity of the interaction between metals and the constituents of calcareous soils, highlights the importance of the study of the soil properties affecting the mobility –and thus the availability– of metals in these soils.

It is generally accepted that to assess the mobility and availability of metals in soils it is not sufficient merely to analyse the total metal content in soils, nor is this a

useful tool for determining potential risks, as soils are dynamic systems. A very delicate equilibrium exists among the metal pseudo-total content (inactive and inert), the metal mobile fraction (effective soluble, very active, bioavailable) and the metal mobilizable/potentially mobile fraction (potentially bioavailable, leachable and partly active) (Gupta *et al.*, 1996). Using chemical-extracting media of different strengths it is possible to differentiate between these fractions. The combination of indirect methods (analytical data from metal extractions and speciation modeling of the soil extracting solution) with direct methods (soil mineralogical investigations) is a useful tool for elucidating soil properties and constituents as well as the chemical processes that affect metal extractability, mobility and availability (Ettler *et al.*, 2007; Pérez-Esteban *et al.*, 2013; Sipos *et al.*, 2008).

The objective of the study was to investigate the mobile and potentially mobile fractions of Cd, Cu, Pb and Zn using a set of extraction methods of different strengths, speciation modeling using Visual Minteq Model, and mineralogical investigations in several metal-spiked periurban calcareous agricultural soils representative of the Mediterranean area. Since the role of soil constituents in metal desorption processes is not well defined for these soils, we placed particular emphasis on the soil constituents and properties affecting metal extractability patterns.

Materials and methods

Study area, soil characteristics and sampling

The study area is part of a periurban axis that combines agricultural activity and the main residential and industrial uses in the Madrid region; it is exposed to atmospheric deposition from transportation and industrial activities. The soil samples come from different plots in the "El Encín" Agricultural Research Station (Alcalá de Henares, Madrid, Spain), at an altitude of 588 m, located on the Henares River on quaternary sediments (IGME, 1990). These alluvial sediments have led to an ancient calcaric Fluvisol (Moreno Merino, 1998) which presents Anthric characteristics today (FAO, 2006) mainly as a result of agricultural use. The average annual temperature is 13 ºC, average annual rainfall is 401 mm $year^{-1}$ and potential evaporation is about 760

mm $year^{-1}$. The site is typical of a Mediterranean pluviseasonal-oceanic bioclimate on an upper meso-Mediterranean low dry bioclimatic belt (Rivas-Martínez, 1987).

A set of ten agricultural soil samples (M1, M2, M3, M4, M5, L1, L2, VL1, VL2 and VL3), differing in their carbonate content, amount of organic matter and textural class, was selected according to information from previous studies (de Santiago-Martín et al., in press; Lafuente *et al.*, 2008). Sampling was done at randomly selected points. To avoid potential bias, 30-40 kg were taken from each sampling point (0-30 cm) and homogenized. Soil samples were air-dried and passed through a 2 mm sieve.

Experimental design

The environmental impact from metal accumulation in periurban agricultural areas was simulated by the addition of a multi-elemental salt solution of metals to the soil samples. Cd, Cu, Pb, and Zn were selected owing to their different speciation, mobility and extractability in soils. Three containers (40 cm wide x 59 cm long x 21 cm high) of 10 kg each were set for each sample: one un-spiked sample with the addition of distilled water; and the other two spiked at two different concentration levels using nitrate salts of metals in aqueous solution: low level (Tt1) (3 mg kg^{-1} of Cd + 140 mg kg^{-1} of Cu + 300 mg kg^{-1} of Pb + 300 mg kg^{-1} of Zn) or high level (Tt2) (20 mg kg^{-1} of Cd + 875 mg kg^{-1} of Cu + 600 mg kg^{-1} of Pb + 2000 mg kg^{-1} of Zn) within the limits proposed by current European legislation (Directive 86/278/EEC).

Each soil sample and the corresponding solution was mixed and left to equilibrate for a period of 12 months at room temperature without cover or drainage. During this equilibration period, the soils were air-dried, mixed and rewetted with distilled water in cycles of about 2 weeks, in order to favour metal redistribution processes into the soil matrix. These processes play a key role in determining the extractability and availability of metals in soils (McLaughlin, 2001).

At the end of the equilibration period (12 months), duplicates were randomly removed from each un-spiked and metal-spiked soil sample for extraction of the metals by means of one-step extraction methods. Different extracting solutions have been used to assess mobile and potentially mobile fractions of metals, both in equilibrium with inactive and inert fractions (Gupta *et al.*, 1996). The mobile fraction was estimated with 0.01M $CaCl_2$, 1M $MgCl_2$, 0.1M $NaNO_3$, and 1M NH_4NO_3-

methods and the potentially mobile fraction was estimated with 5mM DTPA, 0.05M EDTA, 0.5M HNO_3, 0.11M HAc, 10mM LMWOA and 1M NH_4Ac-methods. The analytical data were coupled with mineralogical investigations (Sipos *et al.*, 2008), measurement of the pH in extracts, and speciation modeling using the Visual Minteq model (Ettler *et al.*, 2007; Pérez-Esteban *et al.*, 2013). In order to evaluate the soil sorption capacity, a sorption test was conducted prior to the incubation experiment and the distribution coefficients were calculated.

Analytical methods

All chemicals were obtained from Merck (Germany). All glassware used was pre-washed with an aqueous solution of HNO_3 1:1000 for 24 h and rinsed with distilled water.

Soil physicochemical parameters

According to ISRIC-methods (2002), the following parameters were determined: soil pH in a 1:2.5 soil to water ratio; equivalent $CaCO_3$ –ECC– according to the acid neutralization method; total organic C by the Walkley-Black wet oxidation procedure; particle-size distribution by Robinson's pipette method; cation exchange capacity –CEC– by the ammonium acetate method; crystalline and amorphous Fe and Mn oxide contents by dithionite-citrate extraction; in addition to acid oxalate extraction. The active equivalent $CaCO_3$ or "active lime" –AL– was determined with NH_4-oxalate as described by Drouineau (1942). We used the two-step acid hydrolysis procedure with H_2SO_4 to determine the recalcitrant pool of organic matter –RP– (Rovira and Vallejo, 2000).

Total Ca, Mg, Cd, Cu, Pb, and Zn contents of the soil samples were determined after wet digestion with a mixture of nitro-perchloric-hydrofluoric acids under high-pressure conditions (SISS, 1985). Fe, Mn, Ca, Mg, Cd, Cu, Pb, and Zn concentration in the corresponding extracts was quantified by atomic absorption spectroscopy –AAS– (Analytikjena NovAA 300). All samples were extracted and analysed in duplicate. Quantification limits in mg L^{-1} were: Ca = 1, Mg = 0.05, Fe = 0.5, Mn = 0.2, Cd = 0.2, Cu = 0.2, Pb = 0.5 and Zn = 0.1.

Soil mineralogical analyses

Mineral composition of soil samples was examined by X-ray diffraction (XRD) using an EQ 0434520 31 02 (X'Pert MPD) diffractometer. Un-spiked and metal-spiked (Tt1 and Tt2 levels) soil samples (< 2 mm) were examined on randomly-oriented powders. Separation of the clay fraction of un-spiked soils (< 2 μm) was performed by sedimentation in aqueous suspension previous NH_4OH dispersion and posterior H_2O_2 treatment. The air-dried fraction was examined by saturation with Mg, calcinations at 550 °C for 2 h, and ethylene glycol solvation. Abundance of soil minerals in both fine earth and clay fractions was assessed semi-quantitatively (Bish, 1994).

One-step extraction methods of metals

The procedures to determine extractable metals are described as follows: 0.01 mol L^{-1} $CaCl_2$ solution, 1:5 w/v for 2 h (Van Ranst *et al.*, 1999); 1 mol L^{-1} $MgCl_2$ solution, 1:8 w/v for 1 h (Tessier *et al.*, 1979); 0.1 mol L^{-1} $NaNO_3$ solution, 1:2.5 w/v for 2 h (Gupta and Aten, 1993); 1 mol L^{-1} NH_4NO_3 solution, 1:2.5 w/v for 2 h (DIN, 1995; Legislation Germany); 0.005 mol L^{-1} diethylene triamine pentaacetic acid (DTPA) solution in 0.01 mol L^{-1} $CaCl_2$ solution and 0.01 mol L^{-1} triethanolamine (TEA), 1:2 w/v for 2 h (Lindsay and Norwell, 1978); 0.05 mol L^{-1} ethylene diamine tetra-acetic acid (EDTA) solution, 1:10 w/v for 1 h (Quevauviller *et al.*, 1996); 0.5 mol L^{-1} HNO_3 solution, 1:5 w/v for 30 min (Van Ranst *et al.*, 1999); 0.11 mol L^{-1} CH_3COOH solution (HAc), 1:40 w/v for 16 h (Rauret *et al.*, 1999); 0.01 mol L^{-1} low molecular weight organic acids (LMWOA) solution consisting of acetic, lactic, citric, malic and formic acids with a molar ratio of 4:2:1:1:1, 1:10 w/v for 16 h (Feng *et al.*, 2005); and 1 mol L^{-1} CH_3COONH_4 solution (NH_4Ac) buffered by HAc at pH 7, 1:30 w/v for 4 h in column (Van Ranst *et al.*, 1999).

The supernatant of each extraction was centrifuged at 3500 rpm for 15 min and then filtered. Dilutions were made with the corresponding extraction solution. Cd, Cu, Pb and Zn concentration in the extracts was quantified by AAS.

The computer program Visual Minteq v.3.0 (Gustafsson, 2011) was used to predict the metal speciation in the extracts of three representative soil samples (M1, L1 and VL1 soils). We used the database that comes by default in the Visual Minteq

program. The measured values of pH, metal concentration in extracts, and the concentration of the extractant applied were used as the input data. Cd, Cu, Pb, and Zn were simultaneously entered into the code and the calculations were performed for each individual extract.

Sorption capacity of metals

The metallic solution and each soil sample (1:2 w/v) were mechanically shaken for 24 h, and the supernatant of each extraction was centrifuged (3500 rpm, 15 min) and then filtered. Concentration of Cd, Cu, Pb and Zn in the supernatant was quantified by AAS. The distribution coefficients (K_d) of the metals were calculated (Lafuente *et al.*, 2008) using the equation: $K_d = (M_{sorbed})/(M_{solution})$; where M_{sorbed} is the amount of sorbed metal per unit weight of soil (mg kg^{-1}), and $M_{solution}$ is the amount of metal in solution per unit volume of liquid (mg L^{-1}).

Statistical analysis

Significance of differences of the means (n = 10) of the relative metal extractability was investigated by means of one-way ANOVA using a post-hoc test (Tukey). Before performing the ANOVA test, it was performed a Levene's test in order to analyse the homogeneity of variance.

Correlation coefficients were calculated for metal-spiked (Tt1 and Tt2 level) soil samples to relate the amount of metals extracted with the different methods employed to the soil physicochemical parameters (Pearson's correlation test) and the mineralogical composition (Spearman's correlation test). Since mineralogical properties are semiquantitative variables, we used a nonparametric correlation test, the Spearman's test. Analyses were conducted using SPSS (Statistical Package for the Social Sciences) v.17 (SPSS, Inc.) software.

Results and Discussion

Soil characteristics

Soil physicochemical parameters

The main results of the physicochemical analyses from un-spiked soils are shown in Table 1. All soil samples showed pH values above 8. The ECC content ranged from moderate (M1, M2, M3, M4, and M5 soils) to low (L1 and L2 soils) and very low (VL1, VL2, and VL3 soils). Differences were observed in organic matter – OM– content and particle-size distribution within each group of soil samples. Thus, the OM content ranged from low to high, in all cases showing a high proportion of RP. The textural classes of soils ranged from sandy-loam to sandy-clay loam, with fine sand being the most abundant size fraction. Crystalline Fe oxide content was low. Crystalline Mn and amorphous Fe and Mn oxide content was very low (data not shown). In general, the total Ca and Mg content varied in line with the carbonate fraction.

The total Cu, Pb, and Zn content was similar to that obtained by other authors for agricultural soils in the Mediterranean area (Jiménez Ballesta *et al.*, 2010; Micó *et al.*, 2006; Peris *et al.*, 2007) and in no case exceeded the levels established by the European Union (Directive 86/278/EEC). The total Cd content was lower than the quantification limit.

Mineralogical composition

Examinations by XRD of the un-spiked soil samples indicated high contents of quartz and phyllosilicates in varying proportions (Table 2). The content in feldspars was always very low. Calcite was the predominant mineral in the carbonate group. Dolomite was not detected in L2, VL1 and VL3 soil samples.

The XRD analysis of the metal-spiked soils showed the presence of lead oxide carbonate ($4PbCO_3 \cdot 3PbO$; JCPDS No. 00-017-0732), an intermediate oxycarbonate confirmed by the reflections at 0.364, 0.286, 0.267, 0.215 and 0.165 nm in samples originally characterized as containing calcite and dolomite in their composition – mainly M1, M2 and M3 soils at Tt2 level. Figure 1 shows the XRD patterns from un-spiked and metal-spiked (at Tt2 level) samples of M1 soil.

Table 1. Physicochemical parameters of the un-spiked soil samples.

Soil sample	pH	Carbonate fraction		Organic fraction				Oxide fraction	Particle-size distribution				CEC	Total content			
		ECC	AL	TOC	OM	RP	C/N	Cry-Fe	CS	FS	Silt	Clay		Ca	Mg	Cu	Pb	Zn
		g kg^{-1}		g kg^{-1}		% TOC		g kg^{-1}	g kg^{-1}				cmol$_c$ kg^{-1}	g kg^{-1}		mg kg^{-1}		
M1	8.4	190	77	6	10	69	8	8	111	603	126	161	7	94	11	8	14	55
M2	8.1	148	53	12	20	48	12	9	114	569	124	193	9	94	11	12	56	63
M3	8.1	125	33	18	31	66	10	12	23	592	215	170	11	41	11	10	24	63
M4	8.4	118	23	15	26	54	11	11	95	605	146	154	9	36	9	11	24	62
M5	8.7	117	39	10	17	57	14	8	166	567	129	139	6	99	9	15	24	75
L1	8.2	106	21	18	32	72	11	13	159	590	78	172	7	40	7	12	26	53
L2	8.1	100	42	8	13	75	10	10	112	462	167	259	13	34	9	9	22	55
VL1	8.1	32	15	12	21	71	11	11	45	459	168	328	19	10	6	10	24	62
VL2	8.2	27	15	12	21	74	11	12	99	386	172	344	20	13	6	13	21	71
VL3	8.2	9	6	8	14	52	8	7	245	560	70	124	10	5	4	7	25	45

ECC = equivalent $CaCO_3$; AL = active lime; TOC = total organic C; OM =organic matter; RP = recalcitrant pool; Cry-Fe = crystalline Fe oxides; CS = coarse sand; FS = fine sand; CEC = cation exchange capacity.

Table 2. Mineralogical composition in un-spiked soil samples.

Fraction	< 2 mm						< 2 μm					
Soil Sample	2:1 L	1:1 L	Q	F	C	D	V	M-I	K	Q	C	D
M1	++	tr	++++	++	+++	++	++	+++	+	++	++++	tr
M2	+	tr	++++	+	+++	+	nd	++	+	++	++++	tr
M3	++	+	++++	++	++	tr	nd	++++	++	++++	++	+
M4	+	tr	++++	+	+	tr	nd	++++	+	++++	++	nd
M5	+	tr	++++	+	+	tr	nd	+++	+	+++	++++	nd
L1	++	tr	++++	++	++	tr	nd	++	+	++	++++	+
L2	+	tr	++++	++	++	nd	nd	+++	+	+++	++++	nd
VL1	++	tr	++++	++	tr	nd	+++	++++	+	++++	nd	nd
VL2	+	tr	++++	+	tr	tr	nd	++++	+	++++	nd	nd
VL3	+	tr	++++	++	nd	nd	nd	++++	+	++++	nd	nd

L = layer; Q = Quartz; F = Feldspars; C = Calcite; D = Dolomite; V = Vermiculite; M-I = Mica-Illite; K = Kaolinite. Number of +´s is proportional to abundance: (++++) most abundant, (+) least abundant, (tr) trace, and (nd) not detected.

Table 2 shows the mineralogical composition of the clay fraction of un-spiked soils. The most common phyllosilicate was mica-illite, identified as a trioctahedral mineral based on the intensities of the 001/002 basal reflections (Douglas, 1985). The minerals 1:1 of the kaolinite group displayed lower percentages. Vermiculite was occasionally present (M1 and VL1 soils). With regard to non-phyllosilicate minerals, it is worth noting the presence of quartz, calcite and dolomite in varying proportions.

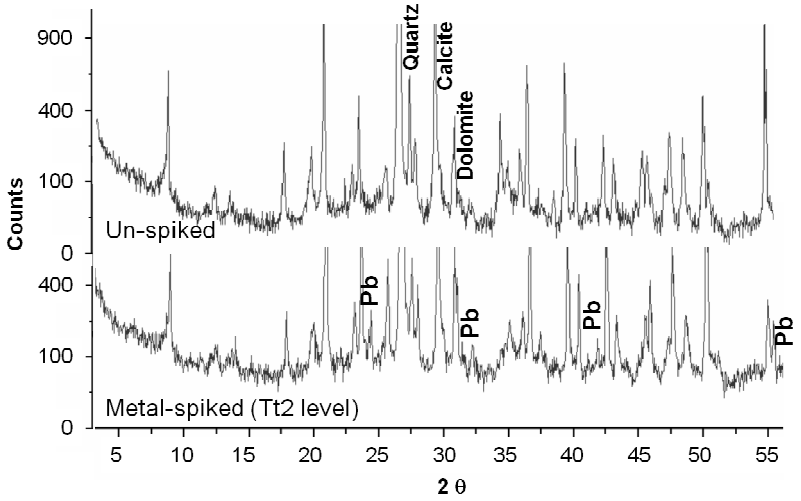

Fig 1. Patterns of X-ray diffraction of the randomly-orientated from un-spiked and metal-spiked (Tt2 level) samples of M1 soil.
Pb = lead oxide carbonate ($4PbCO_3 \cdot 3PbO$).

Metal sorption capacity

The sorption test showed that the metal sorption capacity of the selected soils was very high, ranging from 94 to 100% for Cu, Pb, and Zn, in agreement with the results obtained previously in the study area (de Santiago-Martín *et al.*, in press). The Cd sorption capacity, at about 64-92%, was lower than for other metals, and the lowest percentage was found in VL3 soil, with the highest coarse sand –CS– content. The distribution coefficients (K_d) calculated highlighted that Pb was in all cases the most retained metal, ranging from $8 \cdot 10^3$ to $30 \cdot 10^3$ at Tt1 level and from $0.3 \cdot 10^3$ to $6 \cdot 10^3$ at Tt2 level. Cd was the metal with the lowest K_d value, ranging from 4 to 24 in metal-spiked soils at Tt2 level. Cu and Zn presented intermediate values, ranging from 30 to $8 \cdot 10^2$ for Cu and from 40 to $1 \cdot 10^3$ for Zn. These results indicate *a priori* that these soils would be able to buffer an impact from metal contamination. Nevertheless, high metal extraction percentages were obtained with the one-step extraction methods used, as discussed below.

Extraction efficiency and metal speciation in extracts

Values of Cd, Cu, Pb, and Zn concentration obtained in single chemical extractions in the un-spiked and metal-spiked soil samples are shown in Fig. 2, as a percentage of total metal. It was observed that the highest proportion of metals extracted in metal-spiked soil samples was in all cases with complexing and acidic extractants, and the lowest was with neutral salts. The high metal proportion extracted with complexing agents indicates a high potential metal mobility in these soils.

The Visual Minteq thermodynamic calculations were useful in determining the metal speciation in the extracts, which depends on the pH and chemical composition of the solutions (Supplementary Material). The pH of the extracts yielded lower pH values than for water extracts (Table 3). In the case of acids and chelating agents, the pH values of soil extracts were lower in VL1 soil (with a lower ECC content) than in M1 and L1 soils.

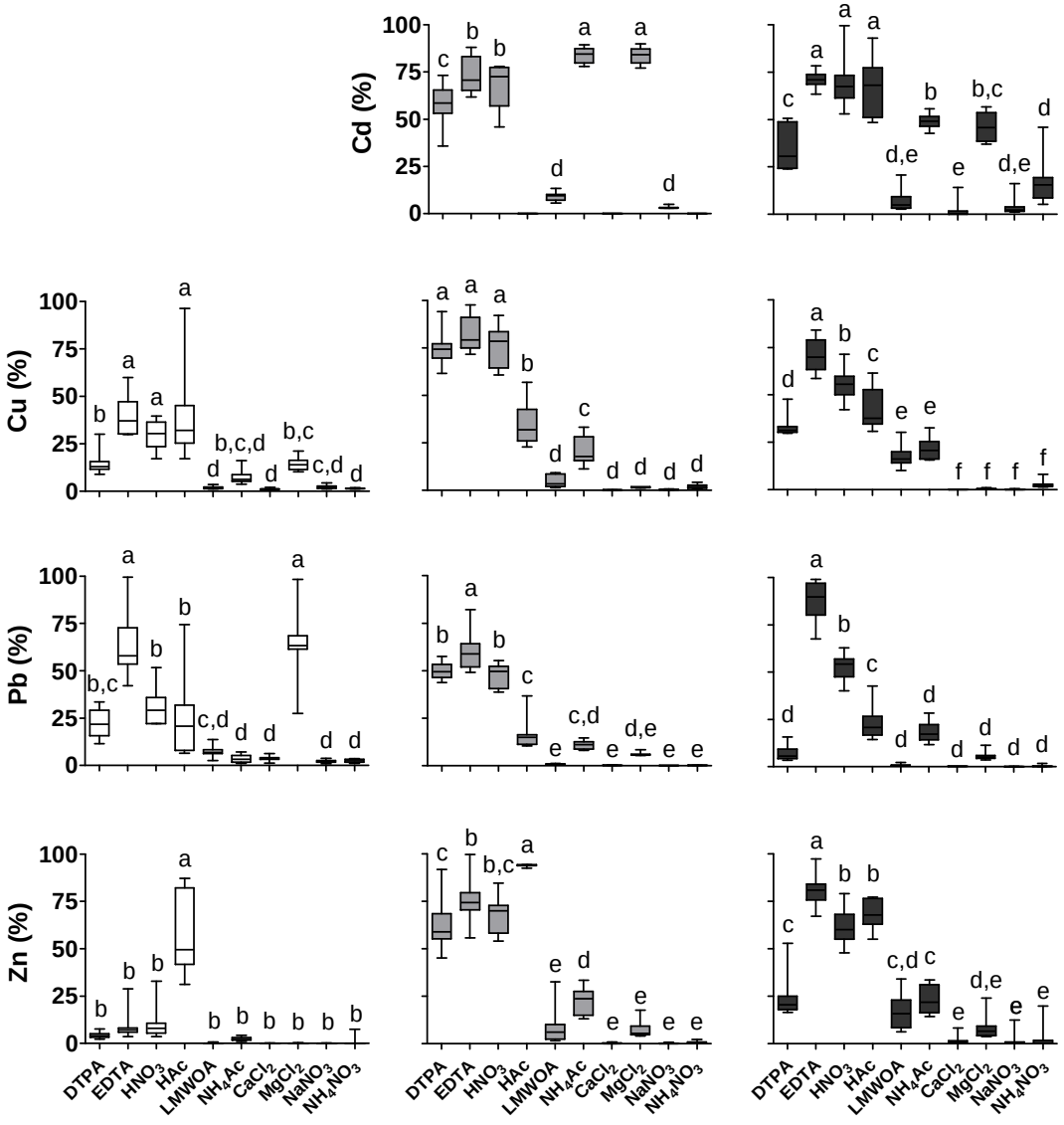

Fig 2. Relative Cd, Cu, Pb and Zn extractability (%) with DTPA, EDTA, HNO_3, HAc, LMWOA, NH_4Ac, $CaCl_2$, $MgCl_2$, $NaNO_3$, and NH_4NO_3-methods in un-spiked (□) and metal-spiked soil samples at Tt1 (■) and Tt2 levels (■). The boxplots show the lower quartile, the median and the upper quartile, with whiskers extending to the most extreme data point (n = 10). Different letters indicate significant differences between extraction methods at $p < 0.05$ after one-way ANOVA. Extractable Cd was lower than the quantification limit in un-spiked soil samples.

Table 3. Values of pH of the individual extracts from un-spiked and metal-spiked (Tt1 and Tt2 levels) M1, L1 and VL1 soil samples.

Soil Sample	Level	DTPA	EDTA	HNO_3	HAc	LMWOA	NH_4Ac	$CaCl_2$	$MgCl_2$	$NaNO_3$	NH_4NO_3
M1	Un-spiked	6.9	6.7	5.0	5.2	7.1	7.4	7.9	7.6	8.1	7.4
	Tt1 level	7.1	6.5	4.3	5.2	6.8	7.3	7.8	7.5	7.9	7.3
	Tt2 level	7.2	6.7	3.9	5.1	6.5	7.3	7.3	7.3	7.5	7.1
L1	Un-spiked	6.9	6.4	1.8	4.7	7.1	7.2	7.7	7.5	7.8	7.2
	Tt1 level	6.9	6.4	1.8	4.7	6.7	7.3	7.5	7.2	7.7	7.1
	Tt2 level	6.9	6.4	1.7	4.6	6.2	7.2	7.2	7.0	7.2	6.9
VL1	Un-spiked	6.8	4.4	0.5	3.8	7.0	7.0	7.7	7.2	7.7	7.1
	Tt1 level	6.9	4.4	0.6	3.8	6.9	7.1	7.7	7.1	7.8	7.2
	Tt2 level	6.9	4.3	0.6	3.8	6.6	7.1	7.4	7.0	7.5	7.1

The high proportion of metal extractable with EDTA (60-82 %) was attributed to its high chelation stability constant (Meers *et al.*, 2007). Similarly, the pH range of our soils favoured the formation of soluble complexes with metals in the EDTA and DTPA-extracts (up to 100 % of Me-$EDTA^{-3}$ and Me-$DTPA^{-2}$). Nevertheless, it should be taken into account that the very high percentage of negatively charged complexes could lead to lower metal extraction in soils with a higher OM content, due to re-adsorption processes in the organic matter (Ettler *et al.*, 2007; Peters, 1999). In HAc extractions, the speciation was dominated by Me-$Acetate^{+}$, Me-$(Acetate)_2$ and Me^{2+}. The highest proportion of Me-$(Acetate)_2$ was in M1 soil, and the highest rate of free metal ion was found in VL1 soil, which was attributed to the different pH value of the soil extracts (Table 3).

The speciation was more complex with LMWOA extractions. Metals were present as Me-$Citrate^{-}$ (up to 97%) due to the higher chelation stability constant of citric acid than of the other organic acids in the mixture employed. The lower stability constants of the citrate complexes with Cd and Pb than with Cu and Zn (Barton and Abadía, 2006) resulted in a lower Cd and Pb extraction percentage at Tt2 level. Moreover, at this level we observed the appearance of other Cd and Pb species in minor proportions (free metal ion, Me-Malate (aq), Me-$Lactate^{+}$ and Me-$Acetate^{+}$) due to different complexing abilities in response to pH (Qin *et al.*, 2004). In NH_4Ac extracts, acetic complexes were the dominant species of Cd, Pb, and Zn, which were

mainly present as Me-(Acetate)$^{-3}$, Me-(Acetate)$_2$ (aq) and Me-Acetate$^+$. The dominant species in the case of Cu were ammine complexes.

Very high amounts of metals were extracted with the HNO_3 method (Fig. 2). In general, free metal ion was the predominant species despite the large difference in the pH of the extractant solution from the various soils (Table 3). This could indicate that the main processes affecting metal desorption with the HNO_3 extraction method could be either the dissolution of some of the soil components, or the metal displacement by excess H^+ from the exchangeable complex (Vidal *et al.*, 2004).

Different patterns were obtained in the case of neutral salt extractions. Metal speciation in $CaCl_2$ and $NaNO_3$ extracts was dominated by the free ionic form, except for Cu and Pb in $NaNO_3$ extracts which were in a hydroxocomplex form at pH 7.8-8.1. Moreover, it should be noted that a similar metal percentage was extracted in un-spiked and in metal-spiked soils, suggesting that metal redistribution within the contact time of this study (12 months) could reduce their concentration in exchangeable positions (McLaughlin, 2001). Metals in $MgCl_2$ extracts were mainly in the form of chlorocomplexes.

In the case of NH_4NO_3 extracts, ammine complexes were the dominant species of Cd, Cu, and Zn, while Pb formed mainly nitric complexes. It should be noted that the calculated ionic strengths for $MgCl_2$ and NH_4NO_3 extract exceeded 1 mol L^{-1}, so the results of this calculation must be considered with caution.

The low metal extraction percentage with neutral salts could be due to the pH range of the soils in the study, since strong binding adsorption surfaces (specific adsorption) may play a relevant role in metal retention. Moreover, it is worth noting that competition mechanisms can be established between the alkaline-earth cations, major cations in these soils, and the cations of the extractants. In contrast, high amount of metals were extracted with $MgCl_2$ which was attributed to its higher salt concentration, the combined effect of complexation by chloride and metal displacement from the exchangeable complex by Mg^{2+} (Meers *et al.*, 2007), and to slight carbonate dissolution (Gleyzes *et al.*, 2002).

Metal extractability patterns

Metal extractability patterns in metal-spiked soils were studied according to the soil physicochemical characteristics (Table 4, 5, 6, 7), and by mineralogical investigations. In this regard, it was observed in general that soil properties and components affecting metal extractability patterns depend on the metal being studied, as discussed below. EDTA is an exception, showing a general extractability pattern for all metals. In all cases, significant negative correlations were observed between EDTA-extractable metals and OM content, mainly with the recalcitrant fraction, showing that re-adsorption processes could occur in organic material.

Cadmium extractability patterns

The key role of the soil carbonate fraction in Cd extractability was evident from the correlation coefficients calculated. Extractable-Cd, mainly with $MgCl_2$, NH_4NO_3, DTPA and LMWOA-methods, showed significant and negative correlations with all the soil constituents measured in relation to the carbonate fraction –ECC, AL, total Ca-Mg contents and the relative proportion of calcite and dolomite– (Table 4). This indicates that the soil carbonate fraction may govern both mobile and potentially mobile fractions of Cd in these soils. Due to the contact time of the incubation experiment (12 months), it would produce a slow diffusion of soluble Cd species into the crystal defects and pores of the lime, resulting in stable bonds (Buekers *et al.*, 2007), and hence decreasing Cd extractability. The positive and significant correlations obtained between acidic extractants and ECC and AL contents could corroborate this hypothesis, since these extractants could total or partially dissolve the carbonate, thereby releasing Cd into the solution (Vidal *et al.*, 2004). This pattern has been observed by other authors when studying Cd desorption by fibrous minerals (Shirvani *et al.*, 2007) and Mn-dioxides (Zaman *et al.*, 2009).

Table 4. Significance correlation analyses calculated between soil physicochemical parameters (Pearson's correlation test) and mineralogical composition (Spearman's correlation test) and extractable Cd in metal-spiked soil samples at Tt1 and Tt2 levels.

Level	Extraction method	ECC	AL	Tot-Ca	Tot-Mg	OM	RP	Cry-Fe	Clay	CEC	Calcite	Dolomite
											< 2 mm	
Tt1	DTPA	-0.609	**-0.811****	**-0.739***	-0.608	0.565	-0.050	0.542	0.410	0.469	-0.539	-0.479
	HNO_3	**0.699***	0.605	0.491	0.573	0.188	-0.035	0.345	-0.091	-0.287	0.365	0.374
	LMWOA	-0.549	**-0.663***	-0.459	**-0.783****	0.092	0.056	-0.242	-0.122	0.072	**-0.663***	-0.492
	$MgCl_2$	**-0.714***	**-0.773****	**-0.721***	**-0.737***	0.167	-0.092	0.175	0.490	0.586	-0.533	-0.610
	$NaNO_3$	-0.471	-0.422	-0.432	**-0.636***	-0.134	0.089	-0.515	-0.317	-0.048	-0.149	-0.400
Tt2	DTPA	0.159	0.311	0.521	-0.069	**-0.715***	0.151	**-0.946*****	-0.383	-0.364	-0.167	0.046
	EDTA	-0.523	-0.313	-0.455	-0.117	-0.205	**-0.649***	0.027	0.492	0.613	-0.477	-0.452
	HNO_3	-0.083	-0.191	-0.114	0.328	**0.793****	0.472	0.461	-0.161	-0.099	0.118	-0.216
	HAc	**0.669***	**0.641***	0.528	0.291	0.101	0.119	-0.195	**-0.738***	**-0.775****	0.421	0.426
	LMWOA	**-0.646***	-0.514	-0.594	**-0.654***	-0.090	-0.279	-0.300	-0.170	0.140	-0.384	**-0.662***
	NH_4Ac	-0.365	-0.523	-0.486	-0.595	0.312	-0.231	0.301	0.037	0.031	-0.458	**-0.754***
	$CaCl_2$	-0.519	-0.512	-0.483	**-0.710***	-0.156	-0.326	-0.398	-0.325	-0.051	-0.489	-0.385
	MgCl2	**-0.672***	**-0.678***	**-0.814****	-0.571	0.367	-0.147	0.617	**0.637***	**0.719***	-0.607	-0.610
	$NaNO_3$	-0.563	-0.543	-0.531	**-0.718***	-0.157	-0.359	-0.381	-0.277	0.018	-0.632	-0.492
	NH_4NO_3	**-0.680***	**-0.653***	**-0.731***	**-0.810****	-0.085	-0.508	-0.157	-0.057	0.220	**-0.650***	**-0.649***

ECC = equivalent $CaCO_3$; AL = active lime; Tot-Ca and Tot-Mg = total Ca and Mg; OM = organic matter; RP = recalcitrant pool; Cry-Fe = crystalline Fe-oxide; CEC = cation exchange capacity. *****, ****** and ******* indicate statistical significance at the probability level $p < 0.05$, $p < 0.01$ and $p < 0.001$

The mineralogical study showed no evidence of formation of Cd-carbonate or oxide, probably due to the fact that the Cd concentrations used were below the quantification limit of the XRD equipment (< 2%). Nevertheless, the negative correlation ($p<0.001$) obtained between crystalline Fe-oxide content and DTPA-extractable Cd at Tt2 level suggested that surface precipitation phenomena could occur on Fe-oxides. On the other hand, the high affinity of Cd for high molecular weight organic acids would lead to the formation of stable organomineral associations (Prokop *et al.*, 2003). This could explain the lower Cd extractability in soils with higher OM content, as shown by the significant and negative correlations between OM content and DTPA-extractable Cd, and between RP content and EDTA-extractable Cd at Tt2 level. Nevertheless, re-adsorption processes of negatively charged Me-DTPA and EDTA complexes in the organic matter should be taken into account (Ettler *et al.*, 2007). In view of these results, it could be concluded that potentially mobile Cd, estimated with EDTA and DTPA, is affected by crystalline Fe oxides and OM.

At Tt2 level, specific adsorption positions may be saturated, leaving a fraction of Cd adsorbed in exchange positions which are easily removable. This could be corroborated by the significant and positive correlations obtained between $MgCl_2$-extractable Cd and clay content and CEC. The significant and negative correlations between HAc-extractable Cd and clay content and CEC may be an artefact of carbonate, as the soils with a lower proportion of clay generally present higher carbonate contents in this study.

Copper extractability patterns

There were significant and positive correlations between NH_4Ac and NH_4NO_3-extractable Cu and ECC, AL and total Ca contents at Tt1 level (Table 5).

Table 5. Significance correlation analyses calculated between soil physicochemical parameters (Pearson's correlation test) and extractable Cu in metal-spiked soil samples at Tt1 and Tt2 levels.

Level	Extraction method	ECC	AL	Tot-Ca	Tot-Mg	OM	RP	Cry-Fe	CS	FS	Clay	CEC
Tt1	DTPA	-0.033	-0.133	-0.053	-0.411	-0.091	0.242	-0.533	**0.906*** **	0.404	**-0.652***	-0.492
	HAc	0.355	0.477	0.453	0.033	**-0.746***	0.086	**-0.787****	0.525	0.326	-0.442	-0.421
	NH_4Ac	**0.751***	**0.819****	**0.882****	0.583	-0.590	0.108	**-0.638***	0.144	0.555	-0.504	**-0.633***
	$MgCl_2$	**0.648***	0.452	0.489	0.321	0.178	-0.220	-0.145	0.405	**0.722***	**-0.753***	**-0.870****
	$NaNO_3$	0.374	0.091	0.224	0.017	0.490	0.045	0.006	0.465	**0.693***	**-0.704***	**-0.751***
	NH_4NO_3	**0.802****	**0.836****	**0.826****	0.444	-0.533	0.117	-0.568	0.344	0.586	-0.582	**-0.727***
Tt2	DTPA	-0.527	-0.486	-0.429	**-0.662***	-0.233	-0.327	-0.520	**0.700***	0.059	-0.349	-0.042
	EDTA	-0.629	-0.391	**-0.638***	-0.478	-0.416	**-0.924*****	-0.043	0.085	**-0.711***	0.536	**0.656***
	HAc	0.162	0.302	0.174	-0.258	-0.427	-0.304	**-0.634***	0.621	0.400	-0.569	-0.454
	$CaCl_2$	-0.438	-0.503	-0.418	**-0.648***	-0.022	-0.225	-0.371	**0.784****	0.199	-0.473	-0.202
	$MgCl_2$	-0.331	-0.464	-0.400	-0.614	0.115	-0.174	-0.247	**0.764***	0.309	-0.543	-0.286
	$NaNO_3$	-0.444	-0.466	-0.404	**-0.642***	-0.091	-0.234	-0.423	**0.767****	0.194	-0.455	-0.179

ECC = equivalent $CaCO_3$; AL = active lime; Tot-Ca and Tot-Mg = total Ca and Mg; OM = organic matter; RP = recalcitrant pool; Cry-Fe = crystalline Fe-oxide; CS = coarse sand; FS = fine sand; CEC = cation exchange capacity. *****, ****** and ******* indicate statistical significance at the probability level $p < 0.05$, $p < 0.01$ and $p < 0.001$.

Since the mineralogical study showed no evidence of the formation of Cu-carbonate, the correlations obtained suggest that Cu extractability is mainly regulated by adsorption processes. In this scenario, ammonium ions could displace the Cu adsorbed from carbonates. The highest correlation coefficients ($p<0.01$) obtained for

AL indicated that the finest carbonate fraction could play a greater role in affecting Cu extractability.

The significant and negative correlations between DTPA, $MgCl_2$ and $NaNO_3$-extractable Cu and clay content and CEC at Tt1 level suggest that Cu could be strongly retained by other soil constituents present in the finest soil fraction <2μm – Fe oxides and phyllosilicates– decreasing Cu extractability. Furthermore, the enhanced adsorption capacity of mica-illite minerals in the presence of Fe (Sipos *et al.*, 2008), together with the high ability of Cu^{+2} to displace the Fe^{+2}, could considerably affect Cu extractability. This could explain the significant and negative correlations obtained between HAc-extractable Cu and the crystalline Fe-oxide content at both contamination levels. The significant and positive correlations observed between EDTA-extractable Cu and CEC at Tt2 level suggest that specific adsorption positions could be saturated at this level, with other sorption processes also taking place through weakly-binding surface sites (Sayen and Guillon, 2009).

Similarly, significant and negative correlations were also obtained between HAc-Cu and OM content at Tt1 level, and between EDTA-Cu and RP at Tt2 level. The higher Cu electronegativity, the predominance of positively-charged Cu hydroxocomplexes (characteristics of the pH range studied) and the degree of humification of the soil samples (C/N ~ 11) would favour the formation of stable associations between Cu and humic substance. These results indicate that the potential mobility of Cu may be governed by both inorganic and organic fractions through organomineral associations (Besnard *et al.*, 2001), probably humate-Fe oxide associations (Sayen and Guillon, 2010; Sipos *et al.*, 2008) at both levels of contamination.

Lead extractability patterns

Few correlations were obtained for extractable Pb (Table 6). At level Tt1, significant and positive correlations were obtained between acidic extractants and the ECC and AL contents, suggesting the total or partial dissolution of the carbonate fraction (Vidal *et al.*, 2004). Nevertheless, significant correlations were not obtained at Tt2 level, despite the formation of lead oxide carbonate observed (Fig. 1).

Table 6. Significance correlation analyses calculated between soil physicochemical parameters (Pearson's correlation test) and mineralogical composition (Spearman's correlation test) and extractable Pb in metal-spiked soil samples at Tt1 and Tt2 levels.

Level	Extraction method	ECC	AL	Tot-Ca	Tot-Mg	RP	Clay	CEC	Dolomite
									< 2 mm
Tt1	DTPA	-0.330	-0.232	-0.327	**-0.680***	0.030	0.122	0.211	0.150
	HNO_3	**0.683***	0.590	0.444	0.457	0.041	-0.369	-0.454	0.369
	HAc	**0.718***	**0.742***	0.492	0.354	0.130	-0.366	-0.445	**0.876****
	LMWOA	-0.330	-0.332	-0.371	**-0.650***	0.081	-0.393	-0.161	-0.060
	$MgCl_2$	-0.430	-0.400	-0.436	**-0.703***	0.201	-0.310	-0.052	-0.099
	$NaNO_3$	**-0.638***	-0.494	-0.595	**-0.642***	-0.102	0.047	0.313	-0.177
	NH_4NO_3	-0.467	-0.245	-0.433	-0.198	0.145	**0.672***	**0.740***	-0.102
Tt2	DTPA	-0.555	-0.345	-0.369	-0.571	-0.272	0.008	0.233	-0.043
	EDTA	-0.475	-0.204	-0.574	-0.463	**-0.914*****	0.460	0.573	-0.077
	HAc	0.537	0.579	0.227	0.144	-0.402	-0.472	-0.477	**0.640***
	LMWOA	-0.425	-0.337	-0.423	**-0.651***	-0.334	-0.328	-0.069	-0.088
	$CaCl_2$	-0.462	-0.392	-0.469	**-0.710***	-0.606	-0.116	0.057	-0.352
	$MgCl_2$	-0.620	-0.596	**-0.657***	**-0.778****	-0.402	-0.167	0.126	-0.336
	$NaNO_3$	-0.525	-0.434	-0.445	**-0.705***	-0.343	-0.302	-0.020	-0.193
	NH_4NO_3	-0.526	-0.482	-0.476	**-0.695***	-0.322	-0.332	-0.045	-0.237

ECC = equivalent $CaCO_3$; AL = active lime; Tot-Ca and Tot-Mg = total Ca and Mg; RP = recalcitrant pool; CEC = cation exchange capacity. *****, ****** and ******* indicate statistical significance at the probability level $p < 0.05$, $p < 0.01$ and $p < 0.001$.

The lack of correlations with soil physicochemical parameters at this level led us to study the relationship with soil mineralogical composition. The significant and positive correlations obtained between the HAc-extractable Pb and the relative proportion of dolomite (< 2mm) at both levels suggest that the Pb extractability pattern with acidic extraction methods is mainly affected by the dolomite phase.

However, extractable Pb with other extraction methods with lower strength (DTPA, LMWOA, $MgCl_2$, NH_4NO_3 and $NaNO_3$) showed significant and negative correlations with the total Mg content. In the pH range of our soils, the nucleation of micro-precipitates of lead oxide carbonate on the surfaces of Mg-rich clay minerals – mainly dolomite– favoured by the incubation time of our experiment (12 months) could explain the lower Pb extractability. This behaviour has been observed by other authors when studying Pb retention on calcite (Rangel-Porras *et al.*, 2010). These results therefore suggest that the potential mobility of Pb depends to some degree on the turnover of inorganic C. Nevertheless, because the intensities of reflections obtained from XRD were not high, it can be expected that –in addition to

precipitation– other soil processes such as adsorption and/or complexing processes affect Pb extractability patterns.

The predominant species in the pH range in the study, $Pb(OH)^+$, would form stable bonds with OM and clay, probably through organomineral associations regulating the retention of Pb. However, the slow Pb desorption from organic matter, in contrast to its rapid initial sorption (Strawn and Sparks, 2000), could explain the scarce correlations obtained with clay and OM. This sorption mechanism may not affect Pb extractability patterns or its potential mobility as high sorption does not necessarily lead to high bond strength at the same time.

On the other hand, NH_4NO_3-extractable Pb correlated positively and significantly with clay content and CEC at Tt1 level, suggesting that a fraction of Pb could be in exchange positions at this level.

Zinc extractability patterns

The Pearson's correlation coefficients calculated showed that particle-size distribution was the main soil characteristic affecting Zn extractability, and no significant correlations were obtained with other soil physicochemical parameters, except the general case of EDTA and OM (Table 7). Thus significant and negative correlations were obtained between extractable-Zn with almost all extractants and clay and silt contents, suggesting that the soil fractions with a higher adsorption capacity are primarily responsible for governing both mobile and potentially mobile Zn fractions.

From the mineralogical study we obtained positive correlations between the relative proportion of kaolinite, mica-illite and quartz (< 2μm), and HNO_3 and EDTA-extractable Zn. This indicates that the presence of these minerals, with a low specific surface area, would favour a higher Zn extractability. The significant and negative correlations obtained between extractable-Zn with several extractants and the total Mg content, mainly at Tt2 level, indicate that lower Zn extractability may be linked to the presence of Mg-rich minerals (Lafuente *et al.*, 2008), probably due to the similarity of ionic radius between Zn^{2+} and Mg^{2+}. The significant and negative correlations obtained between EDTA-extractable Zn and the dolomite relative proportion (< 2μm) at Tt2 level, as well as the increase in Mg in soil solution in the

metal-spiked soils (data not shown), could support this hypothesis. These results therefore highlight the fact that the clay content alone cannot explain the Zn-extractability patterns, since soil mineral composition plays a significant role in controlling its extractability.

Table 7. Significance correlation analyses calculated between soil physicochemical parameters (Pearson's correlation test) and mineralogical composition (Spearman's correlation test) and extractable Zn in metal-spiked soil samples at Tt1 and Tt2 levels.

Level	Extraction method	Tot-Mg	OM	CS	FS	Silt	Clay	CEC	Mica-Illite	Kaolinite	Quartz	Dolomite
									< 2 μm			
Tt1	DTPA	-0.530	0.064	**0.836****	0.246	**-0.710***	-0.506	-0.298	-0.039	-0.406	0.039	-0.220
	EDTA	-0.388	-0.346	-0.197	-0.352	0.258	0.353	0.529	**0.658***	-0.058	0.526	-0.468
	HAc	0.193	0.212	0.425	**0.802****	-0.536	**-0.815****	**-0.821****	-0.540	-0.058	-0.415	0.220
	LMWOA	-0.582	-0.088	**0.856****	0.323	**-0.753***	-0.573	-0.343	-0.230	-0.522	-0.197	-0.165
	NH_4Ac	-0.030	0.184	**0.644***	**0.727***	**-0.827****	**-0.753***	**-0.790****	-0.434	-0.290	-0.421	0.275
	$CaCl_2$	-0.618	0.152	**0.803****	0.324	**-0.753***	-0.532	-0.309	-0.211	-0.406	-0.151	-0.055
	$MgCl_2$	**-0.683***	0.164	**0.765****	0.161	**-0.683***	-0.383	-0.147	-0.020	-0.290	0.086	-0.138
	$NaNO_3$	**-0.680***	-0.050	**0.813****	0.162	**-0.674***	-0.427	-0.166	-0.039	-0.406	-0.026	-0.110
	NH_4NO_3	-0.517	-0.032	**0.858****	0.409	**-0.848****	-0.605	-0.426	-0.368	-0.522	-0.421	0.083
Tt2	DTPA	**-0.662***	-0.130	**0.806****	0.159	**-0.663***	-0.426	-0.164	-0.007	-0.406	0.007	-0.165
	EDTA	-0.108	**-0.634***	0.233	-0.490	0.034	0.270	0.253	0.171	-0.522	0.224	**-0.881****
	HAc	-0.234	0.019	0.552	0.523	-0.517	**-0.657***	-0.587	**0.678***	0.522	**0.737***	-0.275
	LMWOA	-0.615	0.097	**0.744***	0.166	**-0.680***	-0.373	-0.194	0.020	-0.058	-0.053	-0.028
	NH_4Ac	-0.184	0.090	**0.683***	0.539	**-0.780****	-0.629	-0.602	0.164	-0.406	0.164	-0.275
	$CaCl_2$	**-0.667***	-0.113	**0.776****	0.160	-0.630	-0.420	-0.148	-0.388	-0.290	-0.434	0.220
	$MgCl_2$	**-0.674***	0.014	**0.718***	0.106	-0.555	-0.365	-0.081	-0.026	-0.290	0.026	-0.193
	$NaNO_3$	**-0.659***	-0.199	**0.731***	0.116	-0.570	-0.376	-0.087	0.184	-0.058	0.243	-0.083
	NH_4NO_3	**-0.657***	-0.179	**0.737***	0.142	-0.592	-0.393	-0.109	0.250	-0.290	0.243	-0.275

Tot-Mg = total Mg; OM = organic matter; CS = coarse sand; FS = fine sand; CEC = cation exchange capacity. *****, ****** and ******* indicate statistical significance at the probability level $p < 0.05$, $p < 0.01$ and $p < 0.001$.

Conclusions

The combination of indirect and direct methods employed in this study has proved to be a useful tool for investigating the extractability and mobility of Cd, Cu, Pb, and Zn in these calcareous soils. The findings show that the carbonate fraction alone cannot explain the metal extractability patterns in the metal-spiked soils studied, under the experimental conditions used. Instead, the joint action of the soil constituents usually involved in metal sorption-desorption processes (specific and non-specific adsorption, complexing and precipitation) have been shown to govern metal extractability patterns. These patterns were different for each metal and contamination level. The content and composition of the organic and inorganic fraction of soils played the main role in affecting the mobile and potentially mobile fractions of metals. The high metal extraction percentage highlights the possible vulnerability of these soils against an environmental impact from metal accumulation in periurban agricultural areas. Nevertheless, further research work focused on studying soil constituents affecting the potential mobility of metals under real terrestrial environmental conditions is crucial for making reliable predictions in calcareous agricultural soils in the Mediterranean area.

Acknowledgements

This work was supported by the Universidad Complutense in Madrid and the Madrid Autonomous Region through Grant GR58/08, Research Team 950605 and Network CARESOIL, Ref. P2009/AMB-1648. We especially wish to thank Miguel Juanco Ortenbach (Centro Superior de Investigaciones Científicas –CSIC) and Julián Velázquez Cano (Universidad Complutense de Madrid –UCM) for their helpful advice on mineralogical analysis and Ms Pru Brooke-Turner for the revision of the English version of the manuscript.

References

Barton, L., Abadía, J., 2006. Iron nutrition in plants and rhizospheric microorganisms. Springer, Dordrecht.

Batjes, N.H., 2000. Soil Vulnerability to Diffuse Pollution in Central and Eastern Europe SOVEUR Project (Version 1.0). FAO and ISRIC.

Besnard, E., Chenu, C., Robert, M., 2001. Influence of organic amendments on copper distribution among particle-size and density fractions in Champagne vineyard soils. Environ. Pollut. 112, 329-337.

Bish, D.L., 1994. Quantitative x-ray diffraction analysis of soils. In: Amonette J.E. and Zelazny LW (ed.) Quantitative methods in soil mineralogy, SSSA Misc Publ, SSSA, Madison, WI, pp. 267-295.

Bolan, N.S., Adriano, D.C., Curtin, D., 2003. Soil acidification and liming interactions with nutrient and heavy metal transformation and bioavailability. Adv. Agron. 78, 215-278.

Buekers, J., Van Laer, L., Amery, F., Van Buggenhout, S., Maes, A., Smolders, E., 2007. Role of soil constituents in fixation of soluble Zn, Cu, Ni and Cd added to soils. Eur. J. Soil Sci. 58, 1514-1524.

de Miguel, E., Llamas, J.F., Chacon, E., Berg, T., Larssen, S., Røyset, O., Vadset, M., 1997. Origin and patterns of distribution of trace elements in street dust: unleaded petrol and urban lead. Atmos. Environ. 31(17), 2733-2740.

de Santiago-Martín, A., Valverde-Asenjo, I., Quintana, J.R., González-Huecas, C., Lafuente, A.L., in press. Soil properties affecting metal extractability patterns in periurban calcareous agricultural soils in the Mediterranean area. Int. J. Environ. Res.

DIN (Deutsches Institut für Normung, Bodenbeschaffenheit), 1995. Extraktion von Spurenelemente mit Ammonium-nitratlösung, Vornorm DINV 19730, DIN Boden-Chemische Bodenuntersuchungs-verfahren, Berlin, Germany.

Directive 86/278/EEC of 12 June 1986 on the protection of the environment, and in particular of the soil, when sewage sludge is used in agriculture.

Douglas, L.A., 1985. Criteria for vermiculitic and chloritic family classes in soil taxonomy. In: Kittrick JA (ed) Mineral Classification of Soils, SSSA Spec Publ, vol. 16, Soil Science Society of America, Madison, WI.

Drouineau, G., 1942. Dosage rapide du calcaire actif de sols. Ann. Agron. 12, 441-450.

Ettler, V., Mihaljevič, M., Šebek, O., Grygar, T., 2007. Assessment of single extractions for the determination of mobile forms of metals in highly polluted soils and sediments - Analytical and thermodynamic approaches. Anal. Chim. Acta 602(1), 131-140.

European Commission, 2006. Thematic Strategy for Soil Protection. COM (2006) 231, Brussels, Belgium.

FAO, 2006. World reference base for soil resources. A framework for international classification, correlation and communication. FAO, Roma.

Feng, M., Shan, X., Zhang, S., Wen, B., 2005. A comparison of the rhizosphere-based method with DTPA, EDTA, $CaCl_2$, and $NaNO_3$ extraction methods for prediction of bioavailability of metals in soil to barley. Environ. Pollut. 137, 231-240.

Gleyzes, C., Tellier, S., Astruc, M., 2002. Fractionation studies of trace elements in contaminated soils and sediments: a review of sequential extraction procedures. Trends Anal. Chem. 21(6+7), 451-467.

Gupta, S.K., Aten, C., 1993. Comparison and evaluation of extraction media and their suitability in a simple model to predict the biological relevance of heavy metal concentrations in contaminated soils. Int. J. Environ. Anal. Chem. 51, 25-46.

Gupta, S.K., Vollmer, M.K., Krebs, R., 1996. The importance of mobile, mobilisable and pseudo total heavy metal fractions in soil for three-level risk assessment and risk management. Sci. Total Environ. 178, 11-20.

Gustafsson, J.P., 2011. Visual MINTEQ, v.3.0. Royal Institute of Technology, Department of Land and Water Resources Engineering, Stockholm http://www2.lwr.kth.se/English/OurSoftware/Vminteq/index.htm.

Hesterberg, D., Stigliani, W.M., Imeson, A.C., 1992. Chemical time bombs: Linkages to scenarios of socio-economic developments, Executive Report 20. International Institute for Applied Systems Analysis, Laxenburg.

IGME (Instituto Geológico y Minero de España), 1990. Mapa geológico de España nº 535, Escala 1:50.000 (Algete), Madrid.

Iram, S., Ahmad, I., Akhtar, S., 2012. Distribution of heavy metals in peri-urban agricultural areas soils. J. Chem. Soc. Pak. 34(4), 861-869.

ISRIC (International Soil Reference and Information Center), 2002. Procedures for Soil Analysis, 3rd edn. International Soil Reference and Information Center, Wageningen.

Jiménez Ballesta, R., Conde Bueno, P., Martín Rubí, J.A., García Giménez, R., 2010. Pedo-geochemical baseline content levels and soil quality reference values of trace elements in soils from the Mediterranean (Castilla La Mancha, Spain). Eur. J. Geosci. 2(4), 441-454.

Lafuente, A.L., González, C., Quintana, J.R., Vázquez, A., Romero, A., 2008. Mobility of heavy metals in poorly developed carbonate soils in the Mediterranean region. Geoderma 145, 238-244.

Lindsay, W.L., Norvell, W.A., 1978. Development of a DTPA soil test for zinc, iron, manganese and copper. Soil Sci. Soc. Am. J. 42, 421-428.

McLaughlin, M.J., 2001 Aging of metals in soils changes bioavailability. Fact Sheet Environ. Risk Assess. 4, 1-6.

Meers, E., Du Laing, G., Unamuno, V., Ruttens, A., Vangronsveld, J., Tack, F.M.G., Verloo, M.G., 2007. Comparison of cadmium extractability from soils by commonly used single extraction protocols. Geoderma 141, 247-259.

Meng, F., Zhang, J., Shi, Q.Q., Liu, M., 2008. Spatial structure and distribution of heavy metals in agricultural soils of peri-urban area in Pudong of Shanghai, China. Proceedings of SPIE 7145.

Micó, C., Recatalá, L., Peris, M., Sánchez, J., 2006. Assessing heavy metal sources in agricultural soils of an European Mediterranean area by multivariate analysis. Chemosphere 65, 863-872.

Moreno Merino, L., 1998. Estudio de la influencia del suelo sobre la composición de las aguas subterráneas a través de la solución del suelo. Modelo en Fluvisoles calcáricos. Dissertation, Universidad Complutense de Madrid.

Pérez-Esteban, J., Escolástico, C., Moliner, A., Masaguer, A., 2013. Chemical speciation and mobilization of copper and zinc in naturally contaminated mine soils with citric and tartaric acids. Chemosphere 90, 276-283.

Peris, M., Micó, C., Recatalá, L., Sánchez, R., Sánchez, J., 2007. Heavy metal contents in horticultural crops of a representative area of the European Mediterranean region. Sci. Total Environ. 378, 42-48.

Peters, R.W., 1999. Chelant extraction of heavy metals from contaminated soils. J. Hazard. Mater. 66, 151-210.

Prokop, Z., Cupr, P., Zlevorova-Zlamalikova, V., Komarek, J., Dusek, L., Holoubek, I., 2003. Mobility, bioavailability, and toxic effects of cadmium in soil samples. Environ. Res. 91, 119-126.

Qin, F., Shan, X.Q., Wei, B., 2004. Effects of low-molecular-weight organic acids and residence time on desorption of Cu, Cd, and Pb from soils. Chemosphere 57, 253-263.

Quevauviller, Ph., Lachica, M., Barahona, E., Rauret, G., Ure, A., Gómez, A., Muntau, H., 1996. Interlaboratory comparison of EDTA and DTPA procedures prior to certification of extractable trace elements in calcareous soil. Sci. Total Environ. 178, 127-132.

Rangel-Porras, G., García-Magno, J.B., González-Muñoz, M.P., 2010. Lead and cadmium immobilization on calcitic limestone materials. Desalination 262, 1-10.

Rauret, G., Lopez-Sanchez, J.F., Sahuquillo, A., Rubio, R., Davidson, C., Ure, A., Quevauviller, Ph., 1999. Improvement of the BCR three-step sequential extraction procedure prior to the certification of new sediment and soil reference materials. J. Environ. Monit. 1, 57-61.

Rivas-Martínez, S., 1987. Memoria del mapa de series de vegetación de España. 1:400.000. ICONA, Madrid.

Rovira, P., Vallejo, V.R., 2000. Evaluating thermal and acid hydrolysis methods as indicators of soil organic matter quality. Commun. Soil Sci. Plant Anal. 31, 81-100.

Sayen, S., Guillon, E., 2009. Aging effect on the copper sorption on a vineyard soil: Column studies and SEM–EDS analysis. J. Colloid Interface Sci. 331, 47-54.

Sayyad, G., Afyuni, M., Mousavi, S.F., Abbaspour, K.C., Richards, B.K., Schulin, R., 2010. Transport of Cd, Cu, Pb and Zn in a calcareous soil under wheat and safflower cultivation - A column study. Geoderma 154, 311-320.

Shirvani, M., Shariatmadari, H., Kalbasi, M., 2007. Kinetics of cadmium desorption from fibrous silicate clay minerals: Influence of organic ligands and aging. Appl. Clay Sci. 37, 175-184.

Sipos, P., Németh, T., Kovács Kis, V., Mohai, I., 2008. Sorption of copper, zinc and lead on soil mineral phases. Chemosphere 73, 461-469.

SISS (Società Italiana della Scienza del Suolo), 1985. Metodi Normalizzati di Analisi del Suolo. Edagricole, Bologna.

Steinitz, C., del Pozo, C., Vargas-Moreno, J.C., Canfield, T., 2011. Futuros alternativos para los paisajes dinámicos del Corredor del Henares. Fundación Paisaje 2011.

Strawn, D., Sparks, D.L., 2000. Effects of soil organic matter on the kinetics and mechanisms of Pb(II) sorption and desorption in soil. Soil Sci. Soc. Am. J. 64, 144-156.

Tessier, A., Campbell, P.G.C., Bisson, M., 1979. Sequential extraction procedure for the speciation of particulate trace metals. Anal. Chem. 51, 844-851.

Van Ranst, E., Verloo, M., Demeyer, A., Pauwels, J.M., 1999. Manual for the soil chemistry and fertility laboratory. Ghent University, Faculty Agricultural and Applied Biological Sciences, pp. 243.

Vidal, J., Pérez-Sirvent, C., Martínez-Sánchez, M.J., Navarro, M.C., 2004. Origin and behaviour of heavy metals in agricultural Calcaric Fluvisols in semiarid conditions. Geoderma 121, 257-270.

Zaman, M.I., Mustafa, S., Khan, S., Xing, B., 2009. Heavy metal desorption kinetic as affected by of anions complexation onto manganese dioxide surfaces. Chemosphere 77, 747-755.

Supplementary Material

Metal extractability patterns to evaluate (potentially) mobile fractions in periurban calcareous agricultural soils in the Mediterranean area – Analytical and mineralogical approaches

Predicted percentages with Visual Minteq of the different chemical species of Cd, Cu, Pb and Zn in the individual extracts from un-spiked and metal-spiked (Tt1 and Tt2 levels) M1, L1 and VL1 soil samples respect to the total concentrations of the extracts. nd = not detected.

Table S1

DTPA extracts									
Chemical specie %	M1 soil			L1 soil			VL1 soil		
	Un-spiked	Tt1 level	Tt2 level	Un-spiked	Tt1 level	Tt2 level	Un-spiked	Tt1 level	Tt2 level
Cd-DTPA^{-3}	nd	99	99	nd	98	98	nd	99	99
Cu-DTPA^{-3}	98	99	97	98	99	93	98	99	99
Pb-DTPA^{-3}	96	98	98	96	97	96	96	97	97
Zn-DTPA^{-3}	91	95	86	91	91	68	90	92	92
Zn_2-DTPA^{-}	nd	nd	9	nd	nd	25	nd	nd	nd

Table S2

EDTA extracts									
Chemical specie %	M1 soil			L1 soil			VL1 soil		
	Un-spiked	Tt1 level	Tt2 level	Un-spiked	Tt1 level	Tt2 level	Un-spiked	Tt1 level	Tt2 level
Cd-EDTA^{-2}	nd	100	100	nd	100	100	nd	96	94
Cu-EDTA^{-2}	100	100	100	100	100	100	93	93	91
Pb-EDTA^{-2}	100	100	100	100	100	100	99	99	98
Zn-EDTA^{-2}	100	100	100	100	100	100	95	95	93

Table S3

HNO_3 extracts									
Chemical specie	M1 soil			L1 soil			VL1 soil		
%	Un-spiked	Tt1 level	Tt2 level	Un-spiked	Tt1 level	Tt2 level	Un-spiked	Tt1 level	Tt2 level
Cd^{2+}	nd	65	65	nd	65	65	nd	66	66
$CdNO_3^+$	nd	31	31	nd	31	31	nd	30	30
Cu^{2+}	67	67	67	67	67	67	68	68	68
$CuNO_3^+$	32	32	32	32	32	32	31	31	31
Pb^{2+}	24	24	24	24	24	24	25	25	25
$PbNO_3^+$	53	53	53	53	53	53	53	53	53
$Pb(NO_3)_2$ (aq)	23	23	23	23	23	23	22	22	22
Zn^{2+}	72	72	72	72	72	72	72	72	72
$ZnNO_3^+$	27	27	27	27	27	27	26	26	26

Table S4

HAc extracts									
Chemical specie	M1 soil			L1 soil			VL1 soil		
%	Un-spiked	Tt1 level	Tt2 level	Un-spiked	Tt1 level	Tt2 level	Un-spiked	Tt1 level	Tt2 level
Cd^{2+}	nd	nd	18	nd	nd	27	nd	nd	58
$Cd\text{-}Acetate^+$	nd	nd	58	nd	nd	59	nd	nd	40
$Cd\text{-}(Acetate)_2$ (aq)	nd	nd	20	nd	nd	13	nd	nd	2
Cu^{2+}	6	7	7	10	11	12	40	40	39
$Cu\text{-}Acetate^+$	43	43	44	51	51	53	52	52	53
$Cu\text{-}(Acetate)_2$ (aq)	39	39	38	33	32	29	8	8	8
$Cu\text{-}(Acetate)_3^-$	12	12	11	7	6	5	nd	nd	nd
Pb^{2+}	2	2	3	4	4	5	nd	20	20
$Pb\text{-}Acetate^+$	37	38	39	45	46	48	nd	63	63
$Pb\text{-}(Acetate)_2$ (aq)	60	60	59	51	50	47	nd	17	17
Zn^{2+}	37	37	38	45	46	48	77	76	76
$Zn\text{-}Acetate^+$	56	56	55	51	50	48	23	23	23

Table S5

LMWOA extracts									
Chemical specie	M1 soil			L1 soil			VL1 soil		
%	Un-spiked	Tt1 level	Tt2 level	Un-spiked	Tt1 level	Tt2 level	Un-spiked	Tt1 level	Tt2 level
Cd^{2+}	nd	nd	19	nd	nd	32	nd	nd	8
Cd-Citrate^{-}	nd	nd	58	nd	nd	32	nd	nd	83
Cd-Malate (aq)	nd	nd	16	nd	nd	25	nd	nd	6
Cu-Citrate^{-}	nd	97	93	nd	97	92	nd	96	95
Pb^{2+}	1	1	5	1	1	11	1	1	2
Pb-Citrate^{-}	95	95	77	95	94	52	95	95	91
Pb-Malate (aq)	1	1	6	1	1	12	1	1	2
Pb-Lactate^{+}	1	1	4	1	1	9	1	1	1
Pb-Acetate^{+}	1	1	6	1	1	12	1	1	2
Zn^{2+}	nd	nd	3	nd	nd	7	nd	nd	1
Zn-Citrate^{-}	nd	96	93	nd	96	83	nd	96	97

LMWOA = low molecular weight organic acids (acetic, lactic, citric, malic and formic acids).

Table S6

NH_4Ac extracts									
Chemical specie	M1 soil			L1 soil			VL1 soil		
%	Un-spiked	Tt1 level	Tt2 level	Un-spiked	Tt1 level	Tt2 level	Un-spiked	Tt1 level	Tt2 level
Cd-$(Acetate)_3^{-}$	nd	63	62	nd	63	63	nd	63	63
Cd-$Acetate^{+}$	nd	9	9	nd	9	9	nd	9	9
Cd-$(Acetate)_2$ (aq)	nd	27	27	nd	27	27	nd	27	27
$CdNH_3^{2+}$	nd	1	1	nd	nd	nd	nd	nd	1
Cu-$(Acetate)_3^{-}$	nd	8	6	nd	7	16	nd	30	23
Cu-$(Acetate)_2$ (aq)	nd	2	1	nd	2	4	nd	7	5
$Cu(NH_3)_4^{+2}$	nd	43	46	nd	45	32	nd	20	26
$Cu(NH_3)_3^{2+}$	nd	40	39	nd	40	38	nd	31	35
$Cu(NH_3)_2^{2+}$	nd	7	6	nd	7	9	nd	10	9
Pb-$Acetate^{+}$	nd	7	7	7	7	7	nd	7	7
Pb-$(Acetate)_2$ (aq)	nd	93	93	93	93	93	nd	93	93
Zn-$Acetate^{+}$	nd	33	30	37	31	39	nd	42	41
Zn-$(Acetate)_2$ (aq)	nd	35	32	40	34	42	nd	46	44
$Zn(NH_3)_4^{2+}$	nd	10	13	5	12	4	nd	2	3
$Zn(NH_3)_3^{2+}$	nd	11	13	7	12	6	nd	3	4

Table S7

$CaCl_2$ extracts									
Chemical specie	M1 soil			L1 soil			VL1 soil		
%	Un-spiked	Tt1 level	Tt2 level	Un-spiked	Tt1 level	Tt2 level	Un-spiked	Tt1 level	Tt2 level
Cd^{2+}	nd	nd	nd	nd	nd	49	nd	nd	nd
$CdCl^{+}$	nd	nd	nd	nd	nd	48	nd	nd	nd
Cu^{2+}	nd	nd	nd	nd	58	76	nd	nd	nd
$CuOH^{+}$	nd	nd	nd	nd	37	21	nd	nd	nd
Pb^{2+}	38	43	58	46	52	62	46	46	55
$PbCl^{+}$	14	16	22	17	20	23	17	17	21
$PbOH^{+}$	47	40	19	36	27	14	36	36	23
Zn^{2+}	nd	92	96	nd	95	96	nd	nd	95

Table S8

$MgCl_2$ extracts									
Chemical specie	M1 soil			L1 soil			VL1 soil		
%	Un-spiked	Tt1 level	Tt2 level	Un-spiked	Tt1 level	Tt2 level	Un-spiked	Tt1 level	Tt2 level
$CdCl^{+}$	nd	26	26	nd	26	26	nd	26	26
$CdCl_2$ (aq)	nd	74	74	nd	74	74	nd	74	74
Cu^{2+}	26	28	31	28	31	33	31	32	33
$CuCl^{+}$	41	44	47	44	48	50	48	49	50
$CuCl_2$ (aq)	8	8	9	8	9	10	9	9	10
$CuOH^{+}$	23	18	12	18	10	7	11	10	7
$PbCl^{+}$	17	17	17	17	17	17	17	17	17
$PbCl_2$ (aq)	25	25	25	25	25	26	25	25	26
$PbCl_3^{-}$	35	36	36	36	36	36	36	36	36
$PbCl_4^{-2}$	21	21	21	21	21	21	21	21	21
Zn^{2+}	nd	10	10	nd	10	10	nd	10	10
$ZnCl^{+}$	nd	22	22	nd	22	22	nd	22	22
$ZnCl_2$ (aq)	nd	15	15	nd	15	15	nd	15	15
$ZnCl_4^{-2}$	nd	23	23	nd	23	23	nd	23	23
$ZnCl_3^{-}$	nd	29	29	nd	30	30	nd	30	30

Table S9

$NaNO_3$ extracts									
Chemical specie	M1 soil			L1 soil			VL1 soil		
%	Un-spiked	Tt1 level	Tt2 level	Un-spiked	Tt1 level	Tt2 level	Un-spiked	Tt1 level	Tt2 level
Cd^{2+}	nd	nd	91	nd	nd	91	nd	nd	91
$CdNO_3^+$	nd	nd	8	nd	nd	8	nd	nd	8
Cu^{2+}	33	42	63	47	51	75	54	49	64
$CuNO_3^+$	3	4	6	4	5	7	5	5	6
$CuOH^+$	51	43	27	42	33	15	36	40	27
Pb^{2+}	37	43	55	46	50	61	50	47	55
$PbNO_3^+$	16	19	24	20	22	27	22	20	24
$PbOH^+$	44	35	19	32	26	10	27	30	19
Zn^{2+}	nd	89	92	nd	91	92	nd	nd	92
$ZnNO_3^+$	nd	7	7	nd	7	7	nd	nd	7

Table S10

NH_4NO_3 extracts									
Chemical specie	M1 soil			L1 soil			VL1 soil		
%	Un-spiked	Tt1 level	Tt2 level	Un-spiked	Tt1 level	Tt2 level	Un-spiked	Tt1 level	Tt2 level
Cd^{2+}	nd	nd	17	nd	nd	22	nd	nd	18
$CdNH_3^{2+}$	nd	nd	35	nd	nd	31	nd	nd	34
$Cd(NH_3)_2^{2+}$	nd	nd	20	nd	nd	12	nd	nd	19
$CdNO_3^+$	nd	nd	21	nd	nd	28	nd	nd	22
$Cu(NH_3)_2^{2+}$	6	8	14	10	12	21	12	10	15
$Cu(NH_3)_3^{2+}$	41	44	50	46	49	52	49	47	50
$Cu(NH_3)_4^{2+}$	52	48	36	44	38	24	38	43	34
Pb^{2+}	8	8	8	8	8	9	8	8	9
$PbNO_3^+$	48	49	49	49	49	49	49	49	49
$Pb(NO_3)_2$ (aq)	41	41	41	41	41	41	41	41	41
Zn^{2+}	nd	7	16	nd	14	27	nd	10	18
$ZnNH_3^{2+}$	nd	9	15	nd	14	17	nd	12	16
$Zn(NH_3)_2^{2+}$	nd	16	17	nd	17	12	nd	17	16
$Zn(NH_3)_3^{2+}$	nd	32	22	nd	24	11	nd	29	20
$Zn(NH_3)_4^{2+}$	nd	29	13	nd	16	4	nd	23	11
$ZnNO_3^+$	nd	6	16	nd	14	26	nd	9	17

4. Estudio de los patrones de biodisponibilidad de metales en *Lactuca sativa* L.

Apartado 4.1

Significance of organic matter and carbonate contents on lettuce metal uptake in Mediterranean agricultural soils

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Manuscrito en preparación

Resumen

La gestión de los suelos agrícolas contaminados por metales pesados en el área mediterránea es compleja debido a la presencia de dos ligandos principales, carbonato y materia orgánica (MO), los cuales pueden actuar como sumidero o como fuente de contaminantes. Sin embargo, el papel que juegan en la biodisponibilidad de los metales aún no está bien definido. Se diseñó un experimento de bioensayo con lechuga (*Lactuca sativa* L.), empleando un conjunto de suelos agrícolas, que ofrecen un gradiente natural de carbonato y MO, muestreados en la misma región mediterránea. Los suelos fueron artificialmente contaminados, a dos niveles, con una mezcla de Cd, Cu, Pb y Zn, y se incubaron hasta seis meses, teniendo en cuenta los procesos de "aging" o tiempo de contacto, antes del cultivo. Los patrones de biodisponibilidad metálica en las hojas de lechuga se discutieron en función de la disponibilidad metálica en el suelo, mediante métodos complementarios de extracción química, y de las características del suelo, incluyendo la cantidad y la calidad de la MO.

Los resultados mostraron que la acumulación de los metales en las hojas de lechuga podrían ser parcialmente minimizada, dependiendo del metal, por distintas fracciones de suelo: carbonatada (principalmente para el Cd), mineral fina (para todos los metales), óxido de hierro y orgánica recalcitrante (para Cu y Pb). La fracción más lábil de la MO del suelo, sin embargo, mostró promover los procesos de absorción metálica, mostrando el rol dual ejercido por la MO en función de su naturaleza. Cualesquiera que sean las características del suelo, el proceso de biodisponibilidad de los metales en las partes comestibles de la lechuga fue simulado con éxito por el método de extracción química basado en una mezcla de ácidos orgánicos, destinado a simular el ambiente de la rizosfera, al contrario de los consistentes en extractantes de menor ($NaNO_3$) o mayor (DTPA) fuerza.

Abstract

Management of metal contaminated agricultural soils in the Mediterranean area is difficult due to the presence of two main ligands: calcareous and organic matter (OM) which can act as sink or source of pollutants. Yet, the role they both play in governing metal bioavailability is not well defined. A pot experiment with lettuce (*Lactuca sativa* L.) was designed with a set of agricultural soils offering natural gradients of carbonate and OM contents sampled in the same Mediterranean region. Soils were spiked at two levels with a mixture of Cd, Cu, Pb, and Zn and incubated up to six months for aging before cultivation. Metal bioavailability patterns in lettuce leaves and soil metal availability as seen by complementary methods of chemical extractions were then related to soil characteristics including the quantity and quality of OM.

Results showed that metal accumulation in the lettuce leaves could be partially minimized depending on the metal and the soil fractions: carbonate (mainly for Cd), fine minerals (for all metals), Fe-oxide and recalcitrant organic fractions (for Cu and Pb). The most labile fraction of the soil OM, however, showed to promote metal uptake processes assessing the dual role exerted by OM depending on its nature. Whatever the soil characteristics, the process of metal bioavailability for lettuce edible parts was successfully mimicked by the chemical extraction method with a mixture of organic acids aiming at simulating the rhizosphere ambient, contrarily to those using lower ($NaNO_3$) or stronger (DTPA) extractants.

Keywords

Calcareous soils, lettuce, metal bioavailability, natural organic matter, labile and recalcitrant organic matter

Introduction

Concentrations of "urban elements", including Cd, Cu, Pb, and Zn, have increased in recent decades in agricultural Mediterranean soils near urban or industrial areas (de Miguel *et al.*, 1997; Micó *et al.*, 2006; Semlali *et al.*, 2001). Polluting activities may impact the quality of agricultural soils and, in fine, may lead to phytotoxicity or heavy metal transfer to the human diet via metal uptake by crops (Kabata-Pendias and Pendias, 2001; Peris *et al.*, 2007).

Metal absorption by plants depends on a wide range of factors: sorption-desorption processes of metals in soils, metal speciation and the nature of chemical bounds with solid soil phases (Fuentes *et al.*, 2006), competition between metals (Zorrig *et al.*, 2010), reactions of metals in the rhizosphere (Wang *et al.*, 2002) and mechanisms of uptake and translocation by plants (Brun *et al.*, 2001). Most of these factors are related to the soil type and characteristics.

Calcareous soils of the Mediterranean area are expected to favour metal-immobilization, due to the presence of $CaCO_3$ compounds and to soil pH values (Buekers *et al.*, 2007), but also to climatic characteristics such as low rainfall and high evapotranspiration rates resulting in high metal concentrations in the first centimetres of the soil (Plassard *et al.*, 2000). Such a typical elemental cycling in Mediterranean soils with a significant risk of metal accumulation may lead to a possible remobilization either by plant root activity (Sayyad *et al.*, 2010) or by changes in the environment, as a drop in pH (Bolan *et al.*, 2003).

It is, however, difficult to predict such a metal remobilization which may be minimized in addition to carbonates by the action of other soil components like organic matter (OM). Indeed soil organic matter is frequently reported as a soil compound that can buffer metal remobilization while immobilizing metals through the formation of stable metal-humus complexes (Smith, 2009). But in the pH range of calcareous soils and depending on the nature of the OM, metal solubility may be enhanced by the formation of metal-organic complexes in the soil solution raising a potential risk of metal bioavailability (Kaschl *et al.*, 2002; Wong *et al.*, 2007). In the literature, many works aiming at elucidating the role of OM were carried out in a particular case where soils were amended with exogenous organic matter and yet few

studies exist on comparable soils with natural in situ varying proportions of indigenous organic matter. Furthermore, the nature of the OM is rarely taken into account to unravel its role of source and sink of trace metals played in the soils. In this context, it is necessary to assess the way that both content and composition of organic matter affect metal bioavailability in calcareous agricultural Mediterranean soils where often low content of organic matter could be decisive (Micó *et al.*, 2006).

Lettuce is one of the most consumed leafy vegetables of the Mediterranean area, being of particular concern in human dietary uptake. Furthermore legumes like lettuces and endives as well as other similar horticultural plants are often used as indicator crops to test the potential human dietary risk associated with ingesting food crop grown on metal polluted soils (Kabata-Pendias and Pendias, 2001; Zorrig *et al.*, 2012). Considering lettuce as target, the content of metals in the aboveground parts of the plant can be used as an indicator of metal bioavailability, while to quantify metal availability in soils different methods of chemical extraction have been assessed in the literature (Menzies *et al.*, 2007). All these methods aim at differentiating easily exchangeable and mobile metal fractions –extracted with neutral salts– from a pool of potentially mobile metal –extracted with complexing and acidic agents– (Gupta *et al.*, 1996). The results of these extractions used to mimic metal mobility or bioavailability in soils may vary depending on the chemistry of the soil solution especially in carbonate media (González *et al.*, 2007), and their complementary use may bring a gain in elucidation of metal availability.

The aim of the present work was to investigate the role that both the content and nature of soil organic matter play in metal bioavailability patterns, taken into account the calcareous nature of agricultural Mediterranean soils. For that, we sampled a series of agricultural soils offering natural gradients of indigenous carbonate and organic matter contents and used a bioassay experiment with lettuce (*Lactuca sativa* L.). A range of trace metal concentration was set by spiking soils with a mixture of Cd, Cu, Pb, and Zn and by allowing 6 months incubation aging before lettuce culture. Both metal bioavailability as seen by indicators of metal contents in leaves and toxicity patterns for lettuce, and soil metal availability as seen by complementary soil chemical extractions, were related to the characteristics of the soil

series samples to unravel the role of soil constituents, focusing on organic matter and carbonate.

Materials and Methods

Study area and soil sampling

The study area is located in Alcalá de Henares in the central part of the Iberian Peninsula (Madrid, Spain) at an altitude of 588 m. The average annual temperature is about 13 ºC with a low interannual variability, the average annual rainfall is 400 mm y^{-1} and the potential evaporation is about 760 mm y^{-1}. The site is typical of a Mediterranean pluviseasonal-oceanic bioclimate on an upper meso-Mediterranean low dry bioclimatic belt (Rivas-Martínez, 1987). Soil samples were collected from different experimental plots at "El Encín" Agricultural Research Station, located on medium level alluvial terraces from the Henares River on quaternary medium and coarse sandy textured sediments (IGME, 1990). Calcaric Fluvisols developed on such alluvial sediments (Moreno Merino, 1998), currently presenting Anthric characteristics (FAO, 2006) due to agricultural land use. The area makes part of a periurban axis, including agricultural, residential and industrial use in the Community of Madrid, exposed to growing atmospheric emissions from transportation and industrial activities (Steinitz *et al.*, 2011).

We selected ten agricultural soil samples differing in their organic matter (OM) and carbonate contents (named equivalent $CaCO_3$ –ECC) while some differences in their particle-size distribution may occur related to the alluvial character of the parent material. We verified that the OM and ECC contents were not related to each other, in order to avoid co-correlation like that observed in the case of Recatalá *et al.* (2010). Mica-illite minerals were predominant in the phyllosilicate group and calcite in the carbonate one while dolomite was detected only in samples with higher carbonate content (de Santiago-Martín *et al.*, in press). In each plot, bulk samples of about 40 kg were taken in the 0-30-cm surface horizon which was found homogenized due to annual ploughing. Soil samples were air-dried and passed through a 2-mm sieve.

Soil aging after contamination

To mimic a multi-element soil contamination, the selected soil samples were spiked with a mixture of Cd, Cu, Pb and Zn as nitrate salt in solution. Two different concentration levels were chosen as the limits proposed by the Economic European Commission legislation (Directive 86/278/EEC): Tt1 level corresponded to the maximum concentration values of heavy metal content in soils allowed for agriculture (3 mg kg^{-1} of Cd + 140 mg kg^{-1} of Cu + 300 mg kg^{-1} of Pb + 300 mg kg^{-1} of Zn) and Tt2 level corresponded to the semi-maximum concentrations permitted in sewage sludge (20 mg kg^{-1} of Cd + 875 mg kg^{-1} of Cu + 600 mg kg^{-1} of Pb + 2000 mg kg^{-1} of Zn) .

For each soil sample, three containers (40 cm wide x 59 cm long x 21 cm high) were set, 10 kg each, one for the un-spiked sample and the two other for the two different metal levels of spiking. These containers were left aging for a period of 6 months incubation at room temperature without cover or drainage in order to reduce effects of instantaneous availability of metals after spiking (Dahmani-Muller *et al.*, 2001; Jalali and Khanlari, 2008). During this period, samples were air-dried, mixed and rewetted with distilled water in cycles of about 2 weeks, in order to favour metal redistribution into the soil matrix (McLaughlin, 2001). After 6 months, aliquots were randomly taken out for bioassay experiments and for one-step chemical extractions of metals.

Bioassay experiments

Duplicates of un-spiked and metal-spiked soil samples (Tt1 and Tt2 levels) were placed in pots (1.5 kg per pot; 18 cm diameter x 15 cm high) and fertilized with 55.55 ml of Hoagland solution. The differences in nitrogen supply among treatments due to the application of metals as nitrate salts were adjusted by adding KNO_3. Three lettuces (*Lactuca sativa* L.) were planted per pot. Pots were stored randomly in a growth chamber under controlled conditions: 20 ºC air temperature and 12 h light per day. Pots were placed in saucers to prevent cross-contamination from drainage water and watered daily up to 60% of field capacity for 1 month.

Analytical methods

All chemicals were obtained from Merck (Germany) and Panreac (Spain). All glassware used were pre-washed with an aqueous solution of HNO_3 1:1000 for 24 h and rinsed with distilled water.

Soil physicochemical analyses

According to ISRIC-methods (2002), the following parameters were determined: soil pH in a 1:2.5 soil to water ratio; equivalent $CaCO_3$ –ECC– according to the acid neutralization method; total organic C by the Walkley-Black wet oxidation procedure; crystalline and amorphous Fe and Mn oxide contents by the dithionite-citrate extraction followed by an acid oxalate extraction; and particle-size distribution by the Robinson's pipette method. To characterize the nature of the organic carbon, we used the two-step acid hydrolysis procedure with H_2SO_4 to determine labile I and II and recalcitrant pools of organic C, respectively named LPI, LPII and RP (Rovira and Vallejo, 2000).

Major and trace element contents of the soil samples were determined after wet digestion with a mixture of nitro-perchloric-hydrofluoric acids under high-pressure conditions (SISS, 1985). Elements in the extracts were quantified by flame atomic absorption spectrometry (AAS) for Ca, Mg, Fe, Mn, Cd, Cu, Pb and Zn (Analytikjena NovAA 300) or by flame emission spectrometry (AES) for Na and K (Sherwood 410). Quantification limits in mg L^{-1} were: Ca = 1, Mg = 0.05, Fe = 0.5, Mn = 0.2, Cd = 0.2, Cu = 0.2, Pb = 0.5, Zn = 0.1, Na = 1 and K = 1.

One-step chemical extraction methods of metals

Three complementary methods were used according to the strength of the extracting solution used. The easily exchangeable metals expressed as $NaNO_3$-extractable metals were determined after Gupta and Aten (1993) using a solution of 0.1 M $NaNO_3$ in a 1:2.5 soil:solution ratio. The easily extractable metals expressed as low molecular weight organic acids (LMWOA)-extractable metals were determined after Feng *et al.* (2005) using a combined solution of acetic, lactic, citric, malic and formic acids with a molar ratio of 4:2:1:1:1 in a 1:10 soil:solution ratio. The less easily extractable metals expressed as DTPA-extractable metals were determined after

Lindsay and Norwell (1978) using a combined solution of 0.005 M DTPA + 0.01 M $CaCl_2$ + 0.01 M triethanolamine in a 1:2 soil:solution ratio. The supernatant of each extraction was centrifuged at 3500 rpm for 15 min and then filtered. Dilutions were made with the corresponding extracting solution. Cd, Cu, Pb, and Zn concentrations in the extracts were determined by AAS.

Plant analyses

The harvested leaves were abundantly washed with tap water, followed by distilled water, to remove soil particles before been oven dried at 80 ºC for 48 h, and weighed. Oven-dried leaves were ground to powder samples in an electric mill, and digested at 80 ºC with HNO_3:H_2SO_4:$HClO_4$ (5:1:1) at a solid:liquid ratio of 0.5:15 (Allen *et al.*, 1986). The digests were filtered and diluted to 25 ml with distilled water prior to analysis. Concentrations of Cd, Cu, Pb, Zn, Ca, Mg, Na, and K in the digests were determined by AAS or by AES. The mean value of the moisture content ranges of common lettuce plants was employed for converting the results of metal accumulation from dry weight to wet weight (Duckworth, 1966). The Tolerance Index (TI) was calculated as a percentage of leaf wet weight of plants grown in metal-spiked soils with respect to those grown in un-spiked soils (Gisbert *et al.*, 2006).

Statistical analyses

Significance of differences of the means (n = 10) of the biomass, the leaf contents of Cu, Pb and Zn, and the extractable Cu, Pb and Zn concentrations among the three levels (un-spiked and metal-spiked at Tt1 and at Tt2), was investigated by means of one-way ANOVA using a post-hoc test (Tukey). Significance of differences of the means (n = 10) of the TI and the extractable Cd concentrations between metal-spiked Tt1 and Tt2 levels was investigated by means of T-student test.

Pearson's correlation coefficients were calculated to relate the concentrations of metals in the leaves of romaine lettuce plants i) to each other, ii) to soil physicochemical parameters and iii) to extractable metals in the un-spiked and metal-spiked soil samples at Tt1 and Tt2 levels. These analyses were conducted using SPSS (Statistical Package for the Social Sciences) v.17 (SPSS, Inc.) software.

Multivariate analyses were carried out for the un-spiked and metal-spiked soil samples at Tt1 and Tt2 levels to evaluate the metal bioavailability patterns as a function of the soil physicochemical parameters (13 variables, pH and several constituents related to carbonate, organic, and oxide fractions and to particle-size distribution). The statistical procedure was based on Redundancy Analysis –RDA–. We developed a hybrid variant in which the axes 1 and 2 are canonical and the axes 3 and 4 are free (ter Braak, 1994). We performed a standardised RDA, based on a correlation matrix where response and explanatory variables were associated with different units and standardised (with mean 1 and variance 0). The leaves contents of Cd, Cu, Pb and Zn for the plants grown in the un-spiked and metal-spiked soil samples were used as response variables. Soil physicochemical parameters were used as explanatory variables and the Forward Selection method was used to select and rank them, according to their importance in explaining the response variables for each of the four ordinations, and validated by Monte Carlo permutation tests (ter Braak, 1994). Multivariate analyses were performed using CANOCO 4.5 and the biplots were drawn with Canodraw (ter Braak and Smilauer, 2002).

Results and Discussion

Characteristics of initial soil samples

Main characteristics of the soil samples are shown in Table 1. The texture of the soils was sandy-loam and sandy-clay loam, fine sand being the most frequent size fraction in all cases, representing about 50%. All soils showed pH values above 8 consistent with their carbonate nature with a gradient of carbonate fractions ranged from low (Equivalent $CaCO_3$ –ECC– contents about 9 g kg^{-1}) to moderate (ECC about 200 g kg^{-1}). The gradient in total organic carbon (TOC) contents ranged from 6 to 18 g kg^{-1} but with no relation between ECC and TOC. The nature of the organic matter, however, was found different between the samples, as shown by the ratio C/N and the recalcitrant pool (RP) varying from 48 to 75 % of the TOC.

The total metal contents in the selected soils were fairly small, being total Cu = 10.7 ± 2.5; total Pb = 25.9 ± 11.0 and total Zn = 60.4 ± 8.7 mg kg^{-1}. These contents were consistent with contents reported by other authors for low- or unpolluted

agricultural soils of the Mediterranean area (Jiménez Ballesta *et al.*, 2010; Micó *et al.*, 2006; Peris *et al.*, 2007). Pb exhibited the highest range of metal gradient compared to the other trace elements, while the total Cd contents were under our quantification limit and could not be quantified in the initial soil samples.

Table 1. Physicochemical parameters of the un-spiked soil samples.

Nº sample	pH	Carbonate fraction	Organic fraction					Oxide fraction		Particle-size distribution			Total content			
		ECC	TOC	LPI	LPII	RP	C/N	Cry-Fe	Am-Fe	Sand	Silt	Clay	Cd	Cu	Pb	Zn
		g kg^{-1}	g kg^{-1}	% of TOC				g kg^{-1}		g kg^{-1}			mg kg^{-1}			
H1	8.2	106	18.4	10	18	72	11	13.4	0.8	749	78	172	LQL	12.0	25.5	52.9
H2	8.1	125	17.8	10	24	66	10	11.7	0.5	615	215	170	LQL	10.0	23.8	63.3
H3	8.4	118	14.8	20	25	54	11	11.1	0.6	700	146	154	LQL	10.8	24.4	62.0
M1	8.1	32	12.2	13	15	71	11	11.4	0.7	504	168	328	LQL	10.1	24.0	62.1
M2	8.2	27	12.1	9	17	74	11	12.2	0.7	485	172	344	LQL	13.2	21.1	70.7
M3	8.1	148	11.8	40	12	48	12	8.5	0.2	683	124	193	LQL	12.1	55.8	62.7
M4	8.7	117	9.9	18	24	57	14	8	0.2	733	129	139	LQL	15.0	23.7	74.9
L1	8.2	9	8.2	25	23	52	8	7.2	0.4	805	70	124	LQL	7.0	25.4	45.2
L2	8.1	100	7.8	14	11	75	10	10.3	0.3	574	167	259	LQL	8.5	21.5	55.1
L3	8.4	190	5.9	17	14	69	8	8.1	0.2	714	126	161	LQL	8.0	14.0	54.7
Mean	8.3	97	11.9	18	18	64	11	10.2	0.5	656	140	204	-	10.7	25.9	60.4
SD	0.2	58	4.2	9	5	10	2	2.1	0.2	107	44	78	-	2.5	11.0	8.7
CV (%)	2.4	59	35.0	53	29	16	16	20.7	50.4	16	32	38	-	23.2	42.5	14.4

ECC = equivalent $CaCO_3$; TOC = total organic C; LPI = labile pool I; LPII = labile pool II; RP = recalcitrant pool; Cry-Fe = crystalline Fe-oxides; Am-Fe = amorphous Fe-oxides. The total Cd content was in all cases lower than the quantification limit (LQL ~0.2 mg L^{-1}).

Indicators of metal bioavailability and toxicity for lettuce cultured on metal-spiked soils after aging

Figure 1 gives the leaves biomass and the tolerance index obtained after the lettuce cultures. A significant decrease (up to 60%) in the leaf biomass production (dry weight) as well as a lower Tolerance Index (TI) was found only when lettuce were grown in the metal-spiked soil samples at the Tt2 level suggesting toxicity.

These results and can be coupled with the concentrations of major cations determined in the leaves (Fig. 2) as other indicators of toxicity if we hypothesize that mixture of metals added to the soil samples could interfere with the absorption of major cations (Capelo *et al.*, 2012). In our case, and depending on the major cation, such a disorder could be observed whatever the level, Tt1 or Tt2 (case of Ca), only in the case of Tt2 (case of K and Mg), or never (case of Na) (Fig. 2).

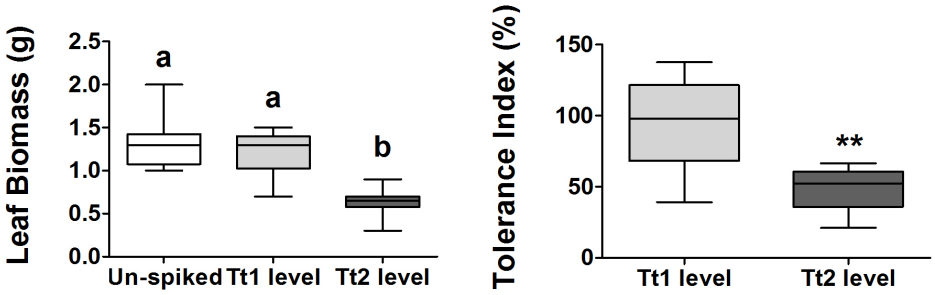

Fig 1. Leaf biomass (dry weight) of lettuce and Tolerance Index in un-spiked (□) and metal-spiked soil samples at Tt1 (■) and Tt2 levels (■). The boxplots show the lower quartile, the median and the upper quartile, with whiskers extending to the most extreme data point. Different letters indicate significant differences of leaf biomass between levels at $p < 0.05$ after one-way ANOVA. *, ** and *** indicate significant differences of Tolerance Index between levels at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, after T-student test.

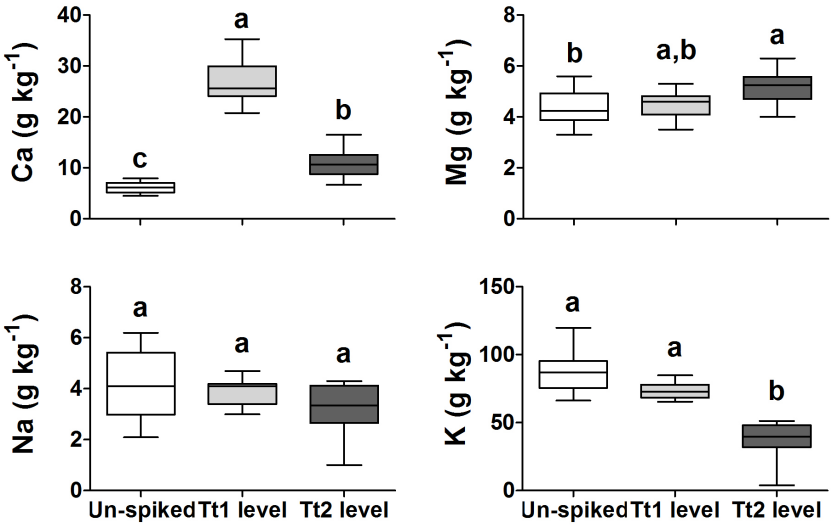

Fig 2. Concentration of major cations in leaves of lettuce plants grown in un-spiked (□) and metal-spiked soil samples at Tt1 (■) and Tt2 levels (■). The boxplots show the lower quartile, the median and the upper quartile, with whiskers extending to the most extreme data point. Different letters indicate significant differences between levels at $p < 0.05$ after one-way ANOVA.

Both the lower Tolerance Index, the decrease in biomass and the nutrient imbalance observed at the Tt2 level of metal-spiked soil highlighted phytotoxicity to lettuce plants, while only the difference in the Ca leaves contents compared to the reference revealed a significant effect on lettuce cultured on soils metal-spiked at the Tt1 level. The fact that an increase of Ca was observed at lower metal contamination had been already noted by Ramos *et al.* (2002). In particular, the increase in Ca content was attributed to a sign of a protective strategy taking place in lettuce to counteract the toxic effects of Cd, blocking Cd transport into roots as a result of the competition of Cd with Ca for Ca-transporters (Clemens, 2006; Zorrig *et al.*, 2010). The decrease of K in leaves observed at the higher Tt2 contamination level (Fig. 2) can be viewed as an alteration of key physiological processes of the plants, as osmotic regulation as suggested by Grattan and Grieve, (1999), and related in our case to the mixture of metals added.

Metal contents in the lettuce leaves are given in Fig. 3. These internal plant values revealed the metal bioavailability and were shown to increase with increasing metal concentration in soils (Fig. 3).

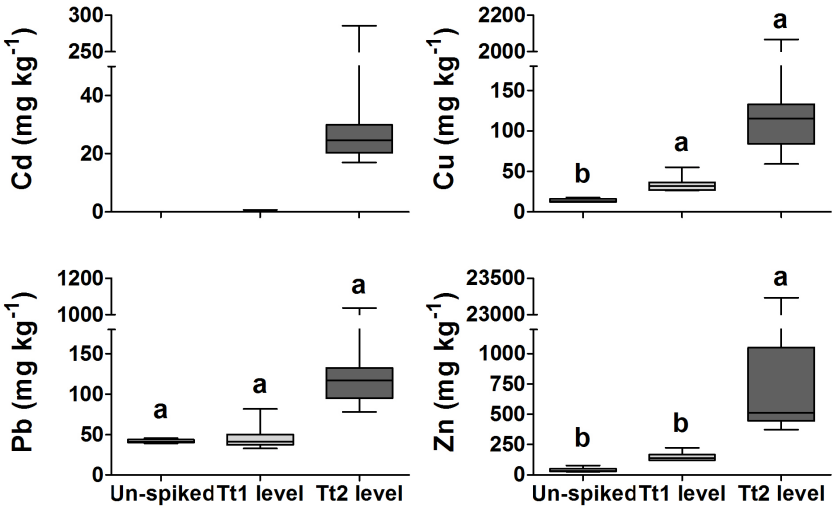

Fig 3. Concentration of metals in leaves of lettuce plants grown in un-spiked (□) and metal-spiked soil samples at Tt1 (■) and Tt2 levels (■). The boxplots show the lower quartile, the median and the upper quartile, with whiskers extending to the most extreme data point. Different letters indicate significant differences of Cu, Pb and Zn content in leaves between levels at $p < 0.05$ after one-way ANOVA. The amount of Cd in leaves was lower than the quantification limit in un-spiked soil samples and in almost all metal-spiked at the Tt1 level.

Compared to the behaviour of the major cations, a wider range of variation in the metal contents in leaves among the different soils was observed (extend of whiskers). This suggests that, together with the plant physiology, some soil constituents play a major role in the transfer of metals from soil to lettuce plants, and in particular the key constituents differentiating the soil samples. The metal contents detected in leaves (DM) were within the range considered excessive or toxic in leaf tissues, at Tt2 level for Cd and at both levels for Cu, Pb and Zn (Kabata-Pendias and Pendias, 2001). Table 2 gives the Pearson's correlation coefficients calculated to relate the concentrations of metals in the leaves of lettuce plants to each other. At Tt1 level, positive and significant correlations were established between Cu and Pb in the lettuce leaves that were lacking for Zn, suggesting metal competitive processes occurring in multi-component systems (Kalis *et al.*, 2006). At Tt2 level, however, all metals were correlated to each other, suggesting that at higher contamination levels plant regulation mechanisms may be overcome.

Table 2. Pearson's correlation coefficients calculated among metal accumulations in lettuce leaves to each other in un-spiked and metal-spiked soil samples at Tt1 and Tt2 levels.

Metal	Un-spiked				Tt1 level				Tt2 level			
	Cd	Cu	Pb	Zn	Cd	Cu	Pb	Zn	Cd	Cu	Pb	Zn
Cd	(a)	(a)	(a)	(a)	(a)	(a)	(a)	(a)	1	**0.997*** **	**0.996*** **	**0.999*** **
Cu		1	**0.887****	**0.913*****		1	**0.975*****	0.627		1	**0.999*****	**0.999*****
Pb			1	**0.829****			1	0.589			1	**0.997*****
Zn				1				1				1

*, ** and *** indicate statistical significance (bolded numbers) at the probability level $p < 0.05$, $p < 0.01$ and $p < 0.001$ (n = 10). (a) cannot be calculated because at least one variable is constant.

Relations between metal bioavailability and soil metal availability patterns

Extractable metal values with $NaNO_3$, LMWOA and DTPA extraction methods in the un-spiked and metal-spiked soil samples at Tt1 and Tt2 levels are shown in Fig. 4. In un-spiked soil samples, extractable metal values with $NaNO_3$ and LMWOA were found very low for Zn, and around 2 - 5 % of the total metal content for Cu and Pb. DTPA extractions gave a slightly higher % of extraction of 12.1 ± 3.5 for Cu, 15.1 ± 4.5 for Pb and only 3.8 ± 1.1 for Zn. For one given type of extraction,

extraction of metals increased with increasing soil metal content except in the case of Pb at Tt2 level for the DTPA extraction which was a surprising result. Apart of this result, these extracted values, however, suggest that metal availability increases when soil metal content increases.

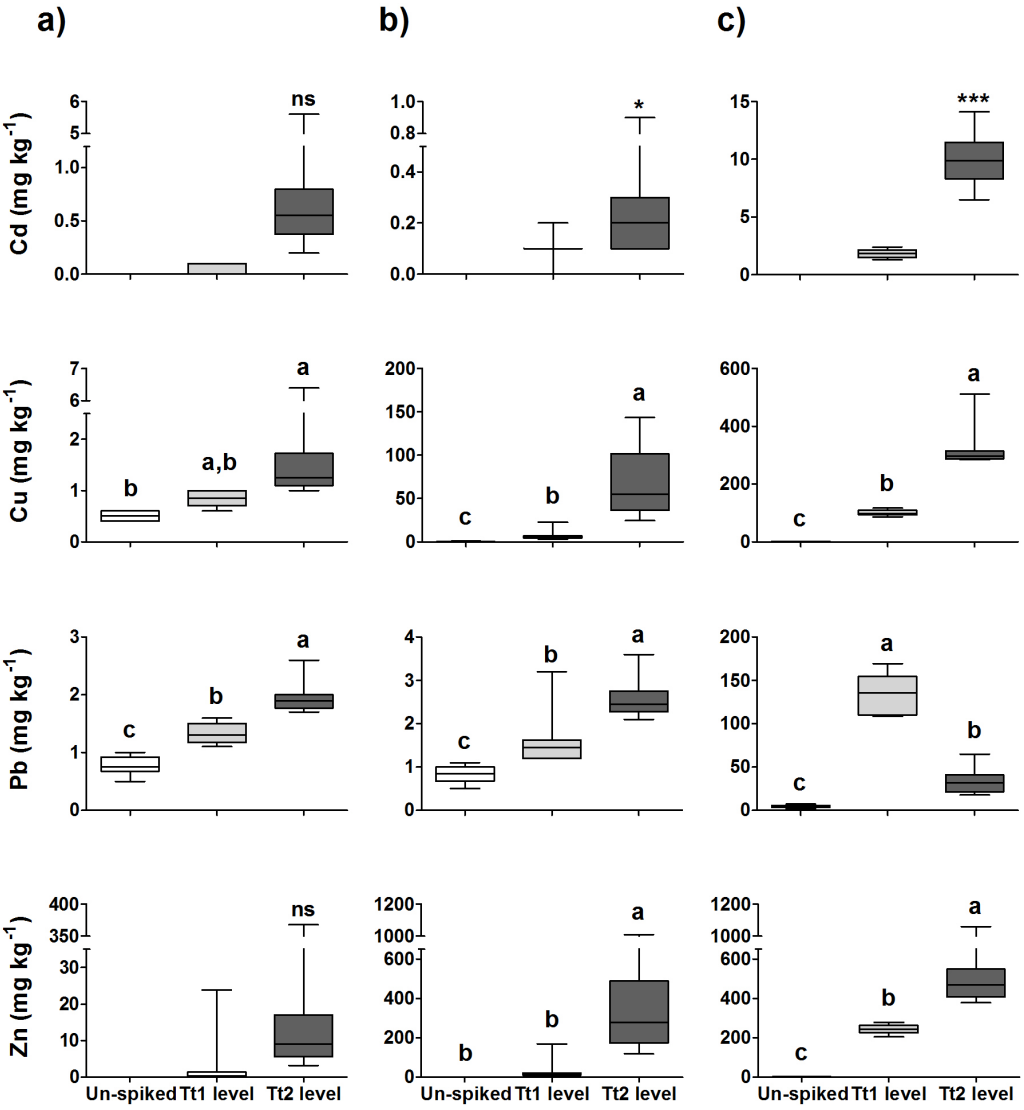

Fig 4. Extractable metal values with $NaNO_3$ (a), LMWOA (b) and DTPA (c) extraction methods in un-spiked (□) and metal-spiked soil samples at Tt1 (■) and Tt2 levels (■). The boxplots show the lower quartile, the median and the upper quartile, with whiskers extending to the most extreme data point. Different letters indicate significant differences of extractable Cu, Pb and Zn between levels at $p < 0.05$ after one-way ANOVA. *, ** and *** indicate significant differences of extractable Cd and $NaNO_3$-Zn between levels at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, and ns indicates no significant differences, after T-student test. Extractable Cd and $NaNO_3$-Zn was lower than the quantification limit in un-spiked soil samples.

For the un-spiked soil samples, our results pointed out that the extraction with organic ligands LMWOA gave rather similar results than the corresponding extracted values with $NaNO_3$. This suggests that for the un-spiked soil mechanisms underlying metal availability is more related to exchangeable metals, while it is necessary to introduce strong organic ligands like DTPA to change metal extractability. For the metal-spiked soils the extraction efficiency followed the strength of the extracting solution: DTPA > LMWOA > $NaNO_3$. Results suggest that metal availability increases when soil metal content increases, but whatever the extracting method used, the percentage of extractable metal was not proportional to the amount of metal added (data not shown). Only in the case of Cd and Zn higher percentages were always extracted in metal-spiked soil samples compared to un-spiked soils. Different patterns were detected for LMWOA-method, depending of the metal, and in the case of DTPA-extractions lower metal percentages were always removed at Tt2 level than at Tt1 level.

In order to relate bioavailability, expressed as the metal contents in leaves, to the soil metal availability, expressed as the metal extracted values, we calculated the Pearson's correlation coefficients as shown in Table 3.

Table 3. Pearson's correlation coefficients calculated between metal accumulations in lettuce leaves and extractable metal concentration values in un-spiked and metal-spiked soil samples at Tt1 and Tt2 levels.

Extraction method	Un-spiked				Tt1 level				Tt2 level			
	Cd	Cu	Pb	Zn	Cd	Cu	Pb	Zn	Cd	Cu	Pb	Zn
$NaNO_3$	(a)	0.606	**0.801****	(a)	(a)	-0.224	-0.049	**0.784****	**0.994*****	**0.983*****	**0.881****	**0.999*****
LMWOA	(a)	-0.024	**0.722***	-0.246	(a)	**0.880****	**0.787****	**0.711***	**0.960*****	0.615	**0.850****	**0.880****
DTPA	(a)	-0.413	-0.333	-0.406	(a)	0.397	0.206	0.317	-0.46	**0.982*****	-0.378	**0.961*****

*, ** and *** indicate statistical significance (bolded numbers) at the probability level $p < 0.05$, $p < 0.01$ and $p < 0.001$ (n = 10). (a) cannot be calculated because at least one variable is constant.

Different patterns were obtained as a function of the contamination level. The method of LMWOA seemed to mimic more accurately the metal bioavailability patterns in romaine leaves of plants grown in metal-spiked soil samples at Tt1 level. The LMWOA-method is commonly used to simulate the plant exudates and

microbial metabolites in the rhizosphere. Plant roots contribute to making metal ions more available to the uptake proteins as they not only acidify the rhizosphere through plasma membrane-localized proton pumps, but actively secrete low-molecular weight compounds that can function as metal chelators (Clemens, 2006). The formation of metal-LMWOA complexes is generally evoked as promoting metal transport into the plants and its subsequent translocation to aboveground parts (Pinto *et al.*, 2004; Zorrig *et al.*, 2010).

At Tt1 level the poor correlations obtained between Cu and Pb in plants and estimates of available metal with the easily exchangeable metals (expressed as $NaNO_3$-extractable metals) have been attributed to strong binding of these metals to soil organic matter and root surface by Black *et al.* (2011). However, lack of significant correlations was obtained with the less easily extractable metals expressed as DTPA-extractable metals at this Tt1 level suggesting that this ligand extracts metals from non-bioavailable fractions for lettuce plants.

A Tt2 level, the extractable metals with the $NaNO_3$-method showed highly significant positive correlations with the metal contents in lettuce leaves, although LMWOA and DTPA-methods also showed some positive and significant correlations. The overcome of the regulatory mechanisms of plants at this level might explain the significant correlations obtained with all the extractants used in almost all cases suggesting that many processes could be related to the metal bioavailability for lettuce depending on the degree of soil metal contamination. It should be interesting to establish whether generic thresholds in soil metal contamination could be given for using specific extraction methods mimicking metal bioavailability (Maderova *et al.*, 2011).

Role of main soil constituents in metal bioavailability for lettuce

The poor correlations between results of chemical extraction aiming at mimicking metal bioavailability and the metal leaf contents emphasis the key role of some soil constituents in the metal phytoavailability for lettuce. With the aim of clarifying the role played by soil constituents, Pearson's correlation analysis between metal bioavailability and soil physicochemical parameters was performed (Table 4).

Table 4. Pearson's correlation coefficients calculated between metal concentrations in lettuce leaves to some soil physicochemical parameters in un-spiked and metal-spiked soil samples at Tt1 and Tt2 levels.

Soil Parameter	Un-spiked				Tt1 level				Tt2 level			
	Cd	Cu	Pb	Zn	Cd	Cu	Pb	Zn	Cd	Cu	Pb	Zn
pH	(a)	0.198	0.170	0.038	(a)	-0.060	-0.158	-0.072	-0.155	-0.123	-0.132	-0.128
ECC	(a)	0.310	-0.019	0.357	(a)	-0.181	-0.228	-0.614	-0.569	-0.540	-0.551	-0.557
TOC	(a)	-0.545	-0.562	-0.507	(a)	-0.331	-0.443	-0.099	-0.297	-0.347	-0.368	-0.319
LPI	(a)	-0.249	-0.344	-0.169	(a)	**0.731***	**0.682***	0.200	0.258	0.275	0.268	0.267
LPII	(a)	-0.489	-0.505	-0.497	(a)	0.137	0.016	0.181	0.279	0.280	0.259	0.290
RP	(a)	0.488	0.585	0.419	(a)	**-0.748***	**-0.639***	-0.281	-0.385	-0.402	-0.385	-0.400
C/N	(a)	-0.224	-0.234	-0.516	(a)	-0.337	-0.481	-0.299	-0.522	-0.524	-0.523	-0.518
Cry-Fe	(a)	-0.154	-0.037	-0.157	(a)	**-0.669***	**-0.682***	-0.149	-0.463	-0.516	-0.523	-0.490
Am-Fe	(a)	-0.208	0.015	-0.152	(a)	-0.329	-0.314	0.215	-0.004	-0.057	-0.066	-0.029
CS	(a)	-0.186	-0.191	-0.243	(a)	**0.762***	**0.712***	0.609	**0.720***	**0.717***	**0.713***	**0.719***
Silt	(a)	0.065	0.112	0.068	(a)	**-0.719***	**-0.675***	-0.467	-0.550	-0.542	-0.532	-0.544
Clay	(a)	0.182	0.488	0.122	(a)	-0.535	-0.436	-0.012	-0.328	-0.347	-0.327	-0.338

*, ** and *** indicate statistical significance (bolded numbers) at the probability level $p < 0.05$, $p < 0.01$ and $p < 0.001$ (n = 10). (a) cannot be calculated because at least one variable is constant. ECC = equivalent $CaCO_3$; TOC = total organic C; LPI = labile pool I; LPII = labile pool II; RP = recalcitrant pool; Cry-Fe = crystalline Fe-oxides; Am-Fe = amorphous Fe-oxides; CS = coarse sand.

Surprisingly, no significant correlations were obtained for un-spiked soil samples. Instead, it was in metal-spiked soils where this statistical analysis allowed us to study these relations. Thus, significant positive correlations were obtained between metal concentrations in leaves and coarse sand (CS) content whatever the level, Tt1 or Tt2 (Cu and Pb), or only in the case of Tt2 (Cd and Zn), highlighting that coarse textures make the metal concentrations in the leaves easier.

Regarding the role played by carbonate and organic fractions, main objective of this study, it became evident that organic material plays a greater role. Thus, although negative correlations were obtained between equivalent $CaCO_3$ (ECC) content and Zn (whatever the level) and Cd, Cu and Pb (at Tt2 level), they were not-significant. Instead, negative correlations were obtained between Cu and Pb bioavailability and the proportion of recalcitrant organic fraction (RP) in metal-spiked soils, being significant at Tt1 level, and accordingly positives with the most labile organic fraction (LPI). Indeed the range of pH of the soils studied favoured organically bound metals mainly through the formation of insoluble complexes with humic acids favoured by interaction with clay minerals (Besnard *et al.*, 2001; Martinez *et al.*, 2010). Moreover, the presence of Fe could enhance the adsorption capacity of

mica-illite minerals (predominant phyllosilicates in these soils) thereby favouring the formation of organomineral associations, probably through humate-Fe oxide associations (Besnard *et al.*, 2001; Sipos *et al.*, 2008). This hypothesis could explain the negative correlations obtained between Cu and Pb bioavailability in metal-spiked soils and the content of crystalline Fe-oxides, being significant at Tt1 level. In this context, metals –Cu and Pb– strongly bound to the humic acids, would allow other metals –Cd and Zn– to bind more easily to transport sites in the cell membrane of roots which would favour its subsequent absorption and translocation (Kalis *et al.*, 2006). These competitive processes for binding to the root surface could explain the positive relationships found between Cu and Pb accumulation in leaves to each other and the lack of relations with Zn at Tt1 level, as stated above (Table 2). The "synergistic effect" between Cu and Pb could also be promoted by competitive processes for binding to organic material, resulting in an increase of Cu and Pb phytoavailability when they are in higher concentrations (Zheljazkov *et al.*, 2006).

Moreover, although the positive correlations with labile organic fraction could simply be an artefact, as opposed to the recalcitrant fraction, it may also be due to the fact that labile organic material can promote metal absorption and/or translocation to aboveground parts through chelation processes (Clemente *et al.*, 2006). So, it is possible that organic fraction plays a dual role as a function of its nature, recalcitrant fraction minimizing the transfer of Cu and Pb to lettuce plants and labile fraction enhancing metal uptake.

In order to further study the affectation of metal bioavailability by these soil constituents, a RDA analysis was made using as response variables the contents of Cd, Cu, Pb and Zn in leaves of lettuce plants grown in un-spiked and metal-spiked soil samples, and as explanatory variables the soil physicochemical parameters (pH and constituents related to carbonate, organic, and oxide fractions and to particle-size distribution). The resulting ordination diagrams are shown in Fig. 5. Eigenvalues for the two first canonical axes formed as a linear combination of the explanatory variables ranged from 0.72 to 0.86, meaning these axes explained up to 86% of the variance, whereas the two other free axes (not shown) represent 0.15 to 0.28. The Monte Carlo permutation test (n=499) indicated that the first canonical axis was statistically significant ($p<0.05$) in one of the three ordinations but when considering

both canonical axes they were together statistically significant ($p<0.05$) in the three analyses. The variables selected for each analysis by the forward selection method are those displayed in the ordination diagrams and were found to be statistically significant.

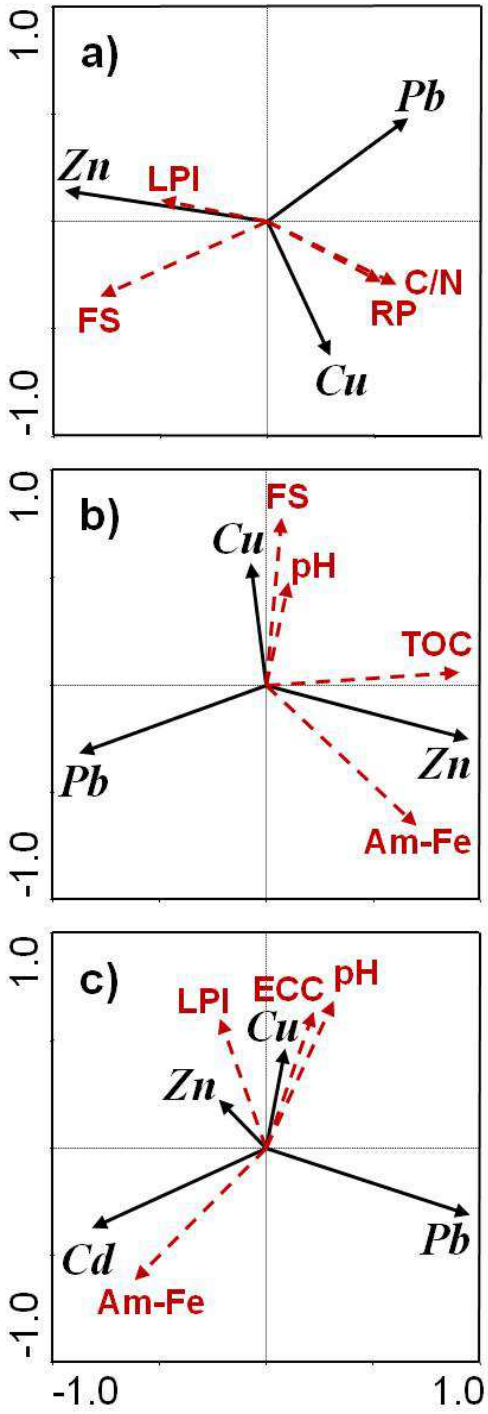

Fig 5. Ordination diagrams from correlation-based RDA with the first two axes for Cd, Cu, Pb and Zn contents in lettuce leaves (italic) as a function of some soil constituents (bold maroon scripts) in un-spiked (a) and metal-spiked soil samples at Tt1 (b) and at Tt2 (c) levels. Each diagram displays the response variables and the most statistically significant explanatory variables.

LPI = labile organic pool I; FS = fine sand; RP = recalcitrant organic pool; Am-Fe = amorphous Fe-oxides; TOC = total organic C; ECC = equivalent $CaCO_3$.

This statistical test, unlike the Pearson's one, allowed us to detect the significant explanatory variables in the un-spiked soils. As shown in the ordination diagram (Fig. 5, a), coarse textures favoured Zn bioavailability in the leaves, showing an opposite pattern for Cu and Pb which were positively related to humic organic material (RP and C/N). This result, although *a priori* surprising since one would expect that humic material exerts a strong metal retention, was attributed to the fact that this soil fraction contributes to increase the cation exchange capacity (CEC) which could favour Cu and Pb bioavailability un-spiked soils. Nevertheless, in metal-spiked soils at Tt1 level, the ordination diagram showed similar results to those obtained by Pearson's correlation analysis. Thus, Cu and Pb bioavailability was negatively affected by the content of TOC and amorphous Fe-oxides, as opposed to Zn. The opposite trend of Zn was attributed to competitive processes stated above.

At Tt2 level, the role played by the reactive oxide phases in minimizing Cu and Pb bioavailability was also highlighted. Regarding organic fraction, positive relations were observed between LPI and Cu and Zn bioavailability in leaves, while the role exerted by this fraction was less evident in the case of Cd and Pb. The positive relations between metal bioavailability and LPI, shown by Pearson's correlations (Table 4) and by RDA diagrams (Fig. 5), could be related to those obtained with metal availability estimated with LMWOA (Table 3). This extractant consists of an organic acid mixture, simulating the rhizosphere ambient, and LPI includes labile organic material, such as organic acids, carbohydrates and proteins, from both plant and microbial origin (Rovira and Vallejo, 2007) probably involved in metal-soil-root interactions and/or in metal translocation.

Finally, results showed that the role played by soil constituents in the process of bioavailability is metal dependant: organic material and in particular labile pools play a role in enhancing metal uptake by lettuce plants, as shown in the metal-spiked soils where the process of bioavailability could be successfully simulated by the LMWOA-method. On the other hand, carbonate fraction showed to play an important role in governing Cd bioavailability by lettuce as was also shown by Recatalá *et al.* (2010). Thus, Cd concentration in leaves of plants grown in metal-spiked soils at Tt2 level was negatively affected by the carbonate fraction and pH, as shown in the ordination diagram (Fig. 5). The contact time of our experiment (6

months) may favour the formation of stable bonds through diffusion and/or coprecipitation of Cd in carbonate fraction, decreasing more Cd availability in soils with higher carbonate content (Buekers *et al.*, 2007; Renella *et al.*, 2004). Nevertheless, it should be taken into account that the retention conferred by carbonates was not entirely effective in preventing Cd uptake since significant amounts of Cd were quantified in edible parts of lettuce plants, at least at the Tt2 level (Fig. 3).

Conclusions

The results of the present work show that the mixture of metals added was phytotoxic to lettuce plants, showing the vulnerability of these soils to pollution in periurban areas. This emphasizes the necessity of paying more attention to the metal environmental impact in calcareous soils and the possibility of entering metals into the human food chain.

Despite the fact that large amounts of metals were quantified in lettuce leaves, metal uptake was minimized by the combined action of carbonate, fine mineral, Fe-oxide and recalcitrant organic fractions, depending on the metal nature. Organic matter, however, was shown to play a dual role depending on its nature, recalcitrant fraction minimizing the transfer of Cu and Pb to lettuce plants and labile fraction enhancing metal uptake. This result was consistent with the suitability of the LMWOA-extraction method of metals from soils in simulating metal bioavailability.

Acknowledgements

This work was supported by the Universidad Complutense in Madrid (UCM) and the Community of Madrid (CAM) through Grant GR58/08, Research Team 950605 and Network CARESOIL, Ref. P2009/AMB-1648. Sampling was done at the "El Encín" Agricultural Research Station under the auspices of a research collaboration agreement signed between the Complutense University of Madrid and IMIDRA in1993.

References

Allen, S.E., Grimshaw, H.M., Rowland, A.P., 1986. Chemical analysis, in: Moore, P.D., Chapman, S.B. (Eds.), Methods in Plant Ecology. Blackwell Scientific Publication, Oxford, London, pp. 285-344.

Besnard, E., Chenu, C., Robert, M., 2001. Influence of organic amendments on copper distribution among particle-size and density fractions in Champagne vineyard soils. Environ. Pollut. 112, 329-337.

Black, A., McLaren, R.G., Reichman, S.M., Speir, T.W., Condron, L.M., 2011. Evaluation of soil metal bioavailability estimates using two plant species (*L. perenne* and *T. aestivum*) grown in a range of agricultural soils treated with biosolids and metal salts. Environ. Pollut. 159, 1523-1535.

Bolan, N.S., Adriano, D.C., Curtin, D., 2003. Soil Acidification and Liming Interactions with Nutrient and Heavy Metal Transformation and Bioavailability. Adv. Agron. 78, 215-272.

Brun, L.A., Maillet, J., Hinsinger, P., Pépin, M., 2001. Evaluation of copper availability to plants in copper-contaminated vineyard soils. Environ. Pollut. 111, 293-302.

Buekers, J., Van Laer, L., Amery, F., Van Buggenhout, S., Maes, A., Smolders, E., 2007. Role of soil constituents in fixation of soluble Zn, Cu, Ni and Cd added to soils. Eur. J. Soil Sci. 58, 1514-1524.

Capelo, A., Santos, C., Loureiro, S., Pedrosa, M.A., 2012. Phytotoxicity of lead on *Lactuca sativa*: effects on growth, mineral nutrition, photosynthetic activity and oxidant metabolism. Fresen. Environ. Bull. 21, 450-459.

Clemens, S., 2006. Toxic metal accumulation, responses to exposure and mechanisms of tolerance in plants. Biochim. 88, 1707-1719.

Clemente, R., Escolar, A., Bernal, M.P., 2006. Heavy metals fractionation and organic matter mineralization in contaminated calcareous soil amended with organic materials. Bioresour. Technol. 97, 1894-1901.

Dahmani-Muller, H., van Oort, F., Balabane, M., 2001. Metal extraction by *Arabidopsis halleri* grown on an unpolluted soil amended with various metal-bearing solids: a pot experiment. Environ. Pollut. 114, 77-84.

de Miguel, E., Llamas, J.F., Chacón, E., Berg, T., Larssen, S., Røyset, O., Vadset, M., 1997. Origin and patterns of distribution of trace elements in street dust: unleaded petrol and urban lead. Atmos. Environ. 31, 2733-2740.

de Santiago-Martín, A., Valverde-Asenjo, I., Quintana, J.R., González-Huecas, C., Lafuente, A.L., in press. Soil properties affecting metal extractability patterns in periurban calcareous agricultural soils in the Mediterranean area. Int. J. Environ. Res.

Directive 86/278/EEC of 12 June 1986 on the protection of the environment, and in particular of the soil, when sewage sludge is used in agriculture.

Duckworth, R.B., 1966. Fruit and Vegetables. Pergamon Press, Oxford, UK.

FAO, 2006. World reference base for soil resources. A framework for international classification, correlation and communication. FAO, Roma.

Feng, M., Shan, X., Zhang, S., Wen, B., 2005. A comparison of the rhizosphere-based method with DTPA, EDTA, $CaCl_2$, and $NaNO_3$ extraction methods for prediction of bioavailability of metals in soil to barley. Environ. Pollut. 137, 231-240.

Fuentes, A., Lloréns, M., Sáez, J., Aguilar, M.I., Pérez-Marín, A.B., Ortuño, J.F., Meseguer, V.F., 2006. Ecotoxicity, phytotoxicity and extractability of heavy metals from different stabilised sewage sludges. Environ. Pollut. 143, 355-360.

Gisbert, C., Clemente, R., Navarro-Aviñó, J., Baixauli, C., Ginér, A., Serrano, R., Walker, D.J., Bernal, M.P., 2006. Tolerance and accumulation of heavy metals by Brassicaceae species grown in contaminated soils from Mediterranean regions of Spain. Environ. Exp. Bot. 56, 19-27.

González, C., Quintana, J.R., Moreno, L., Vázquez, A., Lafuente, A.L., Romero, A., 2007. Applying multivariate methods to soil–solution interactions in carbonate media. Geoderma 137, 352-359.

Grattan, S.R., Grieve, C.M., 1999. Salinity-mineral nutrient relations in horticultural crops. Sci. Hort. 78, 127-157.

Gupta, S.K., Aten, C., 1993. Comparison and evaluation of extraction media and their suitability in a simple model to predict the biological relevance of heavy metal concentrations in contaminated soils. Int. J. Environ. Anal. Chem. 51, 25-46.

Gupta, S.K., Vollmer, M.K., Krebs, R., 1996. The importance of mobile, mobilisable and pseudo total heavy metal fractions in soil for three-level risk assessment and risk management. Sci. Total Environ. 178, 11-20.

IGME (Instituto Geológico y Minero de España), 1990. Mapa geológico de España nº 535. Escala 1:50.000 (Algete), Madrid.

ISRIC (International Soil Reference and Information Center), 2002. Procedures for Soil Analysis, 3th ed. International Soil Reference and Information Center, Wageningen.

Jalali, M., Khanlari, Z.V., 2008. Effect of aging process on the fractionation of heavy metals in some calcareous soils of Iran. Geoderma 143, 26-40.

Jiménez Ballesta, R., Conde Bueno, P., Martín Rubí, J.A., García Giménez, R., 2010. Pedo-geochemical baseline content levels and soil quality reference values of trace elements in soils from the Mediterranean (Castilla La Mancha, Spain). Eur. J. Geosci. 2(4), 441-454.

Kabata-Pendias, A., Pendias, H., 2001. Trace Elements in Soils and Plants, third ed. Boca Ratón, New York.

Kalis, E.J.J., Temminghoff, E.J.M., Weng, L., van Riemsdijk, W.H., 2006. Effects of humic acid and competing cations on metal uptake by *Lolium perenne*. Environ. Toxicol. Chem. 25(3), 702-711.

Kaschl, A., Römheld, V., Chen, Y., 2002. The influence of soluble organic matter from municipal solid waste compost on trace metal leaching in calcareous soils. Sci. Total Environ. 291, 45-57.

Lindsay, W.L., Norvell, W.A., 1978. Development of a DTPA soil test for zinc, iron, manganese and copper. Soil Sci. Soc. Am. J. 42, 421-428.

Maderova, L., Watson, M., Paton, G.I., 2011. Bioavailability and toxicity of copper in soils: Integrating chemical approaches with responses of microbial biosensors. Soil Biol. Biochem. 43(6), 1162-1168.

Martinez, R.E., Sharma, P., Kappler, A., 2010. Surface binding site analysis of Ca^{2+}-homoionized clay-humic acid complexes. J. Coll. Interface Sci. 352, 526-534.

McLaughlin, M.J., 2001. Aging of metals in soils changes bioavailability. Fact Sheet Environ. Risk Assess. 4, 1-6.

Menzies, N.W., Donn, M.J., Kopittke, P.M., 2007. Evaluation of extractants for estimation of the phytoavailable trace metals in soils. Environ. Pollut. 145, 121-130.

Micó, C., Recatalá, L., Peris, M., Sánchez, J., 2006. Assessing heavy metal sources in agricultural soils of an European Mediterranean area by multivariate analysis. Chemosphere 65, 863-872.

Moreno Merino, L., 1998. Estudio de la influencia del suelo sobre la composición de las aguas subterráneas a través de la solución del suelo. Modelo en Fluvisoles calcáricos. PhD thesis, Universidad Complutense de Madrid.

Peris, M., Micó, C., Recatalá, L., Sánchez, R., Sánchez, J., 2007. Heavy metal contents in horticultural crops of a representative area of the European Mediterranean region. Sci. Total Environ. 378, 42-48.

Pinto, A.P., Mota, A.M., de Varennes, A., Pinto, F.C., 2004. Influence of organic matter on the uptake of cadmium, zinc, copper and iron by sorghum plants. Sci. Total Environ. 326, 239-247.

Plassard, F., Winiarski, T., Petit-Ramel, M., 2000. Retention and distribution of three heavy metals in a carbonated soil: comparison between batch and unsaturated column studies. J. Contam. Hydrol. 42, 99-111.

Ramos, I., Esteban, E., Lucena, J.J., Gárate, A., 2002. Cadmium uptake and subcellular distribution in plants of *Lactuca* sp. Cd-Mn interaction. Plant Sci. 162, 761-767.

Recatalá, L., Sánchez, J., Arbelo, C., Sacristán, D., 2010. Testing the validity of a Cd soil quality standard in representative Mediterranean agricultural soils under an accumulator crop. Sci. Total Environ. 409, 9-18.

Renella, G., Adamo, P., Bianco, M.R., Landi, L., Violante, P., Nannipieri, P., 2004. Availability and speciation of cadmium added to a calcareous soil under various managements. Eur. J. Soil Sci. 55, 123-133.

Rivas-Martínez, S., 1987. Memoria del mapa de series de vegetación de España. 1:400.000. ICONA. Madrid.

Rovira, P., Vallejo, V.R., 2000. Evaluating thermal and acid hydrolysis methods as indicators of soil organic matter quality. Commun. Soil Sci. Plant Anal. 31, 81-100.

Rovira, P., Vallejo, V.R., 2007. Labile, recalcitrant, and inert organic matter in Mediterranean forest soils. Soil Biol. Biochem. 39, 202-215.

Sayyad, G., Afyuni, M., Mousavi, S.F., Abbaspour, K.C., Richards, B.K., Schulin, R., 2010. Transport of Cd, Cu, Pb and Zn in a calcareous soil under wheat and safflower cultivation - A column study. Geoderma 154, 311-320.

Semlali, R.M., van Oort, F., Denaix, L., Loubet, M., 2001. Estimating distributions of endogenous and exogenous Pb in soils, using Pb isotopic ratios. Environ. Sci. Technol. 35, 4180-4188.

Sipos, P., Németh, T., Kovács Kis, V., Mohai, I., 2008. Sorption of copper, zinc and lead on soil mineral phases. Chemosphere 73, 461-469.

SISS (Società Italiana della Scienza del Suolo), 1985. Metodi Normalizzati di Analisi del Suolo. Edagricole, Bologna.

Smith, S.R., 2009. A critical review of the bioavailability and impacts of heavy metals in municipal solid waste composts compared to sewage sludge. Environ. Int. 35, 142-156.

Steinitz, C., del Pozo, C., Vargas-Moreno, J. C., Canfield, T., 2011. Futuros alternativos para los paisajes dinámicos del Corredor del Henares. Fundación Paisaje.

Ter Braak, C.J.F.,1994. Canonical community ordination. Part I: basic theory and linear methods. Ecosci. 1, 127-140.

Ter Braak, C.J.F., Smilauer, P., 2002. CANOCO Reference manual and CanoDraw for Windows. User´s guide: Software for Canonical Community Ordination (v. 4.5), Microcomputer Power: Ithaca, NY, USA.

Wang, Z., Shan, X., Zhang, S., 2002. Comparison between fractionation and bioavailability of trace elements in rhizosphere and bulk soils. Chemosphere 46, 1163-1171.

Wong, J.W.C., Li, K.L., Zhou, L.X., Selvam, A., 2007. The sorption of Cd and Zn by different soils in the presence of dissolved organic matter from sludge. Geoderma 137, 310-317.

Zheljazkov, V.D., Craker, L.E., Xing, B., 2006. Effects of Cd, Pb, and Cu on growth and essential oil contents in dill, peppermint, and basil. Environ. Exp. Bot. 58, 9-16.

Zorrig, W., Rouached, A., Shahzad, Z., Abdelly, C., Davidian, J., Berthomieu, P., 2010. Identification of three relationships linking cadmium accumulation to cadmium tolerance and zinc and citrate accumulation in lettuce. J. Plant Physiol. 167, 1239-1247.

Zorrig, W., Shahza, Z., Abdelly, C., Berthomieu, P., 2012. Calcium enhances cadmium tolerance and decreases cadmium accumulation in lettuce (*Lactuca sativa*). Afr. J. Biotechnol. 11(34), 8441-8448.

Apartado 4.2

Carbonate, organic and clay fractions determine metal bioavailability in periurban calcareous agricultural soils in the Mediterranean area

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Manuscrito en preparación

Resumen

Un conjunto de suelos calcáreos periurbanos de uso agrícola del área mediterránea, con un gradiente natural en el contenido y la composición de la materia orgánica (MO), fueron contaminados artificialmente con una mezcla de Cd, Cu, Pb y Zn, en dos niveles –designados como Tt1 y Tt2–, dentro de los valores límites propuestos por la legislación Europea actual, e incubados durante 12 meses. Posteriormente, se llevaron a cabo experimentos de bioensayo con dos variedades de lechuga (*Lactuca sativa* L.), romana e iceberg. Los patrones de biodisponibilidad metálica fueron evaluados en función de los constituyentes del suelo y de los metales extraíbles.

La contaminación metálica condujo a una disminución en la biomasa vegetal (hasta un 50%) y al desequilibrio de los nutrientes. El análisis de redundancia mostró que las fracciones carbonatada, orgánica y mineral fina explicaban hasta el 85% de la varianza de los patrones de biodisponibilidad de los metales en las plantas de lechuga. Las fracciones carbonatada y mineral fina estuvieron negativamente relacionadas con la biodisponibilidad de Cd y Zn en las plantas, respectivamente. La MO explicó la biodisponibilidad de Cu y Pb, aunque los patrones fueron diferentes entre los diferentes tejidos de la planta, en función de la composición orgánica. La fracción orgánica lábil limitó la absorción metálica por las raíces (en el nivel Tt2 para el Pb y en ambos niveles para el Cu) pero favoreció la translocación de Cu y Pb (en ambos niveles). Se obtuvieron correlaciones significativas entre la concentración de los metales en la hojas y los metales extraídos, tanto con sales neutras como con agentes complejantes. La alta tasa de translocación metálica, junto con la considerable influencia de las propiedades del suelo, puede indicar que los valores límite de metales pesados en suelos, propuestos por la actual legislación europea, deberían ser revisados en el caso de sistemas de multi-componentes, y/o establecerse de acuerdo con las características de los suelos.

Abstract

A set of periurban calcareous agricultural Mediterranean soils, with a natural gradient in the content and composition of organic matter (OM), was spiked with a mixture of Cd, Cu, Pb and Zn at two levels –designated Tt1 and Tt2– within the limit values proposed by current European legislation, and incubated for ≤12 months. We conducted bioassay experiments with romaine and iceberg varieties of lettuce (*Lactuca sativa* L.). Metal bioavailability patterns were evaluated as a function of soil constituents and extractable metals.

Metal contamination led to a decrease in plant biomass (up to 50%) and nutrient imbalance. The redundancy analysis showed that the carbonate, OM and fine mineral fractions explain up to 85% of the variance in metal bioavailability patterns in lettuce plants. Carbonate and fine mineral fractions were negatively related to Cd and Zn bioavailability in plants, respectively. OM explained the Cu and Pb bioavailability, although patterns were different between plant tissues as a function of organic composition. The labile fraction limited metal absorption by roots at the Tt2 level for Pb, and at both levels for Cu, but favoured Cu and Pb translocation at both levels. Significant correlations were obtained between metals in leaves and extractable metals with both neutral salts and complexing agents. The high rate of metal translocation, in conjunction with the considerable influence of soil properties, may indicate that the limiting values of metals in soils proposed by the current European legislation should be revised in the case of multi-component systems, and/or be established according to the soil characteristics.

Keywords

Bioavailability, calcareous soils, heavy metals, lettuce, organic matter

Introduction

A substantial proportion of Spain's periurban agricultural areas is threatened by heavy metal contamination caused by the increased urban and industrial expansion in recent years, a situation which is widespread throughout most of the European Mediterranean region (Maas *et al.*, 2010). The increase in metal contents in agricultural soils is a matter of concern due to the possible damage to human health through crop intake (Kabata-Pendias and Pendias, 2001; Peris *et al.*, 2007). This scenario is typical of the southeast of the region of Madrid (central Spain), where calcaric Fluvisols are particularly important due to their high agricultural and economic value.

Mobility patterns in soils differ for each metal, and are strongly influenced by soil parameters such as soil pH, organic matter (OM) content, clay mineralogy, and the concentration and combination of metals present in the soil (Adriano, 2001). In recent works we have conducted research into metal extractability patterns in a set of metal-spiked calcaric Fluvisols in the Madrid region where we reported that metal mobility decreased with contact time due to adsorption and aging processes, and was not only governed by the carbonate fraction but also to a great extent by the organic and clay fractions (de Santiago-Martín *et al.*, in press). However, these processes may be reversed by changes in soil physicochemical properties and/or by the rhizosphere environment, leading to a release of (bio)available metals (Fernández *et al.*, 2005; Liao *et al.*, 2006).

Despite the fact that ~ 45% of European soils have a low OM content, principally in southern Europe (European Commission, 2006), the organic fraction could be a key factor governing these metal remobilisation processes. Organic matter can immobilize metals through the formation of stable metal-humus complexes, but can also enhance their availability to plants through an increase in the cation exchange capacity in soils by providing metal chelates and enhancing the solubility of metals in the soil solution (Clemente *et al.*, 2006; Zeng *et al.*, 2011). This dual role is a function of OM composition, metal characteristics and/or conditions of metal deficiency or excess in plant uptake (Cattani *et al.*, 2006; Inaba and Takenaka, 2005). Nevertheless, the dual role exerted by the organic fraction has not been satisfactorily explained. In calcareous agricultural soils –typically poor in OM content– the carbonate fraction

could be expected to mask the role of OM. However, as reported above, we have observed that metal availability patterns are not explained by the carbonate fraction alone, but rather by the combined action of other soil fractions such as organic matter (de Santiago-Martín *et al.*, in press). Although several authors have demonstrated the role exerted by the organic fraction in metal (bio)availability in organic amended calcareous soils (Clemente *et al.*, 2007; Kaschl *et al.*, 2002), there is scarce information on soils with a natural gradient in OM content.

In order to explore this subject in greater depth, this work focuses on the interactions between metal ions and crop plants in periurban calcareous agricultural soils in the Mediterranean area with a natural gradient in the content and composition of OM. We conducted bioassay experiments with two varieties of lettuce (*Lactuca sativa* L.), romaine and iceberg. Metal bioavailability patterns were evaluated as a function of soil constituents and extractable metals with several methods of different strengths.

Materials and Methods

Study area, soil characteristics and sampling

The soil samples come from different plots in an Agricultural Research Station (Alcalá de Henares, Madrid, Spain) located in the periurban axis that combines agricultural activity and the main residential and industrial uses in this region, at an altitude of 588 m, on quaternary sediments (IGME, 1990). Soils classified as calcaric Fluvisol (Moreno Merino, 1998) present Anthric characteristics today (FAO, 2006). The average annual temperature is 13ºC, average annual rainfall is 401 mm $year^{-1}$ and potential evaporation is about 760 mm $year^{-1}$.

A set of ten soil samples was selected with a differing OM content –high (H1, H2 and H3 soils), moderate (M1, M2, M3 and M4) and low (L1, L2 and L3)– and differing carbonate content and textural class. Some of the properties of the soil samples are shown in Table 1.

Table 1. Physicochemical and mineralogical parameters of un-spiked soil samples.

Soil sample	pH	Carbonate fraction	Organic fraction				Oxide fraction		Particle-size distribution			CEC	Total content			< 2 mm	
		ECC	TOC	LPI	LPII	RP	cryFe	amFe	CS	FS	Silt	Clay		Cu	Pb	Zn	Calcite	Dolomite
		g kg^{-1}	g kg^{-1}	% of TOC			g kg^{-1}		g kg^{-1}				cmolc kg^{-1}	mg kg^{-1}				
H1	8.2	106	18	10	18	72	13.4	0.8	159	590	78	172	6.9	12.0	25.5	52.9	++	tr
H2	8.1	125	18	10	24	66	11.7	0.5	23	592	215	170	10.6	10.0	23.8	63.3	++	tr
H3	8.4	118	15	20	25	54	11.1	0.6	95	605	146	154	8.9	10.8	24.4	62.0	+	tr
M1	8.1	32	12	13	15	71	11.4	0.7	45	459	168	328	19.1	10.1	24.0	62.1	tr	nd
M2	8.2	27	12	9	17	74	12.2	0.7	99	386	172	344	20.4	13.2	21.1	70.7	tr	tr
M3	8.1	148	12	40	12	48	8.5	0.2	114	569	124	193	8.7	12.1	55.8	62.7	+++	+
M4	8.7	117	10	18	24	57	8.0	0.2	166	567	129	139	5.6	15.0	23.7	74.9	+	tr
L1	8.2	9	8	25	23	52	7.2	0.4	245	560	70	124	10.0	7.0	25.4	45.2	nd	nd
L2	8.1	100	8	14	11	75	10.3	0.3	112	462	167	259	13.3	8.5	21.5	55.1	++	nd
L3	8.4	190	6	17	14	69	8.1	0.2	111	603	126	161	7.1	8.0	14.0	54.7	+++	++

ECC = equivalent $CaCO_3$; TOC = total organic C; LPI = labile pool I; PLII = labile pool II; RP = recalcitrant pool; cryFe = crystalline Fe oxides; amFe = amorphous Fe oxides; CS = coarse sand; FS = fine sand; CEC = cation exchange capacity. The total Cd content was in all cases lower than the quantification limit (<0.2 mg L^{-1}).

Number of +´s is proportional to abundance: (++++) most abundant, (+) least abundant, (tr) trace, and (nd) not detected.

Total metal content values were similar to those obtained by other authors for agricultural soils in the Mediterranean area (Jiménez Ballesta et al., 2010; Micó *et al.*, 2006; Peris *et al.*, 2007), and in no case exceeded the levels set by the European Union (Directive 86/278/EEC). Sampling was done at randomly selected points. To avoid potential bias, 30-40 kg were taken from each sampling point (0-30 cm) and homogenized. Soil samples were air-dried and passed through a 2 mm sieve.

Experimental design

The environmental impact from metal accumulation in periurban agricultural areas was simulated by the addition of a multi-elemental salt solution of heavy metals to the soil samples. Cadmium, Cu, Pb, and Zn were selected owing to their different speciation, mobility and bioavailability in soils. Three containers (40 cm wide x 59 cm long x 21 cm high) of 10 kg each were used for each sample: one un-spiked sample with the addition of distilled water; and the other two spiked at two different concentration levels using nitrate salts of heavy metals in aqueous solution: low level (Tt1) (3 mg kg^{-1} of Cd + 140 mg kg^{-1} of Cu + 300 mg kg^{-1} of Pb + 300 mg kg^{-1} of Zn) or high level (Tt2) (20 mg kg^{-1} of Cd + 875 mg kg^{-1} of Cu + 600 mg kg^{-1} of Pb + 2000 mg kg^{-1} of Zn), within the limits proposed by current European legislation (Directive 86/278/EEC).

Each soil sample and the corresponding solution was mixed and left to equilibrate for a period of 12 months at room temperature without cover or drainage. During this equilibration period, the soils were air-dried, mixed and rewetted with distilled water in cycles of about two weeks, in order to favour the metal redistribution processes within the soil matrix. These processes play a key role in determining metal (bio)availability in soils (McLaughlin, 2001). At the end of the equilibration period (12 months), duplicates were randomly removed from each un-spiked and metal-spiked soil sample in order to extract the metals by means of one-step extraction methods, and for the bioassay experiments.

Different extracting solutions were used to assess mobile and potentially mobile fractions of metals (Gupta *et al.*, 1996). The mobile fraction was estimated with 0.01M $CaCl_2$, 1M $MgCl_2$, 0.1M $NaNO_3$, and 1M NH_4NO_3-methods, and the

potentially mobile fraction was estimated with 5mM DTPA, 0.05M EDTA, 0.5M HNO_3, 0.11M HAc, 10mM LMWOA and 1M NH_4Ac-methods.

We selected two varieties of *Lactuca sativa* L. (romaine and iceberg) for the bioassay experiments. For each variety, un-spiked and metal-spiked soil sample duplicates were placed in pots (1.5 kg per pot; 18 cm diameter x 15 cm high) and fertilized with 55.55 ml of Hoagland solution. The differences in nitrogen supply between the levels due to the application of metals as nitrate salts were adjusted by adding KNO_3. Three lettuces were planted per pot. Plants were grown in a growth chamber under controlled conditions: air temperature 20ºC and 12 h light per day. Pots were placed in saucers to prevent cross-contamination from drainage water, and watered daily up to 60% of field capacity for one month.

Analytical methods

All chemicals were obtained from Merck (Germany) and Panreac (Spain). All glassware used was pre-washed with an aqueous solution of HNO_3 1:1000 for 24 h and rinsed with distilled water.

Soil physicochemical and mineralogical analyses

According to ISRIC methods (2002), the following parameters were determined: soil pH in a 1:2.5 soil to water ratio; equivalent $CaCO_3$ –ECC– according to the acid neutralization method; total organic C –TOC– by the Walkley-Black wet oxidation procedure; particle-size distribution by Robinson's pipette method; cation exchange capacity –CEC– by the ammonium acetate method; crystalline and amorphous Fe and Mn oxide contents by dithionite-citrate extraction; in addition to acid oxalate extraction. We used the two-step acid hydrolysis procedure with H_2SO_4 to determine the labile (I and II) and recalcitrant pools of organic matter –LPI, LPII and RP, respectively– (Rovira and Vallejo, 2000). Total Cd, Cu, Pb, and Zn contents of the soil samples were determined after wet digestion with a mixture of nitro-perchloric-hydrofluoric acids under high-pressure conditions (SISS, 1985).

The concentration of Fe, Mn, Cd, Cu, Pb, and Zn in the corresponding extracts was quantified by flame atomic absorption spectroscopy –AAS– (Analytikjena NovAA 300). All samples were extracted and analysed in duplicate.

Mineral composition of soil samples was examined by X-ray diffraction (XRD) using an EQ 0434520 31 02 (X'Pert MPD) diffractometer. Soil samples were examined on randomly-oriented powders. Abundance of soil minerals in fine earth fraction was assessed semi-quantitatively (Bish, 1994).

Chemical extractions of metals

To determine extractable metals we used the following procedures: 0.01 mol L^{-1} $CaCl_2$ (Van Ranst *et al.*, 1999); 1 mol L^{-1} $MgCl_2$ (Tessier *et al.*, 1979); 0.1 mol L^{-1} $NaNO_3$ (Gupta and Aten, 1993); 1 mol L^{-1} NH_4NO_3 (DIN 1995; Legislation Germany); 0.005 mol L^{-1} diethylene triamine pentaacetic acid (DTPA) in 0.01 mol L^{-1} $CaCl_2$ solution and 0.01 mol L^{-1} triethanolamine (TEA) (Lindsay and Norwell, 1978); 0.05 mol L^{-1} ethylene diamine tetra-acetic acid (EDTA) (Quevauviller *et al.*, 1996); 0.5 mol L^{-1} HNO_3 (Van Ranst *et al.*, 1999); 0.11 mol L^{-1} CH_3COOH (HAc) (Rauret *et al.*, 1999); 0.01 mol L^{-1} low molecular weight organic acids (LMWOA) solution consisting of acetic, lactic, citric, malic and formic acids with a molar ratio of 4:2:1:1:1 (Feng *et al.*, 2005), and 1 mol L^{-1} CH_3COONH_4 solution (NH_4Ac) buffered by HAc at pH 7 (Van Ranst *et al.*, 1999). The supernatant of each extraction was centrifuged at 3500 rpm for 15 min and then filtered. Dilutions were made with the corresponding extraction solution. The concentration of Cd, Cu, Pb and Zn in the extracts was quantified by AAS.

Plant analyses

The harvested leaves and roots were washed in abundant tap water followed by distilled water to remove soil particles. Leaf and root samples were then oven dried at 80ºC for 48 h and subsequently weighed. Oven-dried samples were ground to a powder in an electric mill. The powdered samples were digested at 80ºC with HNO_3:H_2SO_4:$HClO_4$ (5:1:1) at a solid:liquid ratio of 0.5:15 (Allen *et al.*, 1986). The digests were filtered and diluted to 25 ml with distilled water prior to analysis. Cadmium, Cu, Pb, Zn, Ca, Mg, Na, and K concentration in the digests was quantified by AAS or by flame emission spectrometry.

The mean value of the moisture content ranges of common lettuce plants was used to convert the results of metal accumulation from dry weight to wet weight

(Duckworth, 1966). The Tolerance Index (TI) was calculated as a percentage of leaf and root wet weight of plants grown in metal-spiked soils compared to those grown in un-spiked soils (Gisbert *et al.*, 2006). The metal translocation factors (TF) were calculated, as the metal content in the leaves divided by the metal content in the roots, for romaine and iceberg lettuce varieties (Abdu *et al.*, 2011).

Data analyses

The significance of the differences in the means (n = 10) of the values for biomass, leaf and root contents of Cu, Pb and Zn and TF values among the three levels (un-spiked and metal-spiked at Tt1 and at Tt2) were investigated by means of one-way ANOVA using a post-hoc test (Tukey). The significance of differences in the means (n = 10) of the values for TI and leaf and root content of Cd and TF values, between metal-spiked Tt1 and Tt2 levels, were investigated by means of a Student's T-test.

Correlation coefficients were calculated for metal-spiked soil samples at Tt1 and Tt2 levels to relate the amount of metals in plant leaves and roots to: a) each other, b) major cations in plants, c) soil physicochemical parameters, d) extractable metals (Pearson's correlation test) and e) mineralogical composition (Spearman's correlation test). Since mineralogical properties are semiquantitative variables, we used a nonparametric correlation test, the Spearman's test. These analyses were conducted using SPSS (Statistical Package for the Social Sciences) v.17 (SPSS, Inc.) software.

Multivariate analysis was carried out in metal-spiked soils at Tt1 and Tt2 levels to investigate the metal bioavailability patterns in lettuce leaves and roots as a function of the soil physicochemical and mineralogical parameters. The statistical procedure was based on Redundancy Analysis (RDA) (ter Braak, 1994). In these analyses the canonical axes obtained in the ordination are a linear combination of the explanatory variables. The content of Cd, Cu, Pb and Zn in romaine and iceberg lettuce leaves and roots were used as response variables. The explanatory variables initially used in the eight ordinations were the most representative for characterising organic, carbonate and oxide fractions, as well as particle-size distribution. We developed a hybrid variant in which axes 1 and 2 are canonical and axes 3 and 4 are

free. The relationship between the eigenvalues of these two types of axes can indicate the relationship between the response and the explanatory matrices.

Additionally, the Forward Selection method was used to select and rank the explanatory variables according to their importance in explaining the metal accumulation patterns in leaves and roots for each of the eight ordinations (ter Braak, 1994). This selection of variables was validated through Monte Carlo permutation tests. We performed a standardised RDA, based on a correlation matrix. Response and explanatory variables, which were associated with different units, were standardised (with mean 1 and variance 0). Correlation biplots were used, focusing on variables rather than samples. The ordination diagram obtained provides a clear view of the correlation between the metals accumulated in leaves and roots and the selected (best-ranked) explanatory variables. Multivariate analyses were performed using CANOCO 4.5, and the biplots were drawn with Canodraw (ter Braak and Smilauer, 2002).

Results and Discussion

Metal bioavailability and toxicity for lettuce leaves and roots

In general, leaf and root biomass decreased by up to 50% with increasing metal contamination, and was more pronounced in the iceberg variety at the Tt2 level (Fig. 1), highlighting the phytotoxicity caused by the metal mixture added. For the same contamination level, the variability in the soils of the Tolerance Index –TI– values calculated (60-86% at Tt1 level and 46-64% at Tt2 level) showed that this phytotoxicity is highly affected by soil constituents and properties.

It was also observed that the nutrient balance in leaves and roots was altered in plants grown in metal-spiked soils (Fig. 2). The leaf and root contents of Ca, Mg and Na showed an increase –not significant in the case of Ca– in romaine and iceberg lettuce leaves and roots with increasing metal concentration in soils, although values for the root-to-leaf Na and Mg translocation factor (TF) decreased.

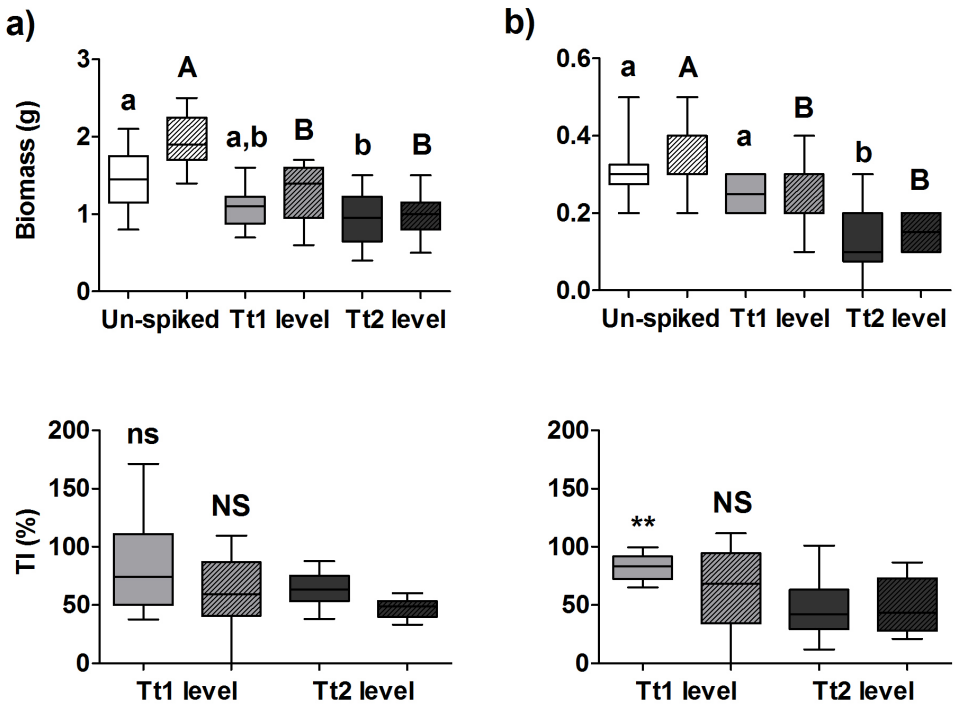

Fig 1. Leaf biomass (dry weight) and Tolerance Index –TI– of leaves (a) and roots (b) of romaine (□, ▨, ■) and iceberg (▨, ▨, ■) varieties of lettuce plants grown in un-spiked (□, ▨) and metal-spiked soil samples at Tt1 (▨, ▨) and Tt2 levels (■, ■). The boxplots show the lower quartile, the median and the upper quartile, with whiskers extending to the most extreme data point. Different letters, in lowercase for romaine and uppercase for iceberg varieties, indicate significant differences of leaf and root biomass between levels at $p < 0.05$ after one-way ANOVA. *, ** and *** indicate significant differences of TI between levels at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, after T-student test.

In contrast, the leaf and root content of K and K-TF values significantly decreased at the Tt2 level, and significant negative correlations were obtained between all metals and K contents in romaine leaves at this same level (Table 2). Increased Ca levels resulting from a protective strategy to resist the toxic effects of metals such as Cd (Zorrig *et al.*, 2010) may produce a decrease in K uptake (Grattan and Grieve, 1999). These results indicate that the mixture of metals used induced a nutrient imbalance that could affect key physiological processes in plants such as osmotic regulation. Moreover, the biomass reduction observed may be the consequence of metal interaction with essential elements, which can modulate the resultant metal toxicity (Wu *et al.*, 2007).

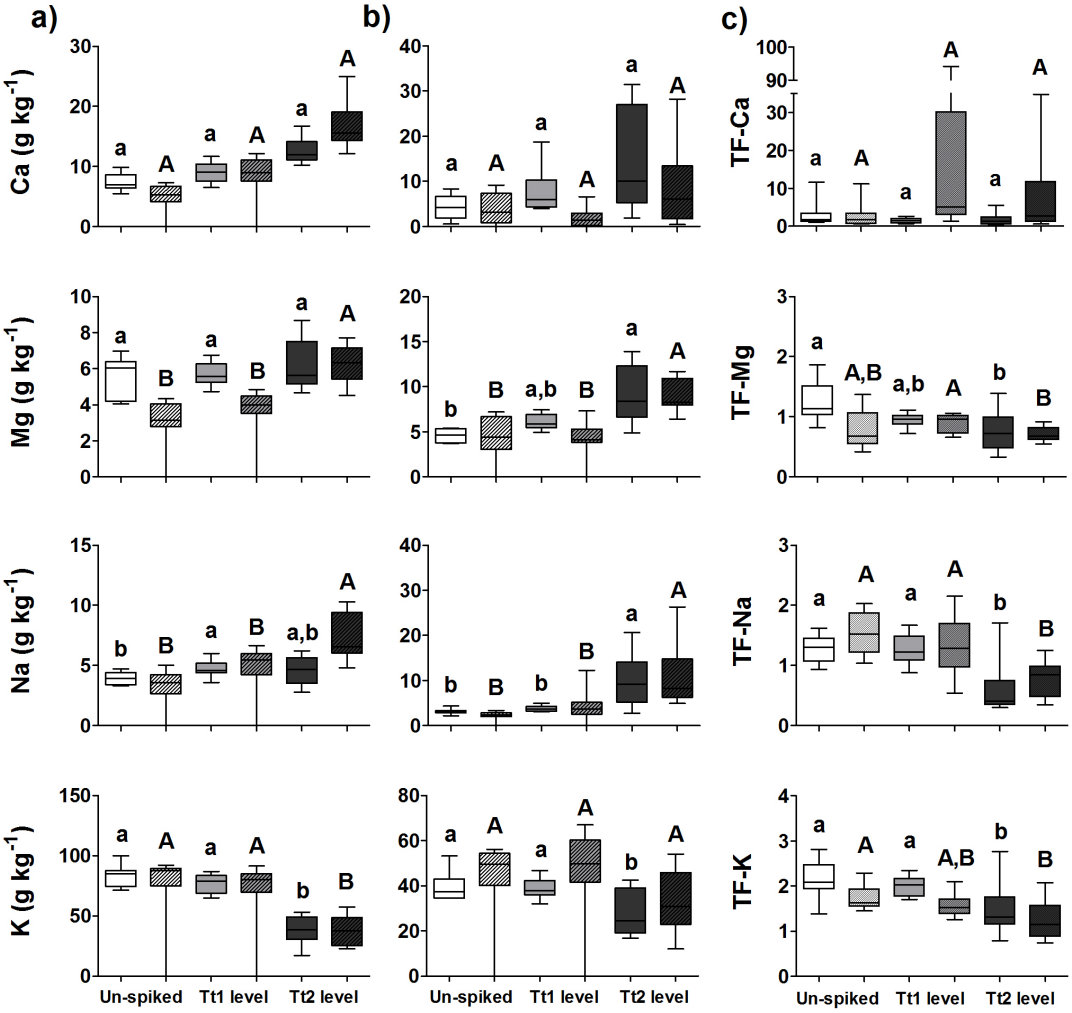

Fig 2. The contents of Ca, Mg, Na, and K in leaves (a) and roots (b) and translocation factor values (c), TF, of romaine (□, ▒, ■) and iceberg (▨, ▨, ▨) varieties of lettuce plants grown in un-spiked (□ , ▨) and metal-spiked soil samples at Tt1 (▒, ▨) and Tt2 levels (■, ▨). The boxplots show the lower quartile, the median and the upper quartile, with whiskers extending to the most extreme data point. Different letters, in lowercase for romaine and uppercase for iceberg varieties, indicate significant differences among levels at $p < 0.05$ after one-way ANOVA.

Table 2. Pearson's correlation coefficients calculated between the amount of metals in leaves and roots of lettuce plants to each other and to K in plants in metal-spiked soil samples at Tt1 and Tt2 levels.

		Tt1 level								Tt2 level							
		Cd		Cu		Pb		Zn		Cd		Cu		Pb		Zn	
		Romaine	Iceberg	Romaine	Iceberg	Romaine	Iceberg	Romaine	Iceberg	Romaine	Iceberg	Romaine	Iceberg	Romaine	Iceberg	Romaine	Iceberg
Content in leaves	Cd	1	1							1	1						
	Cu	0.320	-0.015	1	1					**0.971*** **	**0.944*** **	1	1				
	Pb	-0.065	0.090	**0.743***	**0.881****	1	1			**0.961*** **	**0.734***	**0.997*** **	**0.893*** **	1	1		
	Zn	**0.656***	**0.939*** **	**0.813****	0.122	0.528	0.270	1	1	**0.985*** **	**0.979*** **	**0.966*** **	**0.804****	**0.978*** **	**0.975*** **	1	1
	K	**-0.673***	0.005	0.483	-0.539	-0.065	**-0.704***	-0.099	0.015	**-0.669***	-0.399	**-0.776****	-0.561	**-0.758***	-0.434	**-0.663***	-0.436
Content in roots	Cd	1	1							1	1						
	Cu	0.407	0.412	1	1					**0.783****	0.501	1	1				
	Pb	**0.643***	**0.804****	0.593	0.045	1	1			**0.855****	**0.732***	**0.976*** **	0.248	1	1		
	Zn	0.613	0.588	**0.659***	**0.859****	**0.717***	0.098	1	1	**0.657***	**0.902*** **	0.409	**0.826****	0.298	0.312	1	1
	K	-0.376	-0.235	-0.158	-0.112	0.309	0.237	0.002	-0.001	-0.414	-0.050	-0.143	-0.056	-0.021	0.329	-0.184	-0.038

*, ** and *** indicate statistical significance (bolded numbers) at the probability level $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively (n = 10). (a) cannot be calculated because at least one variable is constant.

The contents of Cd, Cu, Pb and Zn were generally higher in roots than in leaves of both lettuces, although dissimilar patterns were observed between the varieties (Fig. 3). It is worth noting the different contents of Cd between the two varieties –higher in romaine lettuce– which was attributed to diverse genotypic strategies based on Cd sequestration (Wu *et al.*, 2007).

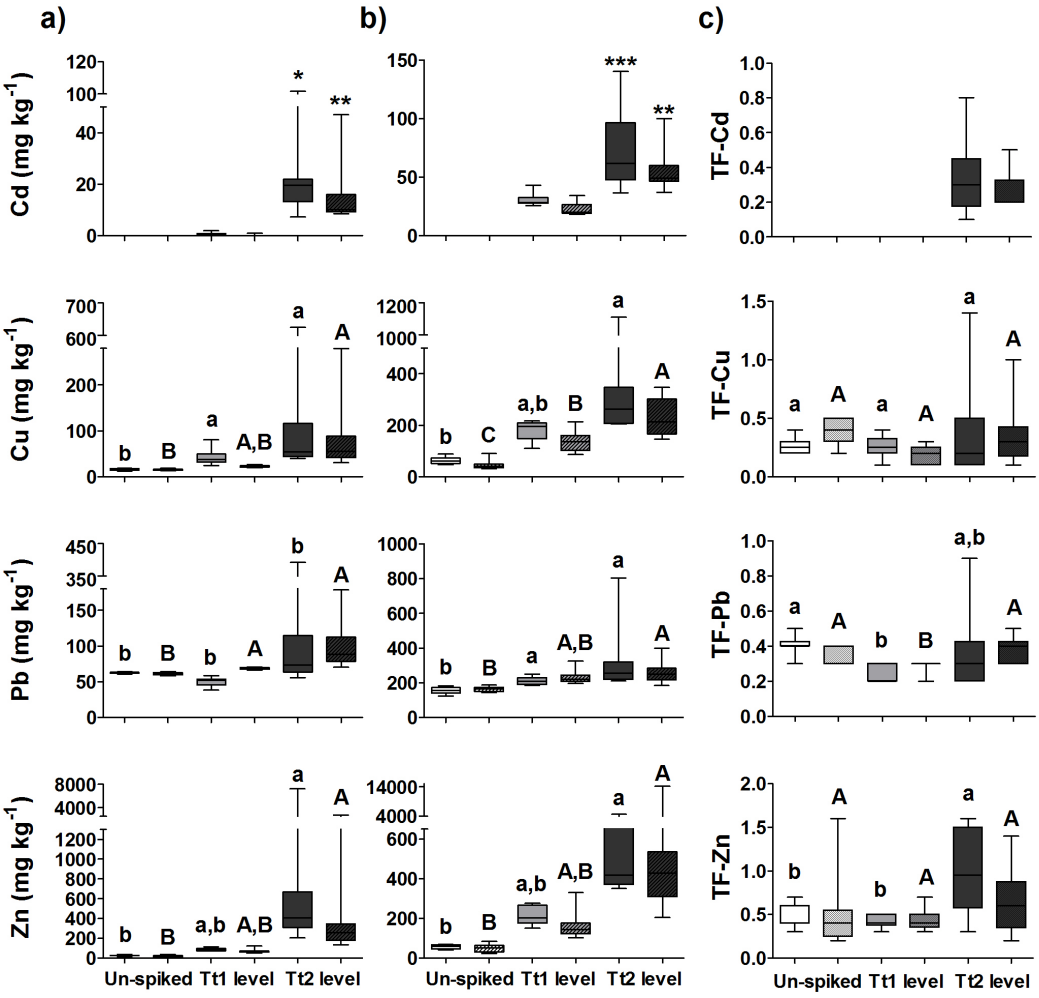

Fig 3. The contents of Cd, Cu, Pb, and Zn in leaves (a) and roots (b) and translocation factor values (c), TF, of romaine (□, ▒, ■) and iceberg (▨, ▨, ■) varieties of lettuce plants grown in un-spiked (□ , ▨) and metal-spiked soil samples at Tt1 (▒, ▨) and Tt2 levels (■, ■). The boxplots show the lower quartile, the median and the upper quartile, with whiskers extending to the most extreme data point. Different letters, in lowercase for romaine and uppercase for iceberg varieties, indicate significant differences among levels for Cu, Pb and Zn at $p < 0.05$ after one-way ANOVA. *, ** and *** indicate significant differences between levels for Cd at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, after T-student test. The amount of Cd in leaves was lower than the quantification limit in un-spiked soil samples.

As expected, metal content in lettuce plants rose with increasing metal concentration in soils. Metal contents in plants was Zn>Cu≥Pb>Cd, with the highest transfer percentage value for Cd, predominantly in the romaine variety. Pearson's correlation analysis showed that Cd and Zn contents in leaves were correlated with Cd and Zn contents in roots, respectively (data not shown), while no correlations were found in the case of Cu and Pb. This suggested that internal transport of Cd and Zn was probably accomplished by diffusion, while in the case of Cu and Pb there may be a significant restriction on the internal transport of metals from the roots towards the aboveground parts (Dahmani-Muller *et al.*, 2001), or a strong adsorption onto the root cell wall components due to the high ambient pH (Chaignon *et al.*, 2003). Nevertheless, as regards TF values, we observed that there was a high metal translocation to edible parts, with the highest values for Zn-TF found mainly at the Tt2 level (Chakravarty and Srivastava, 1997). This high metal translocation meant that the limit values considered excessive or toxic to leaf tissues were, in general, exceeded (Kabata-Pendias and Pendias, 2001). Moreover, Cd (at Tt2 level) and Pb (whatever the level) contents in lettuce leaves expressed on a wet weight basis (data not shown) reached values that exceeded the European Union limits for leafy vegetables (466/2001/EEC).

On the other hand, Pearson's correlation analysis showed positive and significant correlations between the contents of Cd and Zn, and between Cu and Pb in romaine and iceberg leaves at the Tt1 level (Table 2). In a multicomponent system there is competitive binding of the metal ions to the root surface, and when strongly binding metals such as Cu and Pb are bound to humic acid, other metals –Cd and Zn– can bind more easily to transport sites in the cell membrane of roots, and subsequently be absorbed and translocated (Kalis *et al.*, 2006). Similarly, there may be an interaction –including a synergism mechanism– between Cd and Zn (Podar and Ramsey, 2005; Zorrig *et al.*, 2012). Nevertheless, as regards metal contents in roots, positive and significant correlations were observed between Cd and Pb and between Cu and Zn-MTF values at the Tt1 level. In this work, apoplastic Cu and Pb –bound to the root cell walls– was not removed, and root concentration could be overestimated (Cattani *et al.*, 2006). In fact, a high metal content adsorbed by the roots does not necessarily imply a high metal uptake, since metals such as Pb could

present greater adsorption concentrations either through precipitation in roots in the form of lead phosphate or carbonate, or due to a lack of appropriate Pb carriers (Kalis *et al.*, 2006). At the Tt2 level, the higher metal concentration of the mixture could overcome the plant regulation mechanisms, as shown by the positive and significant correlations obtained among all metals in leaves and roots.

Metal bioavailability patterns in lettuce roots as a function of soil parameters

Eigenvalues for the two first canonical axes, formed as a linear combination of the explanatory variables, ranged from 0.63 to 0.85 (Table 3). The Monte Carlo permutation test (n=499) indicated that the first canonical axis was statistically significant ($p<0.05$) in six of the eight ordinations.

Table 3. Eigenvalues obtained for the canonical and the free axes in the eight RDA analyses performed and significance of the first and all canonical axes based of Monte Carlo permutations test.

Plant tissue	Level	Lettuce variety	Axes eigenvalues				P-value (Monte Carlo)	
			Canonical axes		Free axes		First canonical	All canonical
			1	Sum 1+2	3	Sum 3+4		
Roots	Tt1	Romaine	0.41	0.63	0.28	0.36	0.226	0.048
		Iceberg	0.51	0.79	0.16	0.20	0.072	0.020
	Tt2	Romaine	0.61	0.83	0.12	0.16	0.004	0.002
		Iceberg	0.54	0.85	0.09	0.14	0.030	0.002
Leaves	Tt1	Romaine	0.68	0.79	0.15	0.20	0.038	0.020
		Iceberg	0.66	0.80	0.15	0.20	0.012	0.004
	Tt2	Romaine	0.54	0.66	0.19	0.29	0.012	0.010
		Iceberg	0.63	0.80	0.16	0.19	0.032	0.020

Nevertheless, when both canonical axes were considered together they were statistically significant ($p<0.05$) in the eight analyses. The two free axes (3 and 4), representing the variance not explained by the canonical axes, presented eigenvalues from 0.14 to 0.36, suggesting that the canonical axes can successfully explain the metal bioavailability patterns in roots (Table 3 and Fig. 4).

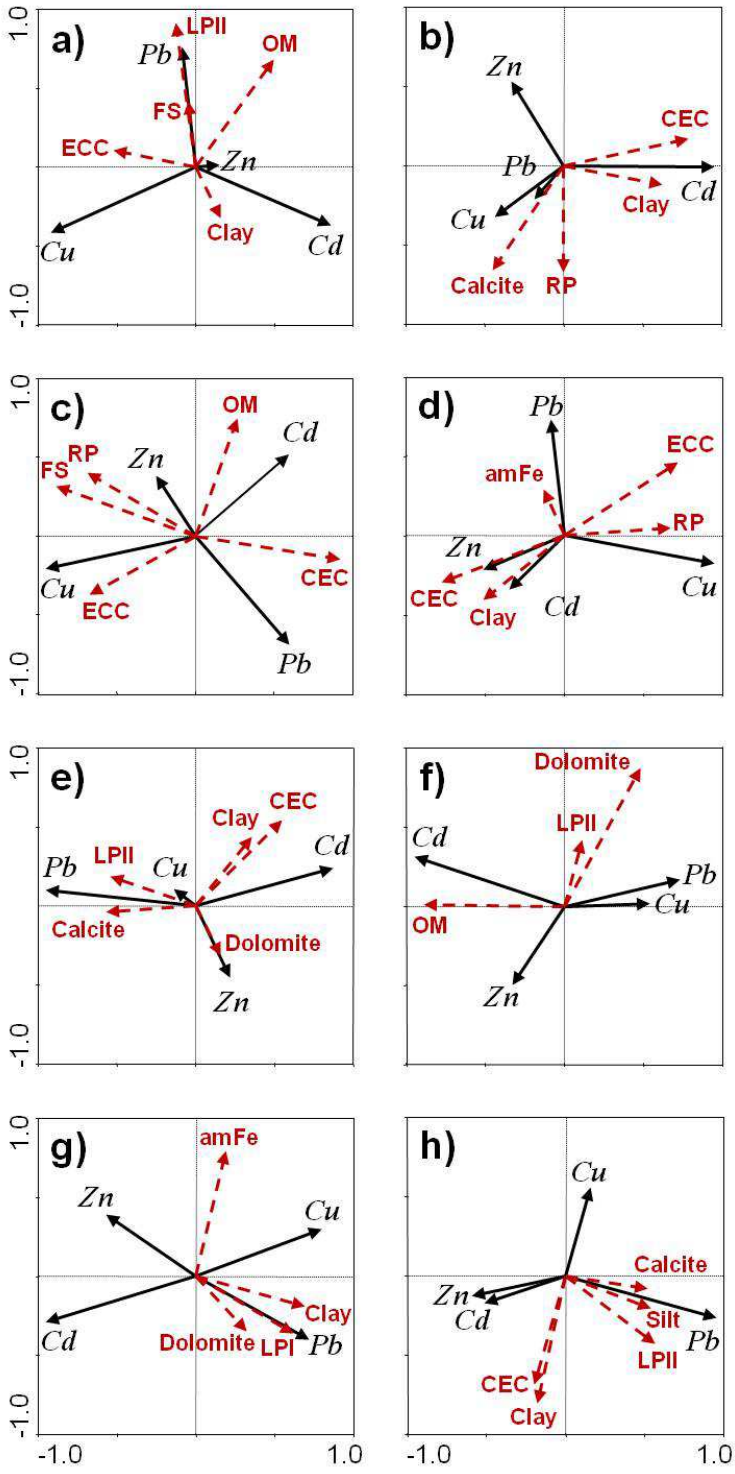

Fig 4. Ordination diagrams from correlation-based RDA with the first two axes for Cd, Cu, Pb and Zn contents (italic) in lettuce romaine roots (a and b), iceberg roots (c and d), romaine leaves (e and f) and iceberg roots (g and h) as a function of some soil constituents (bold maroon scripts) in metal-spiked soils at Tt1 (a, c, e and g) and at Tt2 levels (b, d, f and h). Each diagram displays the response variables and the most statistically significant explanatory variables. LPI = labile pool I; LPII = labile pool II; RP = recalcitrant pool; OM = organic matter; ECC = equivalent $CaCO_3$; FS = fine sand; CEC = cation exchange capacity; am-Fe = amorphous Fe-oxides.

The explanatory variables selected for each analysis were those with the greatest contributions to the regression model (considering the conditional effects once the other variables have been incorporated in the model) chosen by the forward selection method. These variables are shown in the ordination diagrams, and were found to be statistically significant.

Although the two plant varieties showed contrasting abilities to accumulate metals, the soil properties and constituents explaining the metal bioavailability patterns were similar for both (Fig. 4).

The ordination diagrams, together with Pearson's and Spearman's correlation analyses, showed that the carbonate fraction explained the Cd bioavailability patterns, with negative relationships between the ECC content and the relative proportion of calcite and dolomite (< 2mm) and Cd accumulated in the leaves and roots of romaine and iceberg lettuces grown in metal-spiked soils at the Tt1 and Tt2 levels (Table 4 and Fig. 4). This pattern could be explained by either the Cd retention capacity exerted by the carbonates, thereby reducing the Cd toxicity in plants (Recatalá *et al.*, 2010); or by the high Ca concentrations in the soil solution in these soils which compete with Cd for sorption sites and uptake; or both. Hence we observed a slight increase in the amount of Ca in the leaves and roots of plants grown in metal-spiked soils (Fig. 2), as mentioned above, as well as in the soil solution (data not shown).

Conversely, the ordination diagrams showed that Cu and Pb contents in romaine and iceberg roots were positively related to the carbonate fraction at both levels for Cu and at the Tt2 level for Pb. In previous works we observed the formation of lead oxide carbonate in these soils under the same experimental conditions (data not published). No evidence was found for the formation of Cu-carbonate, although adsorption processes onto carbonates may occur. In fact, Spearman's correlation analysis showed significant negative correlation between Pb content in iceberg roots and the relative proportion of calcite (< 2mm). Therefore, although plants could increase the content of soluble Cu and Pb by partial dissolution of carbonates (Sayyad *et al.*, 2010), this positive relationship with the carbonate fraction, contrary to the relationship obtained for Cd, was attributed to the competitive process between Cd and these metals to bind to the root surface.

		Tt1 level								Tt2 level							
		Cd		Cu		Pb		Zn		Cd		Cu		Pb		Zn	
		Romaine	Iceberg	Romaine	Iceberg	Romaine	Iceberg	Romaine	Iceberg	Romaine	Iceberg	Romaine	Iceberg	Romaine	Iceberg	Romaine	Iceberg
Leaves	ECC	**-0.653***	-0.578	-0.063	-0.267	0.290	-0.193	-0.179	-0.468	-0.400	-0.550	-0.456	-0.360	-0.443	-0.087	-0.490	-0.498
	Calcite	**-0.754***	-0.528	-0.214	-0.150	0.253	-0.118	-0.460	-0.553	-0.291	-0.557	-0.384	-0.382	-0.360	-0.145	-0.419	-0.472
	LPI	-0.268	-0.140	-0.002	0.467	-0.239	0.552	-0.039	-0.123	0.310	0.494	0.351	0.290	0.351	0.067	0.392	0.415
	LPII	-0.320	-0.166	-0.359	0.051	0.139	-0.293	-0.308	-0.252	0.119	0.099	0.142	0.267	0.150	0.604	0.099	0.163
	RP	0.377	0.206	0.195	-0.404	0.108	-0.278	0.196	0.241	-0.264	-0.345	-0.304	-0.376	-0.312	-0.538	-0.291	-0.357
	cryFe	-0.157	-0.560	-0.505	0.334	-0.298	0.037	-0.472	-0.420	-0.456	-0.411	-0.580	-0.514	-0.593	-0.503	-0.483	-0.488
	CS	0.536	**0.715***	0.605	-0.293	0.449	-0.089	**0.864****	**0.673***	**0.704***	**0.645***	**0.686***	**0.657***	**0.676***	0.494	**0.750***	**0.692***
	Clay	-0.032	-0.373	-0.532	**0.771***	**-0.767****	0.586	-0.505	-0.370	-0.439	-0.290	-0.324	-0.458	-0.307	-0.417	-0.357	-0.338
Root	ECC	-0.543	**-0.729***	0.051	-0.046	-0.166	-0.566	-0.167	-0.350	-0.385	-0.505	0.158	0.221	0.033	-0.080	-0.430	-0.490
	Calcite	-0.531	**-0.765***	-0.296	-0.215	-0.280	**-0.695***	-0.487	-0.448	-0.056	-0.340	0.380	0.223	0.352	-0.177	-0.323	-0.428
	LPI	-0.138	0.146	0.104	-0.401	-0.061	0.460	-0.289	-0.325	0.158	0.256	-0.231	-0.332	-0.131	-0.064	0.321	0.373
	LPII	-0.224	-0.251	-0.113	-0.359	0.442	-0.168	-0.024	-0.111	-0.420	-0.228	-0.447	-0.536	-0.344	0.138	0.028	0.099
	RP	0.226	0.024	-0.019	0.524	-0.192	-0.275	0.234	0.324	0.264	0.053	0.494	0.622	0.356	-0.081	-0.194	-0.281
	cryFe	-0.302	-0.219	-0.287	**-0.688***	-0.051	-0.059	0.229	-0.506	-0.355	-0.471	-0.326	-0.521	-0.368	-0.397	-0.536	-0.520
	CS	**0.740***	0.368	0.575	**0.780***	0.593	-0.027	0.512	**0.810****	0.211	0.589	0.162	0.581	0.190	0.578	**0.739***	**0.726***
	Clay	-0.096	0.165	-0.103	**-0.708***	-0.295	0.574	-0.030	-0.617	-0.067	-0.439	-0.184	**-0.659***	-0.148	**-0.641***	-0.391	-0.368

Table 4. Correlation coefficients calculated between the amount of metals in leaves and roots of lettuce plants to some soil physicochemical parameters (Pearson's correlation test) and to relative proportion of calcite (Spearman's correlation test) in metal-spiked soil samples at Tt1 and Tt2 levels.

*, ** and *** indicate statistical significance (bolded numbers) at the probability level $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively (n = 10). (a) cannot be calculated because at least one variable is constant.

ECC = equivalent $CaCO_3$; LPI = labile pool I; LPII = labile pool II; RP = recalcitrant pool; cryFe = crystalline Fe-oxides; CS = coarse sand.

Instead, the ordination diagrams showed that OM –despite its low content– was the most important factor explaining Cu and Pb bioavailability patterns, thus highlighting the key role played by this fraction in governing Cu and Pb uptake in calcareous soils (Chiu *et al.*, 2006; Recatalá *et al.*, 2011; Wang *et al.*, 2010). As mentioned above, assuming that there is competitive binding of the metal ions to the root surface, the presence of OM decreases the adsorption of Cu and Pb to the roots, which would be strongly bound by organic matter (Fernández *et al.*, 2005; Kalis *et al.*, 2006; Qian *et al.*, 2012).However, the organic fraction appears to exert the opposite effects, depending on its composition in Cu and Pb bioavailability patterns in leaves and roots. Pb bioavailability patterns in roots at the Tt1 level were negatively related to the recalcitrant organic fraction (RP), or, accordingly, positively related to the labile fractions (LPII), suggesting a strong binding to humic acids at this level (Clemente *et al.*, 2006; Zeng *et al.*, 2011). Nevertheless, Cu bioavailability patterns in roots were negatively related to OM content and to LPII or, accordingly, positively related to RP. This can be attributed to the fact that although labile OM facilitates Cu mobility in soils through the formation of organic complexes, these complexes were not available for plant uptake by roots (Cattani *et al.*, 2006). Conversely, labile OM could lead to enhanced translocation of Cu and Pb from roots to leaves in chelated forms inside the plant, probably with citric and oxalic acids (Inaba and Takenaka, 2005; Kidd, 2009; Liao *et al.*, 2006). This could explain the positive relationships obtained between Cu and Pb bioavailability in leaves, and LPI and LPII.

These results could therefore indicate that the labile fraction of organic matter plays a dual role in these soils, limiting metal absorption by roots in the case of Cu, but favouring metal translocation to leaves, in the case of Cu and Pb. At the Tt2 level, these bioavailability patterns were similar in both leaves and roots of romaine and iceberg plants, except in the case of Pb bioavailability in roots. At this level, positive relationships were established between Pb content in roots and RP, and were more evident for romaine plants. This may be due to competitive processes between Cu and Pb in binding to humic acids, resulting in an increased bioavailability of Cu and Pb when these metals are present in higher concentrations (Zheljazkov *et al.*, 2006). Another explanation for the positive relationship between Cu and Pb bioavailability in roots and RP –at both levels for Cu and at the Tt2 level for Pb– was given by Wang *et*

al. (2004). These authors obtained positive correlations between OM content and Cu bioavailability in the roots of several vegetables, which was attributed to the fact that metals combined with organic matter can be decomposed by microorganisms and then absorbed by plants. In any case, the high affinity of Cu and Pb for organic matter could be a key factor governing their bioavailability by romaine and iceberg lettuce plants.

Pearson's correlation analysis showed that the contents of Zn in romaine and iceberg leaves and roots was negatively correlated to fine mineral fractions and, accordingly, positively correlated to coarse mineral fractions at both the Tt1 and Tt2 levels (Table 4). In previous research we assessed the soil constituents and properties affecting the potential mobility of metals in these soils under these experimental conditions, and reported that silt content conditioned the Zn-extractability patterns with LMWOA and DTPA-methods (de Santiago-Martín *et al.*, in press). In the present work we have demonstrated that soil constituents affecting Zn potential mobility also influence Zn uptake by lettuce plants. This pattern was less evident in the ordination diagram, although we could corroborate the positive relationship between Cd and Zn in both romaine and iceberg lettuce, unlike that of Cu and Pb, as discussed above.

Metal bioavailability patterns in lettuce leaves and roots as a function of metal availability in soils

As shown by the Pearson's correlation coefficients calculated (Table 5), some one-step extraction methods related to both mobile and potentially mobile metal fractions explained the metal bioavailability patterns in leaves and roots of both varieties. As expected, the most significant correlations were obtained between metal bioavailability in leaves and extractable metals with neutral salts related to the metal mobile fraction. This showed that neutral salts could be an appropriate extraction method for ecotoxicological purposes in assessing the risk of metals entering the food chain (Menzies *et al.*, 2007).

Table 5. Pearson's correlation coefficients calculated between metal contents in lettuce leaves and roots and extractable metal values in metal-spiked soils at Tt1 and Tt2 levels.

Level	Extraction method	Cd				Cu				Pb				Zn			
		Romaine		Iceberg		Romaine		Iceberg		Romaine		Iceberg		Romaine		Iceberg	
		Leaves	Roots	Leaves	Roots	Leaves	Roots	Leaves	Roots	Leaves	Roots	Leaves	Roots	Leaves	Roots	Leaves	Roots
Tt1	DTPA	0.427	0.408	0.174	0.437	0.542	0.499	-0.433	**0.882****	-0.369	0.214	0.557	0.569	**0.766****	**0.664***	**0.746***	**0.948*****
	EDTA	-0.207	-0.337	-0.237	-0.037	0.251	0.160	0.282	-0.067	**-0.732***	-0.422	**0.703***	**0.807****	-0.052	-0.201	0.284	-0.008
	HNO_3	**-0.669**	**-0.725***	**-0.814****	-0.440	-0.243	-0.394	0.185	-0.339	0.120	-0.241	-0.292	0.022	-0.396	-0.374	-0.598	-0.644
	HAc	(a)	(a)	(a)	(a)	0.173	0.528	0.014	0.532	-0.111	0.184	0.315	-0.210	-0.032	-0.264	-0.648	-0.502
	LMWOA	**0.731***	**0.718***	**0.678***	**0.805****	0.550	0.427	-0.276	0.465	0.093	**0.685***	0.177	0.262	**0.816****	0.452	**0.881****	**0.987*****
	NH_4Ac	0.083	0.275	0.203	0.347	0.134	0.118	-0.318	0.331	-0.315	0.436	0.350	0.474	0.559	0.420	0.202	0.515
	$CaCl_2$	(a)	(a)	(a)	(a)	0.454	0.162	-0.605	0.359	0.291	0.317	-0.118	-0.379	**0.768****	0.609	**0.826****	**0.961*****
	$MgCl_2$	0.527	0.605	0.432	**0.776***	0.390	0.259	-0.562	0.171	0.056	0.567	0.123	0.306	0.714*	**0.676***	**0.819****	**0.946*****
	$NaNO_3$	**0.778****	**0.896*****	**0.985*****	**0.721***	0.467	0.012	-0.619	0.423	-0.279	0.431	0.414	0.482	**0.758***	0.513	**0.926*****	**0.982*****
	NH_4NO_3	(a)	(a)	(a)	(a)	0.194	0.337	-0.239	0.334	**-0.691***	-0.563	0.499	0.511	**0.738***	0.505	**0.863****	**0.956*****
Tt2	DTPA	0.386	0.417	0.305	0.381	**0.926*****	-0.008	**0.953*****	0.269	**0.886****	0.457	0.582	0.529	**0.969*****	**0.939*****	**0.966*****	**0.965*****
	EDTA	-0.078	-0.259	0.149	-0.025	0.289	-0.517	0.317	-0.618	0.290	-0.488	0.489	0.100	0.091	0.071	0.123	0.102
	HNO_3	-0.197	-0.023	-0.218	0.119	0.015	-0.531	0.140	-0.297	0.029	-0.439	0.003	0.021	-0.048	-0.145	-0.016	-0.038
	HAc	0.079	-0.493	-0.081	-0.058	0.621	-0.232	**0.717***	0.166	0.208	-0.245	**0.678***	0.504	0.394	0.272	0.386	0.370
	LMWOA	**0.894*****	0.539	**0.848****	**0.842****	0.052	-0.174	0.040	-0.237	**0.947*****	0.159	**0.807****	**0.842****	**0.741***	**0.664***	**0.740***	**0.714***
	NH_4Ac	0.219	-0.303	0.172	0.116	0.272	-0.324	0.456	0.070	**0.668***	-0.167	**0.729***	**0.693***	0.510	0.459	0.469	0.463
	$CaCl_2$	**0.966*****	0.473	**0.962*****	**0.865****	**0.888****	0.010	**0.920*****	0.356	0.627	-0.386	0.613	0.525	**0.983*****	**0.954*****	**0.976*****	**0.979*****
	$MgCl_2$	-0.064	-0.020	0.038	-0.026	**0.808****	-0.049	**0.875****	0.339	**0.857****	0.037	**0.681***	**0.767****	**0.939*****	**0.902*****	**0.940*****	**0.937*****
	$NaNO_3$	**0.945*****	0.459	**0.981*****	**0.859****	**0.937*****	0.068	**0.945*****	0.351	**0.960*****	0.170	**0.772****	**0.828****	**0.991*****	**0.972*****	**0.996*****	**0.996*****
	NH_4NO_3	**0.863****	0.343	**0.907*****	**0.728***	0.398	-0.134	0.484	-0.056	**0.952*****	0.162	**0.760***	**0.828****	**0.994*****	**0.974*****	**0.993*****	**0.995*****

*, ** and *** indicate statistical significance (bolded numbers) at the probability level $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively (n = 10). (a) cannot be calculated because at least one variable is constant.

Nevertheless, it is particularly worth noting that numerous significant correlations were obtained between metal bioavailability in leaves and roots and HAc, NH_4Ac, DTPA and LMWOA methods, which estimate the potentially mobile fractions of metals (Gupta *et al.*, 1996). As discussed above, metal fractions estimated *a priori* as being potentially mobile could be bioavailable in these soils, and therefore successfully simulated by extraction methods with a higher strength.

Metal bioavailability in soils is the result of the combined effect of root-soil-microbe interaction occurring in the soil of the rhizosphere. Hence the suitability of acetic acid was attributed to the fact that it is one of the main components of the organic acids present in the rhizosphere and is involved in metal adsorption by roots (Wang *et al.*, 2004; Han *et al.*, 2005). The DTPA method has been proved suitable for simulating metal uptake in lettuce plants in these soils, as previously reported for neutral and near-alkaline soils (Feng *et al.*, 2005). The positive relationships between metal bioavailability in leaves and roots and LMWOA-extractable metals was attributed to the fact that the LMWOA method simulates the plant exudates and microbial metabolites in the rhizosphere zone where metal-LMWOA complexes may promote the transport of metal into the plants and its subsequent translocation from the roots to the aboveground parts (Liao *et al.*, 2006; Pinto *et al.*, 2004; Zorrig *et al.*, 2010). The suitability of this method corresponds to the positive relationship found between the Cu and Pb contents in leaves and the labile organic fraction.

Finally, extraction methods with a stronger extraction capacity, such as EDTA and HNO_3 methods, have been shown to be unsuitable for simulating the bioavailability of metals to lettuce plants in the current context, probably because they may extract metals from non-labile soil fractions (Feng *et al.*, 2005). Nevertheless, these methods may be suitable for potential risk predictions in the longer term, in relation to the turnover of organic and inorganic C affecting the storage capacity, mobility and (bio)availability of metals in soils.

It should be noted that different behaviours were obtained for Cd and Zn on the one hand, and for Cu and Pb on the other. In the case of Cd and Zn, the extraction methods that were suitable for simulating the metal bioavailability patterns in roots were also suitable for leaves. Nevertheless, in the case of Cu and Pb we observed different results for leaves and roots and for the two levels of

contamination, as previously discussed. At the Tt1 level, no satisfactory correlations were obtained with extractable metals. At the Tt2 level, Cu and Pb contents in the leaves of both varieties was positive and significantly correlated with some one-step extraction methods, while correlations with Pb in roots were scarce, and non-existent in the case of Cu. The variety of factors involved in Cu and Pb uptake (added as a multi-element salt solution) such as the competitive binding to the root surface and the strong binding to soil organic matter and root surface (Black *et al.*, 2011), together with the impossibility of distinguishing between metal adsorption and absorption patterns in roots (Kalis *et al.*, 2006), hindered the elucidation of the uptake pattern of each individual metal.

Conclusions

The results obtained in the present work show that despite the high metal sorption capacity conferred by the carbonates, the limit values considered excessive or toxic to leaf tissues were exceeded in all cases. This indicates the significant risk caused by the high but finite metal retention capacity in these soils, inasmuch as the retention conferred by carbonates has proved not to be entirely effective. The content and composition of the organic fraction plays a key role in governing metal bioavailability patterns in both romaine and iceberg lettuce varieties, despite the low organic matter content. The organic fraction exerted contrary effects depending on its composition in Cu and Pb bioavailability patterns in the leaves and roots of lettuce plants. The labile organic fraction played a dual role in these soils, in some cases limiting Cu and Pb absorption by roots, but in all cases favouring metal translocation to leaves.

Metal bioavailability patterns in the edible parts of lettuce in these soils have been successfully simulated by neutral salts related to the metal mobile fraction. It is particularly worth highlighting the suitability of several extraction methods related to potentially mobile fractions of metals, such as complexing agents. Although the metal mixture was added at levels that are consistent with European regulations (Directive 86/278/EEC), the considerable phytotoxicity and the high metal contents in the edible parts of the lettuce suggest that the values in the current European legislation should be revised when contamination is produced by a mixture of heavy metals and/or should be established according to soil characteristics.

Acknowledgements

This work was supported by the Universidad Complutense in Madrid and the Madrid Autonomous Region through Grant GR58/08, Research Team 950605 and Network CARESOIL, Ref. P2009/AMB-1648. We especially wish to thank Folkert van Oort and Isabelle Lamy (Pessac, INRA) for their helpful advice in the development of the present work and Ms Pru Brooke-Turner for the revision of the English version of the manuscript.

References

Abdu, N., John, O., Agbenin, J.O., Buerkert, A., 2011. Phytoavailability, human risk assessment and transfer characteristics of cadmium and zinc contamination from urban gardens in Kano, Nigeria. J. Sci. Food Agric. 91(15), 2722-2730.

Adriano, D.C., 2001. Trace Elements in Terrestrial Environments, Biogeochemistry, Bioavailability and Risk of Metals, second ed. Springer, New York.

Allen, S.E., Grimshaw, H.M., Rowland, A.P., 1986. Chemical analysis, in: Moore, P.D., Chapman, S.B. (Eds.), Methods in Plant Ecology. Blackwell Scientific Publication, Oxford, London, pp. 285-344.

Bish D.L., 1994. Quantitative x-ray diffraction analysis of soils. In: Amonette JE and Zelazny LW (ed) Quantitative methods in soil mineralogy, SSSA Misc Publ, SSSA, Madison, WI, pp. 267-295.

Black, A., McLaren, R.G., Reichman, S.M., Speir, T.W., Condron, L.M., 2011. Evaluation of soil metal bioavailability estimates using two plant species (*L. perenne* and *T. aestivum*) grown in a range of agricultural soils treated with biosolids and metal salts. Environ. Pollut. 159, 1523-1535.

Cattani, I., Fragoulis, G., Boccelli, R., Capri, E., 2006. Copper bioavailability in the rhizosphere of maize (Zea mays L.) grown in two Italian soils. Chemosphere 64, 1972-1979.

Chaignon, V., Sanchez-Neira, I., Herrmann, P., Jaillard, B., Hinsinger, P., 2003. Copper bioavailability and extractability as related to chemical properties of contaminated soils from a vine-growing area. Environ. Pollut. 123, 229-238.

Chakravarty, B., Srivastava, S., 1997. Effect of cadmium and zinc interaction on metal uptake and regeneration of tolerant plants in linseed. Agric. Ecosyst. Environ. 61, 45-50.

Chiu, K.K., Ye, Z.H., Wong, M.H., 2006. Growth of *Vetiveria zizanioides* and *Phragmities australis* on Pb/Zn and Cu mine tailings amended with manure compost and sewage sludge: A greenhouse study. Bioresour. Technol. 97, 158-170.

Clemente, R., Escolar, A., Bernal, M.P., 2006. Heavy metals fractionation and organic matter mineralization in contaminated calcareous soil amended with organic materials. Bioresour. Technol. 97, 1894-1901.

Clemente, R., Paredes, C., Bernal, M.P., 2007. A field experiment investigating the effects of olive husk and cow manure on heavy metal availability in a contaminated calcareous soil from Murcia (Spain). Agric. Ecosyst. Environ. 118, 319-326.

Commission Regulation (EEC) No 466/2001 of 8 March 2001 setting maximum levels for certain contaminants in foodstuffs.

Dahmani-Muller, H., van Oort, F., Balabane, M., 2001. Metal extraction by Arabidopsis halleri grown on an unpolluted soil amended with various metal-bearing solids: a pot experiment. Environ. Pollut. 114, 77-84.

de Santiago-Martín, A., Valverde-Asenjo, I., Quintana, J.R., González-Huecas, C., Lafuente, A.L., in press. Soil properties affecting metal extractability patterns in periurban calcareous agricultural soils in the Mediterranean area. Int. J. Environ. Res.

DIN (Deutsches Institut für Normung, Bodenbeschaffenheit), 1995. Extraktion von Spurenelemente mit Ammonium-nitratlösung, Vornorm DINV 19730, DIN Boden-Chemische Bodenuntersuchungs-verfahren, Berlin, Germany.

Directive 86/278/EEC of 12 June 1986 on the protection of the environment, and in particular of the soil, when sewage sludge is used in agriculture.

Duckworth, R.B., 1966. Fruit and Vegetables. Pergamon Press, Oxford, UK.

European Commission, 2006. Thematic Strategy for Soil Protection. COM (2006) 231, Brussels, Belgium.

FAO, 2006. World reference base for soil resources. A framework for international classification, correlation and communication. FAO, Roma.

Feng, M., Shan, X., Zhang, S., Wen, B., 2005. A comparison of the rhizosphere-based method with DTPA, EDTA, $CaCl_2$, and $NaNO_3$ extraction methods for prediction of bioavailability of metals in soil to barley. Environ. Pollut. 137, 231-240.

Fernández, M.D., Cagigal, E., Vega, M.M., Urzelai, A., Babín, M., Pro, J., Tarazona, J.V., 2005. Ecological risk assessment of contaminated soils through direct toxicity assessment. Ecotoxicol. Environ. Saf. 62, 174-184.

Gisbert, C., Clemente, R., Navarro-Aviñó, J., Baixauli, C., Ginér, A., Serrano, R., Walker, D.J., Bernal, M.P., 2006. Tolerance and accumulation of heavy metals by Brassicaceae species grown in contaminated soils from Mediterranean regions of Spain. Environ. Exp. Bot. 56, 19-27.

Grattan, S.R., Grieve, C.M., 1999. Salinity-mineral nutrient relations in horticultural crops. Sci. Hort. 78, 127-157.

Gupta, S.K., Aten, C., 1993. Comparison and evaluation of extraction media and their suitability in a simple model to predict the biological relevance of heavy metal concentrations in contaminated soils. Int. J. Environ. Anal. Chem. 51, 25-46.

Gupta, S.K., Vollmer, M.K., Krebs, R., 1996. The importance of mobile, mobilisable and pseudo total heavy metal fractions in soil for three-level risk assessment and risk management. Sci. Total Environ. 178, 11-20.

Han, F., Shan, X.Q., Zhang, J., Xie, Y.N., Pei, Z.G., Zhang, S.Z., Zhu, Y.G., Wen, B., 2005. Organic acids promote the uptake of lanthanum by barley roots. New Phytologist 165, 481-492.

IGME (Instituto Geológico y Minero de España), 1990. Mapa geológico de España nº 535. Escala 1:50.000 (Algete), Madrid.

Inaba, S., Takenaka, C., 2005. Effects of dissolved organic matter on toxicity and bioavailability of copper for lettuce sprouts. Environ. Internat. 31, 603- 608.

ISRIC (International Soil Reference and Information Center), 2002. Procedures for Soil Analysis, 3th ed. International Soil Reference and Information Center, Wageningen.

Jiménez Ballesta, R., Conde Bueno, P., Martín Rubí, J.A., García Giménez, R., 2010. Pedo-geochemical baseline content levels and soil quality reference values of trace elements in soils from the Mediterranean (Castilla La Mancha, Spain). Eur. J. Geosci. 2(4), 441-454.

Kabata-Pendias, A., Pendias, H., 2001. Trace Elements in Soils and Plants, third ed. Boca Ratón, New York.

Kalis, E.J.J., Temminghoff, E.J.M., Weng, L., van Riemsdijk, W.H., 2006. Effects of humic acid and competing cations on metal uptake by *Lolium perenne*. Environ. Toxicol. Chem. 25(3), 702-711.

Kaschl, A., Römheld, V., Chen, Y., 2002. The influence of soluble organic matter from municipal solid waste compost on trace metal leaching in calcareous soils. Sci. Total Environ. 291, 45-57

Kidd, P., Barceló, J., Bernal, M.P., Navari-Izzo, F., Poschenrieder, C., Shilev, S., Clemente, R., Monterroso, C., 2009. Trace element behaviour at the root–soil interface: Implications in phytoremediation. Environ. Exp. Bot. 67, 243-259.

Liao, Y.C., Chang Chien, S.W., Wang, M.C., Shen, Y., Hung, P.L., Das, B., 2006. Effect of transpiration on Pb uptake by lettuce and on water soluble low molecular weight organic acids in rhizosphere. Chemosphere 65, 343-351.

Lindsay, W.L., Norvell, W.A., 1978. Development of a DTPA soil test for zinc, iron, manganese and copper. Soil Sci. Soc. Am. J. 42, 421-428.

Maas, S., Scheifler, R., Benslama, M., Crini, N., Lucot, E., Brahmia, Z., Benyacoub, S., Giraudoux, P., 2010. Spatial distribution of heavy metal concentrations in urban, suburban and agricultural soils in a Mediterranean city of Algeria. Environ. Pollut. 158, 2294-2301.

McLaughlin, M.J., 2001. Aging of metals in soils changes bioavailability. Fact Sheet Environ. Risk Assess. 4, 1-6.

Menzies, N.W., Donn, M.J., Kopittke, P.M., 2007. Evaluation of extractants for estimation of the phytoavailable trace metals in soils. Environ. Pollut. 145, 121-130.

Micó, C., Recatalá, L., Peris, M., Sánchez, J., 2006. Assessing heavy metal sources in agricultural soils of an European Mediterranean area by multivariate analysis. Chemosphere 65, 863-872.

Moreno Merino, L., 1998. Estudio de la influencia del suelo sobre la composición de las aguas subterráneas a través de la solución del suelo. Modelo en Fluvisoles calcáricos. PhD thesis, Universidad Complutense de Madrid.

Peris, M., Micó, C., Recatalá, L., Sánchez, R., Sánchez, J., 2007. Heavy metal contents in horticultural crops of a representative area of the European Mediterranean region. Sci. Total Environ. 378, 42-48.

Pinto, A.P., Mota, A.M., de Varennes, A., Pinto, F.C., 2004. Influence of organic matter on the uptake of cadmium, zinc, copper and iron by sorghum plants. Sci. Total Environ. 326, 239-247.

Podar, D., Ramsey, M.H., 2005. Effect of alkaline pH and associated Zn on the concentration and total uptake of Cd by lettuce: comparison with predictions from the CLEA model. Sci. Total Environ. 347, 53-63.

Qian, Y., Gallaghe, F.J., Feng, H., Wu, M., 2012. A geochemical study of toxic metal translocation in an urban brownfield wetland. Environ. Pollut. 166, 23-30.

Quevauviller, Ph., Lachica, M., Barahona, E., Rauret, G., Ure, A., Gómez, A., Muntau, H., 1996. Interlaboratory comparison of EDTA and DTPA procedures prior to certification of extractable trace elements in calcareous soil. Sci Total Environ 178, 127-132.

Rauret, G., Lopez-Sanchez, J.F., Sahuquillo, A., Rubio, R., Davidson, C., Ure, A., Queqauviller, Ph., 1999. Improvement of the BCR three-step sequential extraction procedure prior to the certification of new sediment and soil reference materials. J. Environ. Monit. 1, 57-61.

Recatalá, L., Sánchez, J., Arbelo, C., Sacristán, D., 2010. Testing the validity of a Cd soil quality standard in representative Mediterranean agricultural soils under an accumulator crop. Sci. Total Environ. 409, 9-18.

Recatalá, L., Sacristán, D., Arbelo, C., Sánchez, J., 2011. Can a Single and Unique Cu Soil Quality Standard be Valid for Different Mediterranean Agricultural Soils under an Accumulator Crop? Water, Air, Soil Pollut. 223(4), 1503-1517.

Rovira, P., Vallejo, V.R., 2000. Evaluating thermal and acid hydrolysis methods as indicators of soil organic matter quality. Commun. Soil Sci. Plant Anal. 31, 81-100.

Sayyad, G., Afyuni, M., Mousavi, S.F., Abbaspour, K.C., Richards, B.K., Schulin, R., 2010. Transport of Cd, Cu, Pb and Zn in a calcareous soil under wheat and safflower cultivation - A column study. Geoderma 154, 311-320.

SISS (Società Italiana della Scienza del Suolo), 1985. Metodi Normalizzati di Analisi del Suolo. Edagricole, Bologna.

Ter Braak, C.J.F., 1994. Canonical community ordination. Part I: basic theory and linear methods. Ecosci. 1, 127-140.

Ter Braak, C.J.F., Smilauer P., 2002. CANOCO Reference manual and CanoDraw for Windows. User´s guide: Software for Canonical Community Ordination (v. 4.5), Microcomputer Power: Ithaca, NY, USA.

Tessier, A., Campbell, P.G.C., Bisson, M., 1979. Sequential extraction procedure for the speciation of particulate trace metals. Anal. Chem. 51, 844-851.

Van Ranst, E., Verloo, M., Demeyer, A., Pauwels, J.M., 1999. Manual for the soil chemistry and fertility laboratory. Ghent University, Faculty Agricultural and Applied Biological Sciences, pp. 243.

Wang, Z.W., Zhang, S.Z., Shan, X.Q., 2004. Effects of low-molecular-weight organic acids on uptake of lanthanum by barley roots. Plant Soil 261, 163-170.

Wang, X., Chen, X., Liu, S., Ge, X., 2010. Effect of molecular weight of dissolved organic matter on toxicity and bioavailability of copper to lettuce. J. Environ. Sci. 22(12), 1960-1965.

Wu, F., Zhang, G., Dominy, P., Wu, H., Bachir, D.M.L., 2007. Differences in yield components and kernel Cd accumulation in response to Cd toxicity in four barley genotypes. Chemosphere 70, 83-92.

Zeng, F., Ali, S., Zhang, H., Ouyang, Y., Qiu, B., Wu, F., Zhang, G., 2011. The influence of pH and organic matter content in paddy soil on heavy metal availability and their uptake by rice plants. Environ. Pollut. 159, 84-91.

Zheljazkov, V.D., Craker, L.E., Xing, B., 2006. Effects of Cd, Pb, and Cu on growth and essential oil contents in dill, peppermint, and basil. Environ. Exp. Bot. 58, 9-16.

Zorrig, W., Rouached, A., Shahzad, Z., Abdelly, C., Davidian, J., Berthomieu, P., 2010. Identification of three relationships linking cadmium accumulation to cadmium tolerance and zinc and citrate accumulation in lettuce. J. Plant Physiol. 167, 1239-1247.

Zorrig, W., Shahza, Z., Abdelly, C., Berthomieu, P., 2012. Calcium enhances cadmium tolerance and decreases cadmium accumulation in lettuce (*Lactuca sativa*). Afr. J. Biotechnol. 11(34), 8441-8448.

5. Estudio de la perturbación de las propiedades microbiológicas del suelo

Apartado 5.1

Metal contamination disturbs biochemical and microbial properties of calcareous agricultural soils of the Mediterranean area

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Archives of Environmental Contamination and Toxicology (in press)

Resumen

Las características del clima mediterráneo y el contenido en carbonato son factores clave que rigen la acumulación de metales pesados en el suelo. Además, el bajo contenido de materia orgánica podría limitar la capacidad de las poblaciones microbianas para lidiar con el estrés resultante. Por ello, se estudiaron los efectos de la contaminación metálica en una combinación de parámetros biológicos en suelos con estas características. Con este objetivo, los suelos fueron contaminados artificialmente con una mezcla de Cd, Cu, Pb y Zn, dentro de los valores límites propuestos por la legislación Europea actual, y se incubaron durante 12 meses. A continuación, se midieron un conjunto de parámetros bioquímicos (actividades fosfatasa, ureasa, β-galactosidasa, arilsulfatasa, y deshidrogenasa) y microbiológicos (concentración de ADN fúngico y bacteriano, mediante PCR cuantitativa).

Todas las actividades enzimáticas se vieron fuertemente alteradas por la contaminación metálica, mostrando la siguiente secuencia de inhibición: fosfatasa (30-64%) < arilsulfatasa (38-97%) ≤ ureasa (1-100%) ≤ β-galactosidasa (30-100%) < deshidrogenasa (69-100%). La elevada variabilidad entre los suelos se atribuyó a la diferente proporción de las fracciones minerales finas, orgánica, y óxido de Fe cristalino, así como de cationes divalentes en la solución del suelo. La disminución de la concentración de ADN fúngico en los suelos contaminados fue despreciable, mientras que la disminución del ADN bacteriano fue aproximadamente 1-54% en el nivel más bajo y 2-69% en el nivel más alto de contaminación. La menor disminución de ADN bacteriano se produjo en los suelos con mayores contenidos en materia orgánica, arcilla, y carbonato. Finalmente, teniendo en cuenta la fuerte inhibición de los parámetros biológicos medidos, así como la alteración de la relación de ADN fúngico/bacteriano, se proporciona una fuerte evidencia de que una perturbación en el sistema podría alterar procesos clave de suelo, incluso dentro de los valores límite de contaminación propuestos por la actual Directiva Europea. Estos valores límite deberían ser establecidos de acuerdo con las características del suelo y/o revisados cuando la contaminación se produce por una mezcla de metales pesados.

Abstract

Mediterranean climate characteristics and carbonate are key factors governing soil heavy metal accumulation and the low organic matter content could limit the ability of microbial populations to cope with resulting stress. We studied the effects of metal contamination on a combination of biological parameters in soils having these characteristics. With this aim, soils were spiked with a mixture of Cd, Cu, Pb, and Zn, at the two limit values proposed by current European legislation, and incubated for up to 12 months. Then, we measured biochemical (phosphatase, urease, β-galactosidase, arylsulfatase, and dehydrogenase activities) and microbial (fungal and bacterial DNA concentration by quantitative PCR) parameters.

All the enzyme activities were strongly affected by metal contamination, showing the inhibition sequence: phosphatase (30-64%) < arylsulfatase (38-97%) ≤ urease (1-100%) ≤ β-galactosidase (30-100%) < dehydrogenase (69-100%). The high variability among soils was attributed to the different proportion of fine mineral fraction, organic matter, crystalline Fe oxides and divalent cations in soil solution. The decrease of fungal DNA concentration in metal-spiked soils was negligible, while the decrease of bacterial DNA was about 1-54% at the lowest level and 2-69% at the highest level of contamination. The lowest bacterial DNA decrease occurred in soils with the highest organic matter, clay and carbonate contents. Finally, regarding the strong inhibition of the biological parameters measured, and the alteration of the fungal/bacterial DNA ratio, we provide strong evidence that a disturbance on the system could alter key soil processes, even within the limiting values of contamination proposed by the current European Directive. These limiting values should be established according to soil characteristics and/or revised when contamination is produced by a mixture of heavy metals.

Keywords

biological parameters, calcareous soil, enzyme activity, heavy metals, quantitative PCR

Introduction

Heavy metal accumulation in agricultural areas has increased in recent decades as a result of agricultural, industrial and urban activities (Roca-Perez *et al.*, 2010). This metal accumulation in agricultural soils may result in adverse environmental and human health impacts, including metal contamination of groundwater and accumulation in food crops. The alteration of microbial communities has often been proposed as an easy and sensitive indicator of anthropogenic effects on soil ecology (Renella *et al.*, 2005). However, while most of the studies on microbial disturbance by metals mainly refer to Northern European countries, there is less information in Southern Europe, particularly in the Mediterranean area.

The nature and degree of inhibition of soil microorganisms by metals are strongly related to soil properties and components (Speir *et al.*, 1992). In this sense, Mediterranean soils usually show relatively high pH and carbonate content and are typically low in organic matter content. Low rainfall and high evapotranspiration, characteristic of the Mediterranean climate, limit metal mobility. In addition, metal retention capacity in soils is increased due to carbonate content, which results in an increased metal accumulation in the first few centimetres of soil. In this sense, we observed a high metal retention capacity (> 90 %) in calcareous agricultural soils in the Mediterranean area (Lafuente *et al.*, 2008). However, it has been reported that there can be a significant metal mobility and availability even in calcareous soils of the Mediterranean area with significant implications for the risk of contamination (Plassard *et al.*, 2000; Sayyad *et al.*, 2010). Therefore, this high but finite capacity to store potentially mobilizable metals leads to considering these soils as the most vulnerable soils (Batjes, 2000). As organic matter plays a key role as a precursor for enzyme synthesis and in physical stabilization (Tabatabai, 1994), the microbial activity of these soils with low organic matter content is often very low (Bastida *et al.*, 2006). This could limit the response of soils to metal contamination and prevent microbial populations from coping with the stress resulting from heavy metal contamination (Moreno *et al.*, 2009). Therefore, we highlight the necessity of paying particular attention to microbial disruption by metal contamination in calcareous agricultural soils of the Mediterranean area with low organic matter contents.

Biochemical parameters such as extracellular enzyme activities are good sensors of the long-term contamination effects of soils by heavy metals (Kızılkaya *et al.*, 2004) and are recognized as a more sensitive bio-indicator than plants and animals of natural and anthropogenic disturbances (Hinojosa *et al.*, 2004). Extracellular enzymes are usually dependent on the state of the organism (active, inactive or dead), with a temporal lag between cell death and the decrease in enzyme activity (Brookes, 1995). By contrast, dehydrogenase activity, which depends on the parent microbial cell, is considered as a good indicator of overall microbial activity (Obbard, 2001). In addition to biochemical parameters, several techniques have been used to quantify the soil microbial biomass, such as real-time quantitative PCR (qPCR). Since the natural variability of soil properties could be a decisive impediment to the use of simple biological parameters alone (Nannipieri *et al.*, 1990), using a combination of these parameters for diagnostic purposes could provide a better picture of the status of soil processes and functioning (Acosta-Martínez *et al.*, 2003).

Although all the metal added could be sorbed, it is necessary to know the efficiency of the retention studying if there is any biological disturbance. Therefore, in the present work, we focus on the study of the disruption of a combination of biological parameters by metal contamination in calcareous agricultural soils in the Mediterranean area with low amounts of organic matter. With this aim, soils with these characteristics were metal-spiked with a mixture of Cd, Cu, Pb and Zn at two different levels and incubated for up to 12 months and some biochemical (phosphatase, urease, β-galactosidase, arylsulfatase, and dehydrogenase activities) and microbial (total, fungal and bacterial DNA concentration) parameters of soils were monitored. Special attention was made to soil properties and components affecting patterns of biological inhibition.

Materials and Methods

Soil characteristics and sampling

The soil samples of the present study come from different plots at "El Encín" Agricultural Research Station, in Alcalá de Henares (Madrid, Spain). The area is located on medium level alluvial terraces by the Henares River on quaternary

sediments, mainly medium and coarse sands (IGME, 1990). These alluvial sediments have caused an ancient calcaric Fluvisol (Moreno Merino, 1998); to currently present Anthrosol characteristics (FAO, 2006) mainly as a result of agricultural use. The site is typical of a Mediterranean pluviseasonal-oceanic bioclimate on an upper meso-Mediterranean low dry bioclimatic belt (Rivas-Martínez, 1987). We selected ten calcareous agricultural soil samples with a natural gradient in organic matter content (ranging from 10 to 32 g kg^{-1}), with a wide range of variation in its carbonate content (from 9 to 190 g kg^{-1}) and in its particle-size distribution –sandy-loam and sandy-clay loam textural classes– (Table 1).

Table 1. Physicochemical parameters of uncontaminated soil samples.

Soil sample	pH	EC	OM	RP	ECC	CS	FS	Silt	Clay	cry-Fe	Total P	CEC	Soil solution		Total content		
													Ca	Mg	Cu	Pb	Zn
		dS m^{-1}	g kg^{-1}	% of TOC	g kg^{-1}							$cmol_c$ kg^{-1}	mg kg^{-1}				
H1	8.2	0.1	32	72	106	159	590	78	172	13	0.6	6.9	249	44	12.0	25.5	52.9
H2	8.1	0.5	31	66	125	23	592	215	170	12	0.8	10.6	718	151	10.0	23.8	63.3
H3	8.4	0.2	26	54	118	95	605	146	154	11	1.0	8.9	243	46	10.8	24.4	62.0
M1	8.1	0.1	21	71	32	45	459	168	328	11	0.4	19.1	489	63	10.1	24.0	62.1
M2	8.2	0.2	21	74	27	99	386	172	344	12	0.6	20.4	610	96	13.2	21.1	70.7
M3	8.1	0.2	20	48	148	114	569	124	193	9	1.4	8.7	341	26	12.1	55.8	62.7
M4	8.7	0.1	17	57	117	166	567	129	139	8	2.1	5.6	308	31	15.0	23.7	74.9
L1	8.2	0.2	14	52	9	245	560	70	124	7	0.5	10.0	402	29	7.0	25.4	45.2
L2	8.1	0.2	13	75	100	112	462	167	259	10	0.8	13.3	338	35	8.5	21.5	55.1
L3	8.4	0.2	10	69	190	111	603	126	161	8	0.7	7.1	338	35	8.0	14.0	54.7

EC = electrical conductivity; OM =organic matter; RP = recalcitrant pool of total organic carbon (TOC); ECC = equivalent $CaCO_3$; CS = coarse sand; FS = fine sand; cry-Fe = crystalline Fe oxides; CEC = cation exchange capacity. The total Cd content was in all cases lower than the quantification limit (<0.2 mg L^{-1}).

Total metal content values were similar to those obtained by other authors for agricultural soils in the Mediterranean area (Jiménez Ballesta *et al.*, 2010; Micó *et al.*, 2006; Peris *et al.*, 2007) and in no case exceeded the levels set by the European Union (Directive 86/278/EEC). These samples included a mixture of 30-40 kg of the soil surface horizons (0-30 cm) that have not been differentiated because of the agricultural activity. Soil samples were air-dried and passed through a 2 mm sieve.

Experimental design

Metal accumulation in agricultural soils was simulated by spiking soils with a mixture of Cd, Cu, Pb, and Zn in order to mimic a multi-element contaminated soil. Three containers (40 cm wide x 59 cm long x 21 cm high) were set for each sample, 10 kg each, one for uncontaminated sample with addition of distilled water and the other two were spiked at two different concentration levels using nitrate salts of heavy metals in aqueous solution: low level (Tt1) (3 mg kg^{-1} of Cd + 140 mg kg^{-1} of Cu + 300 mg kg^{-1} of Pb + 300 mg kg^{-1} of Zn) or high level (Tt2) (20 mg kg^{-1} of Cd + 875 mg kg^{-1} of Cu + 600 mg kg^{-1} of Pb + 2000 mg kg^{-1} of Zn) in line with the limits proposed by current European legislation (Directive 86/278/EEC).

Each soil sample and the corresponding solution was mixed and left to equilibrate for a period of 12 months at room temperature without cover or drainage. During this equilibration period, the soils were air-dried, mixed and rewetted with distilled water in cycles of about 2 weeks in order to favour metal redistribution into the soil matrix (McLaughlin, 2001). At the end of equilibration period, duplicates were randomly taken out from each uncontaminated and metal-spiked soil samples to determine biochemical and microbial parameters.

We selected the following soil enzyme activities because of their wide use in the assessment of biochemical disruption by metals: phosphatase (PHOS), urease (URE), β-galactosidase (BGAL), arylsulfatase (ARYS), and dehydrogenase (DHA) activities. In order to evaluate the microbial biomass, a real-time quantitative PCR (qPCR) was carried out for quantification of DNA from fungi and bacteria in the soils.

Analytical methods

Soil physicochemical analyses

Soil pH was analyzed in a 1:2.5 soil to water ratio (method 4-1, ISRIC, 2002). Total organic C was quantified by wet oxidation according to the Walkley and Black method (method 5-1, ISRIC, 2002). We used the two-step acid hydrolysis procedure with H_2SO_4 to determine recalcitrant (RP) pool of organic matter (Rovira and Vallejo, 2000). Equivalent $CaCO_3$ (ECC) was determined according to the acid neutralization method (method 7-1, ISRIC, 2002). Particle-size distribution was determined by the

Robinson's pipette method, following dispersion with sodium hexametaphosphate (method 3, ISRIC, 2002). The crystalline and amorphous Fe and Mn oxide contents were determined by dithionite-citrate extraction followed by acid oxalate extraction (methods 12-1.1 and 12-2, ISRIC, 2002). The cation exchange capacity (CEC) was determined by the ammonium acetate method (method 9, ISRIC, 2002). The major cations in soil solution were extracted in a 1:5 soil to water ratio and electrical conductivity was measured (method 13-1, ISRIC, 2002).

Total P, Cd, Cu, Pb and Zn contents of the soil samples were determined after wet digestion with a mixture of nitro-perchloric-hydrofluoric acids under high-pressure conditions (SISS, 1985). DTPA-extractable metals were determined by shaking 20 g of soil with 40 ml of 0.005 M DTPA + 0.01 M $CaCl_2$ + 0.01 M triethanolamine (TEA) for 2 h (Lindsay and Norwell, 1978). Elements in the corresponding extracts were quantified by atomic absorption spectroscopy for Fe, Mn, Ca, Mg, Cd, Cu, Pb, and Zn, by flame emission spectrometry for Na and K and by inductively coupled plasma-atomic emission spectroscopy for P. All analyses were performed in duplicate.

Biochemical analyses

Soil samples were incubated at 60% water holding capacity and 25 ºC in the dark for 7 days. Moisture levels were maintained gravimetrically every 2 days using distilled water. The determination of PHOS, BGAL, ARYS, and URE activities were performed, respectively, according to the methods of Tabatabai and Bremner (1970), Dick *et al.* (1996), and Sinsabaugh *et al.* (2000). Briefly, soil solutions were prepared in triplicate with distilled water (1:6.25 w:v), stirring at 10250 rpm, and incubated with substrates (3 pseudo-replicates): 4-nitrophenylphosphate (0.05 M, 90 min at 37 ºC for PHOS, Sigma), 4-nitrophenylgalactopyranoside (0.02 M, 3 h at 37 °C for BGAL, Sigma), 4-nitrophenylsulfate (25 mM, 4 h at 37 °C for ARYS, Sigma), and urea (0.4 M, 3 h at 25 °C for URE). For PHOS, BGAL, and ARYS activities the reaction was stopped with 0.5 M $CaCl_2$ and 0.1 M Tris pH 12 and the para-nitrophenol (PNP) released was measured at 405 nm with a Xenius microplate luminometer (SAFAS, Monaco). For URE activity, NH_4^+ released was determined with a HACH reagent (610 nm). The concentration of product released was compared to para-

nitrophenol standards (Sigma) for ARYS, BGAL and PHOS activities or to NH_4Cl standards (Sigma) for the URE activity. Soil DHA activity was assayed using the method of Schaefer (1963). Soil triplicates were mixed with distilled water (1:1 w:v) and 0.12 M 2,3,5-triphenyltetrazolium chloride (TTC, Sigma) and incubated 16 h at 37 ºC. The reaction was stopped with ethanol (5 vol.), incubated for 2 h in the dark at 37 ºC and the tubes centrifuged 5 min at 6000 rpm. Absorbance of supernatants was measured at 485 nm (KONTRON Spectrophotometer). The concentration of product released was compared with 1,3,5-triphenyl formazan (Sigma) standards. Activities were expressed in mU g^{-1} dry soil. One U of activity was defined as the amount of enzyme that catalyzed 1 μmol substrate in 1 min.

Microbial analyses

Total genomic DNA was extracted in duplicate from 0.5 g of soil using Fast DNA kit for soil (MP Biomedical). rDNA was then amplified according to Gangneux *et al.* (2011) in 10 μl final qPCR mixture containing the following total amounts: 5 ng of total soil DNA, 12.5 pmoles of each primer, 5 μl of iq-SYBR®Green super Mix (Biorad). For bacterial 16S-rDNA, the forward primer used was S-BCT 5'-CAG GCC TAA CAC ATG CAA GTC-3' (63f, Marchesi *et al.*, 1998) and the reverse primer was AS-BCT1 5'-CTG CTG CCT CCC GTA GG-3' (341F, Muyzer *et al.*, 1993). For fungal 18S-rDNA, the forward primer used was S-FUN1 5'-GGA AAC TCA CCA GGT CCA GA-3' (nu-SSU-1196, Borneman and Martin, 2000) and reverse primer was AS-FUN1 5'-ATT GCA ATG CYC TAT CCC CA -3' (nu-SSU-1536, Borneman and Martin, 2000). All cycles were programmed on a CFX apparatus (Biorad) to perform 35 cycles consisting of: 95 °C for 40 s, 64 °C for 45 s and 72 °C for 30 s with bacterial rDNA and 95 °C for 10 s, 62 °C for 30 s and 72 °C for 30 s with fungal rDNA. Total DNA concentration was measured with Smart Spec3000 at 260 nm (Biorad). The quality of the DNA extracted was checked by the ratios 260/280.

Statistical analysis

Pearson's correlation coefficients were calculated to relate the physicochemical parameters to DTPA-extractable metals and to the biochemical and microbial

parameters, using SPSS (Statistical Package for the Social Sciences) v.17 (SPSS, Inc.) software.

Results and Discussion

Extractable metals with DTPA-method

The percentage values of metal DTPA-extractions are shown in Fig. 1. The lowest extraction percentages were in the case of Pb, showing that this metal could be less available. The extractable Pb percentage was so similar among soils that establishing a Pb extractability pattern was not possible. The ECC content showed to play a relevant role in affecting metal extractability, mainly in the case of Cd, since significant and negative correlation was obtained between DTPA-extractable Cd and ECC content at Tt1 level (Table 2). We also observed that soils with higher proportions of coarse mineral fractions (L1 soil) or with minor proportions of fine fractions (H1 soil) showed the highest metal extractability. The significant and positive correlations obtained at both levels between DTPA-extractable Cu and Zn and the coarse sand content corroborated this observation.

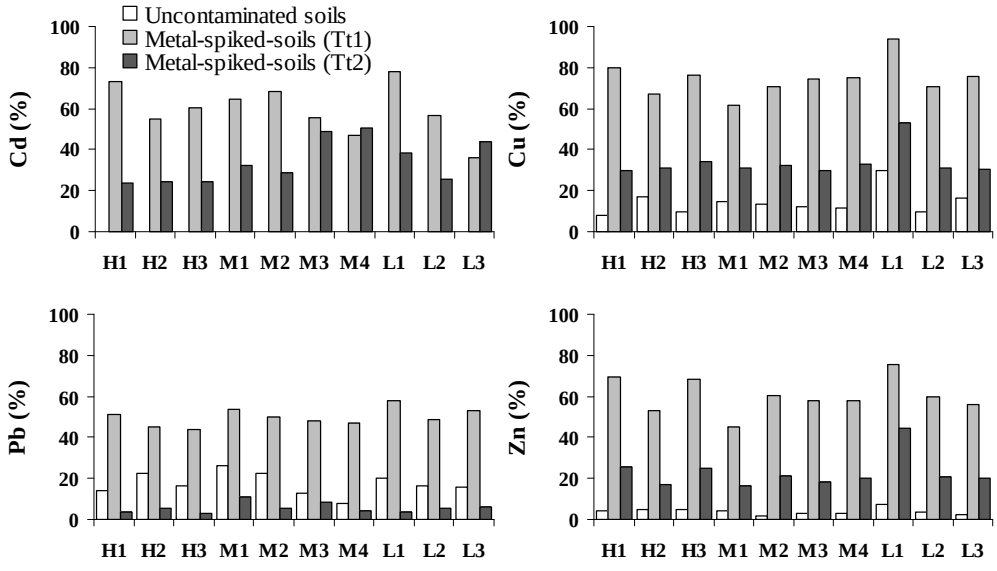

Fig 1 . Relative metal extractability (%) (DTPA-extractable metal vs. total metal content) in uncontaminated and metal-spiked soil samples. Tt1 = low metal amount, Tt2 = high metal amount.

Table 2. Pearson's correlation coefficients calculated between phosphatase (PHOS), urease (URE), β-galactosidase (BGAL), arylsulfatase (ARYS) enzyme activity values, bacterial DNA amounts, Fungal/Bacterial DNA ratio (F/B), DTPA-extractable metals and some soil physicochemical parameters in uncontaminated (Un.) and metal-spiked soil samples (Tt1 = low metal amount, Tt2 = high metal amount).

Level	Parameter	EC	OM	ECC	CS	cry-Fe	Total P	SS Mg	Total Pb
Un.	PHOS	0.019	0.393	-0.333	-0.066	0.503	**-0.684***	-0.008	-0.064
	ARYS	**0.839****	**0.739***	0.030	-0.490	0.501	-0.251	**0.841****	-0.106
	BGAL	-0.039	0.383	**-0.732***	-0.060	0.513	**-0.697***	0.131	-0.017
	URE	0.091	0.499	-0.278	-0.231	0.400	-0.375	0.027	0.249
	Bacterial DNA	0.255	0.241	-0.229	-0.230	0.063	0.271	0.221	0.039
	F/B DNA ratio	0.292	0.275	0.309	-0.098	-0.037	0.077	0.032	**0.742***
Tt1	DTPA-Cd	-0.109	0.382	**-0.808****	0.340	0.383	-0.510	0.063	0.117
	DTPA-Cu	-0.309	-0.226	-0.155	**0.906*****	-0.478	-0.014	-0.486	0.040
	DTPA-Zn	-0.246	0.031	-0.194	**0.778****	-0.126	-0.075	-0.376	0.006
	PHOS	0.211	0.569	-0.119	-0.058	**0.656***	-0.568	0.192	-0.250
	ARYS	**0.786****	**0.759***	0.067	-0.558	**0.634***	-0.267	**0.853****	-0.233
	BGAL	0.012	0.314	**-0.349**	-0.297	**0.780****	-0.612	0.253	-0.491
	URE	**0.697***	0.418	-0.001	**-0.872****	**0.634***	-0.243	**0.791****	-0.292
	Bacterial DNA	0.371	0.605	0.216	-0.247	0.297	0.513	0.351	0.082
	F/B DNA ratio	0.357	0.073	0.286	0.141	-0.169	0.132	0.025	0.521
Tt2	DTPA-Cu	-0.126	-0.323	-0.575	**0.710***	-0.501	-0.213	-0.234	-0.083
	DTPA-Zn	-0.232	-0.213	-0.478	**0.821****	-0.367	-0.270	-0.334	-0.102
	PHOS	0.199	0.475	-0.418	-0.222	**0.667***	**-0.653***	0.234	-0.200
	ARYS	**0.635***	**0.728***	-0.008	**-0.744***	**0.819****	-0.400	**0.843****	-0.225
	BGAL	-0.151	0.550	-0.468	-0.120	**0.684***	-0.586	0.092	-0.199
	URE	-0.244	-0.292	0.518	-0.063	-0.490	**0.688***	-0.490	**0.675***
	Bacterial DNA	0.383	**0.690***	0.061	-0.265	0.372	0.334	0.598	-0.080
	F/B DNA ratio	0.102	-0.288	0.370	-0.030	-0.254	0.342	-0.279	0.506

EC = electrical conductivity; OM =organic matter; ECC = equivalent $CaCO_3$; CS = coarse sand; cry-Fe = crystalline Fe oxides; SS Mg = Mg in soil solution. *, ** and *** indicate statistical significance at the probability level $p < 0.05$, $p < 0.01$ and $p < 0.001$ (n = 10).

Biochemical and microbial parameters

Biochemical parameters

Enzyme activities in uncontaminated soils were in general favoured in soils with higher recalcitrant OM content and higher proportion of fine mineral fractions (Fig. 2).

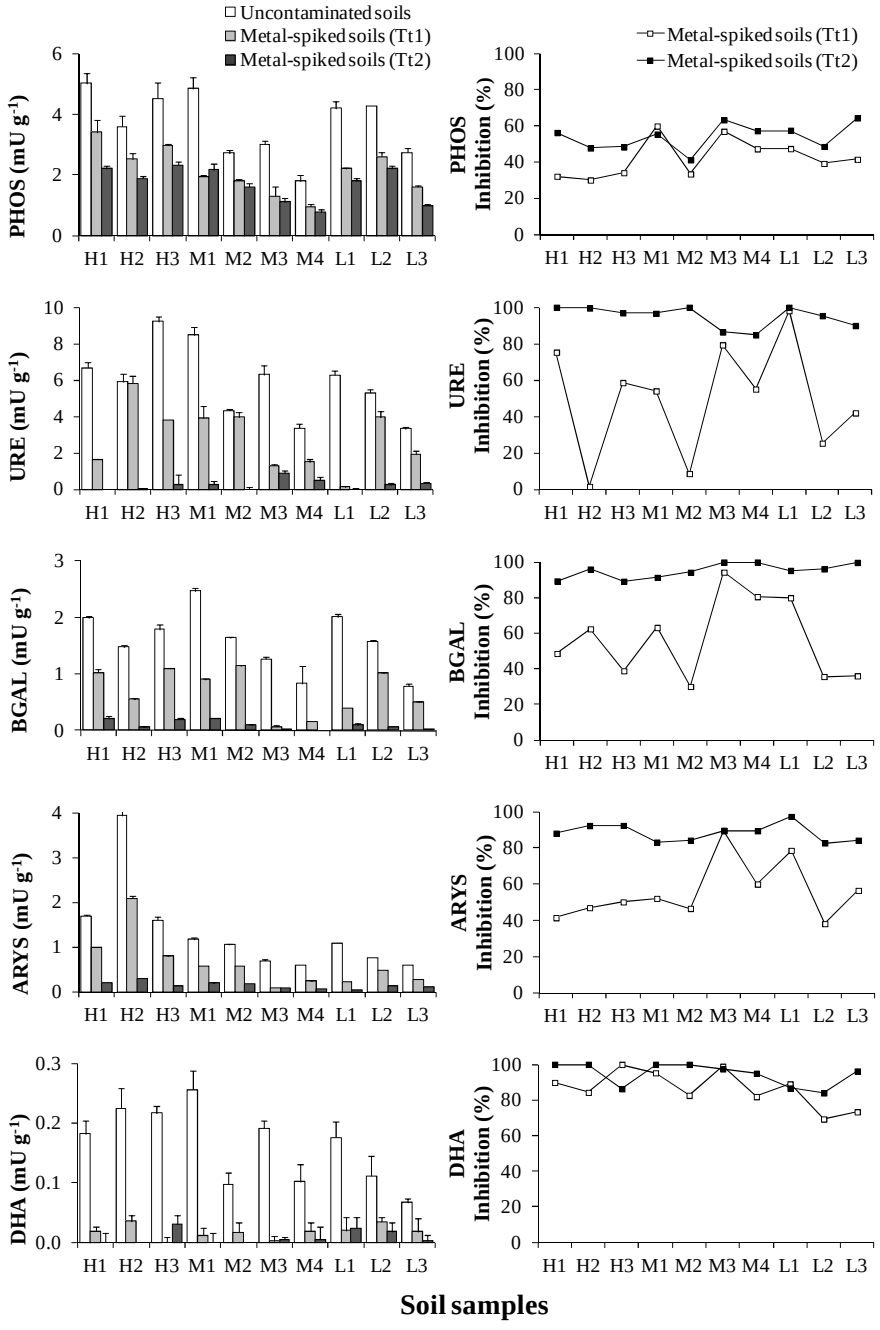

Fig 2. Phosphatase (PHOS), urease (URE), β-galactosidase (BGAL), arylsulfatase (ARYS), and dehydrogenase (DHA) activity values in uncontaminated and metal-spiked soil samples, and inhibition % (metal-spiked vs. uncontaminated soil samples). Tt1 = low metal amount, Tt2 = high metal amount. Vertical bars are Standard Errors.

Thus, high values of PHOS, URE, and BGAL activities in uncontaminated soils were obtained in M1 soil (71% recalcitrant fraction, 328 g kg^{-1} clay) and of ARYS activity in H2 soil (66% recalcitrant fraction, 215 g kg^{-1} silt). Accordingly, the lowest enzyme activities were often measured in soils with lower proportions of fine mineral fractions (M4 and L3 soils). The protective role exerted by the humus-hydrolase and/or clay-humus-hydrolase complexes against proteases (Burns *et al.*, 1972) could explain the relevance of these constituents in preventing the inhibition of enzyme activity.

As expected, all the enzyme activities decreased in metal-spiked soils respect to uncontaminated soils, although with different patterns (Fig. 2). These different inhibition patterns could be explained by the possible development of metal resistant microorganisms (Kunito *et al.*, 1999), which release enzymes poorly affected by high concentrations of metals in soil solution (Kunito *et al.*, 1998). Moreover, despite the high metal retention capacity characteristic of these soils, clear differences in the enzyme activity affectation were observed according to the level of contamination employed. In general, the sequence of inhibition percentage of enzyme activities, from the lowest to the highest values, was: PHOS (30-60%) < URE (1-98%) ≤ ARYS (38-90%) ≤ BGAL (30-94%) < DHA (69-100%) at Tt1 level and PHOS (41-64%) < ARYS (83-97%) < URE (85-100%) < BGAL (89-100%) < DHA (84-100%) at Tt2 level.

Hydrolase activities in many cases reached values close to 100% inhibition, mainly in M3, M4 and L1 soils, which could suggest that the active centre of the enzymes may be inactivated by the metals, preventing them from carrying out their hydrolytic action. In this sense, M3, M4 and L1 soils showed the lowest recalcitrant pool of OM. At the range of pH of soils of the present study, metals could complex with OM, being immobilized but still able to interact with the enzymes maybe with enzyme sulphydral groups (Marzadori *et al.*, 2000). In any case, the results highlighted that at Tt1 level, on one hand, not all the enzyme activities were affected in the same way, and, on the other hand, there was great variability among the different soils in the present study. In the case of metal-spiked soil samples at Tt2 level, the inhibition percentage of enzyme activities was so high and so similar among soils that establishing a clear discrimination among soil properties was not possible. The

uniformity of results at Tt2 level showed that the level of contamination of the mixture of metals added was the most important factor inhibiting the enzyme activity regardless of the soil components. Therefore, the discussion of the results from now on will be based on Tt1 level, except for PHOS activity.

PHOS activity and the total P showed a significant negative relationship (Table 2). Thus, the lowest PHOS activity was in M4 soil, with the highest total P content, with the highest PHOS activity being found in H1 soil, with the lowest total P content. This pattern could be attributed to regulation mechanisms of enzyme production by means of feed-back processes (Olander and Vitousek, 2000). In metal-spiked soil samples, PHOS activity was the least inhibited, suggesting that this enzyme, or the organisms that produce it, are less sensitive to the mixture of metals employed in this work, than for the other activities studied. The inhibition percentage of this enzyme was quite similar at both contamination levels, although it was slightly lower in soils with the highest OM content, favouring the persistence of total and immobilized PHOS activity (Pascual *et al.*, 2002).

The strong inhibition observed on URE, BGAL, and ARYS activities would indicate a disruption of key biological processes such as N mineralization / transformation, cellulose degradation, and organic S turnover. This is consistent with the results obtained by other authors for soils with slightly alkaline pH, who reported that URE, BGAL, and ARYS activities are sensitive bio-indicators of soil degradation caused by metal contamination (Hinojosa *et al.*, 2004; Belyaeva *et al.*, 2005; Martínez-Iñigo *et al.*, 2009). Regarding soil properties affecting the range of inhibition of these enzyme activities, we must highlight the great variability found among different soils, mainly in the case of URE activity at Tt1 level (Fig. 2).

In general, the inhibition percentage of URE, BGAL and ARYS activities at this level was higher in M3, M4, and L1 soils. These soils present a group of common characteristics that could enhance the greater inhibition of these enzymes. These soils have the highest coarse sand content and lower contents of recalcitrant OM, which could limit the physical stabilization of the enzymes in the medium through the formation of the clay-humus-hydrolase complexes mentioned above. The high inhibition in these soils would indicate that these soil constituents, and their properties, are essential for the enzyme protection to metal contamination in these

calcareous soils of the Mediterranean area. We observed that these M3, M4 and L1 soils had lower content of crystalline Fe oxide which could favour the formation of these complexes (Gianfreda *et al.*, 1995). This hypothesis could be corroborated by the significant and positive correlations obtained between these soil constituents and the enzyme activity values (Table 2). Similarly, the possible formation of metal-organic complexes could increase metal retention (Renella *et al.*, 2004) and mitigate enzyme inhibition by metals, and/or the organisms that produce them. In this sense, crystalline Fe oxides could bind and retain metals, making them less available to microorganisms.

It should be noted that the lowest inhibition percentage of URE activity at Tt1 level was produced in H2 and M2 soils, being about 90 fold less than in L1 soil, with the highest inhibition. This behaviour could be attributed to the higher content of divalent cations, Ca^{2+} and Mg^{2+}, in soil solution in both H2 and M2 soils. The presence of $CaCO_3$ in soils has been shown to lead to biological stabilization of both particulate organic carbon and humus, probably because Ca^{2+} provides bridges between negatively charged organic functional groups and negatively charged clay surfaces (Krull *et al.*, 2001). This fact could explain the positive and significant correlations obtained between Mg in soil solution and the electrical conductivity and the URE activity values (Table 2).

Our results showed a strong inhibition of DHA activity, suggesting a drop in soil microbial numbers. However, because metals such as Cu inhibit formazan colour development (Chander and Brookes, 1991), we calculated Pearson's correlations between total DNA concentration and DHA activity values (data not shown). A relationship was only established in the uncontaminated soil samples, making our results inconclusive.

Regarding DTPA extractions (Fig. 1), although we did not observe the same patterns between DTPA-extractable metals and enzyme activities, it appears that the same soil components and properties may be involved in both metal sorption and protection of enzyme activity. Since toxicity depends on the soil properties, the differences in these soil properties between samples explain the differences in the response to heavy metal contamination. On the other hand, as the toxicity occurs by the effect of the combination of various metals (Renella *et al.*, 2003), defining which

metals employed had the greatest effect on the different enzyme activities is not feasible under our experimental conditions and is not the subject of this work.

Microbial parameters

Total DNA concentration decreased in metal-spiked soil samples compared to uncontaminated ones (Fig. 3), with values of about 11-49% at Tt1 level and about 26-55% at Tt2 level. This high decrease reveals a disturbance of the microbial community, the main source of enzymes, which plays a crucial role in maintaining the main soil nutrient cycles. As applications of heavy metal concentrations to European standard levels (Directive 86/278/EEC) resulted in significant negative impacts on the microbial community, these reference values should be established according to soil characteristics (Moreno *et al.*, 2009). Nevertheless, as it seems that it is quite difficult to establish ranges according to soil characteristics and pH may be proper for a first discrimination, standards should be reviewed when there is more than one heavy metal contaminating soils, due to the fact that the adverse effects could increase.

Fungal-DNA represented about 9%, ranging from 3 to 17% of the total DNA quantified in the different uncontaminated soil samples. Although a high decrease occurred in some metal-spiked soil samples, we observed the same proportion of fungal-DNA amount in metal-spiked soils as in uncontaminated soils (8-9% of the total DNA). Similar results were obtained by Anderson *et al.* (2008) when studying fungal community composition after addition of sewage sludge containing heavy metals in a concentration below the maximum permissible limits in current United Kingdom legislation.

Bacterial-DNA in uncontaminated soil samples was about 82% of the total DNA quantified, ranging from 62 to 100% for the different soil samples (Fig. 3).

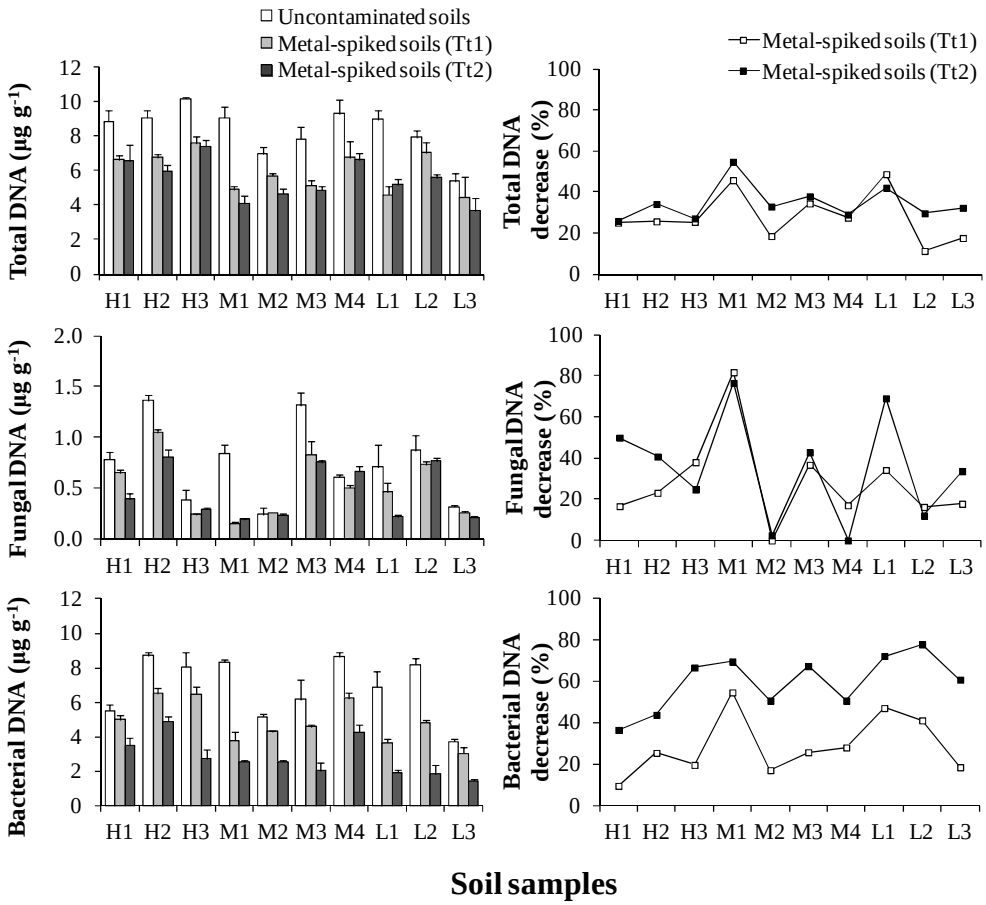

Fig 3. Total, fungal and bacterial DNA amounts in uncontaminated and metal-spiked soil samples and decrease % (metal-spiked vs. uncontaminated soil samples). Tt1 = low metal amount, Tt2 = high metal amount. Vertical bars are Standard Errors.

Bacterial biomass decreased strongly in metal-spiked soil samples, suggesting a higher sensitivity of the bacterial community to metals than the fungal community (Vig *et al.*, 2003). On the one hand, the soil fungal community shows higher adaptation capacity to stress conditions (Chander and Dyckmans, 2001), probably due to a variety of intrinsic and inducible properties of fungi that can ensure survival (Gadd, 2007). These processes of adaptation to metal stress may be accompanied by the exclusion of metal-sensitive fungal strains (Baldrian, 2003), finding different fungal populations but not necessarily different total fungal population size. On the other hand, the soil pH decreased by one unit in metal-spiked soil samples at Tt2 level as a result of metal salts added (data not shown). Since fungi generally exhibit wider pH ranges for optimal growth, it is possible that competitive interactions between

fungi and bacteria could stimulate the fungal community, inhibiting the bacteria (Rousk *et al.*, 2010).

The decrease in the amount of bacterial DNA was about 20% at Tt1 level (1-54%), and about 50% at Tt2 level (2-69%), showing high variability among soil samples. The smallest bacterial DNA decrease was produced in soils with the highest OM (H1, H2, and H3 soils), clay (M2 soil) and carbonate content (L3 soil). Pearson's correlation analysis corroborated the positive relationship between the OM content and the amount bacterial DNA (Table 2). As in the case of enzyme activities, these constituents play a key role in preventing the decrease of bacterial biomass, through physical stabilization and/or reduction of metal availability (Martínez-Iñigo *et al.*, 2009; Moreno *et al.*, 2009). However, no relationship was observed between patterns of hydrolytic enzyme activities and bacterial biomass, which has been previously reported (Piotrowska *et al.*, 2006). Trasar-Cepeda *et al.* (2008) attributed this lack of relation to the fact that most of the enzyme activities in cropped soils are not associated with active microorganisms and must therefore be stabilized by soil components, such as OM. In any case, our results showed that the soil properties and constituents that favour survival and persistence of microbial communities and enzyme activities to metal contamination are similar in these calcareous agricultural soils.

The fungal/bacterial ratio (Fig. 4) increased in some metal-spiked soils, mainly at Tt2 level, therefore involving a disturbance on the system. This disturbance could alter key soil processes such as the fungal decomposition of recalcitrant OM, in which fungi and bacteria are involved through complex interactions, both competitive and mutualistic (de Boer *et al.*, 2005). By Pearson's correlation analysis, we obtained positive correlations between the total Pb content and the fungal/bacterial ratio, being significant in uncontaminated soils (Table 2). This could be attributed to the fact that microbial population existing in soils with slightly higher amounts of Pb could more successfully resist an environmental impact from metal accumulation.

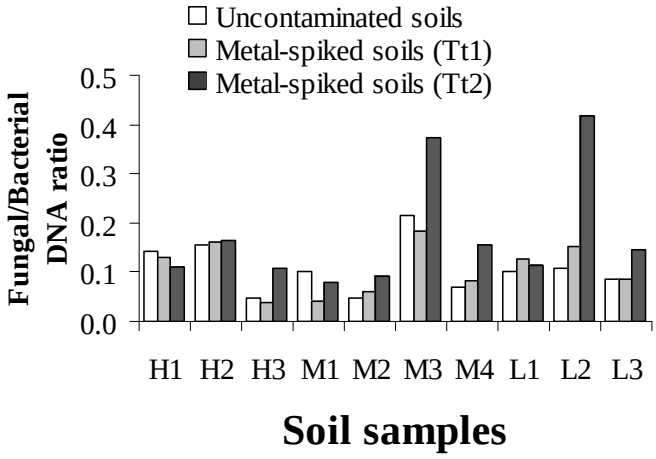

Fig 4. Fungal/Bacterial DNA ratios in uncontaminated and metal-spiked soil samples. Tt1 = low metal amount, Tt2 = high metal amount.

Even if microbial and fungal communities may be modified during the equilibration period due to drying-rewetting cycles, we consider that the higher impact is due to metal contamination by the extinction of species that lack sufficient tolerance to stress and the increase of the population of resistant species (Brandt *et al.*, 2010). The emergence of opportunistic organisms could be our case, since the decrease of bacterial biomass was much higher than that reached by total and fungal biomass.

Conclusions

We conclude that the mixture of metals added at levels consistent with European regulations to calcareous agricultural soils of the Mediterranean area resulted in a large inhibition of the biochemical and microbial parameters studied, under our experimental conditions. Our results hence highlight that although these soils have a high metal binding capacity; it does not mean that they are completely effective preventing adverse effects. These strong adverse effects, as well as the alteration of the fungal/bacterial ratio, could be decisive for microbial function and soil processes. Although pH could be the main characteristic that governs metal retention, the efficiency of this retention was controlled by other soil characteristics. Thus, mineral fraction, crystalline Fe oxides, carbonate, and organic matter were the main soil components affecting the persistence of enzyme activities and survival of bacterial and fungal populations to metal contamination in soils with these characteristics.

Since our results provide evidence that these soils could not withstand an environmental impact from metal accumulation and that the biochemical and microbial parameters are strongly affected, we believe that even at low concentrations a mixture of heavy metals could produce adverse effects on microfauna and hence the single limits should be revised when contamination is produced by a mixture of heavy metals. Nevertheless, further research work under field conditions is necessary.

Acknowledgements

This work was supported by the PESSAC department (INRA), Universidad Complutense de Madrid (UCM) and the Community of Madrid (CAM) through Grant GR58/08, Research Team 950605, and Network CARESOIL, Ref. P2009/AMB-1648. The authors gratefully acknowledge the help of Christelle Marrauld (PESSAC, INRA) in biochemical analyses and the comments and suggestions of the editor and the anonymous referees.

References

Acosta-Martínez, V., Zobeck, T.M., Gill, T.E., Kennedy, A.C., 2003. Enzyme activities and microbial community structure in semiarid agricultural soils. Biol. Fertil. Soils 38, 216-227.

Anderson, I.C., Parkin, P.I., Campbell, C.D., 2008. DNA- and RNA-derived assessments of fungal community composition in soil amended with sewage sludge rich in cadmium, copper and zinc. Soil Biol. Biochem. 40(9), 2358-2365.

Baldrian, P., 2003. Interactions of heavy metals with white-rot fungi. Enzyme and Microb. Technol. 32, 78-91.

Bastida, F., Moreno, J.L., Hernández, T., García, C., 2006. Microbiological degradation index of soils in a semiarid climate. Soil Biol. Biochem. 38, 3463-3473.

Batjes, N.H., 2000. Soil Vulnerability to Diffuse Pollution in Central and Eastern Europe SOVEUR Project (Version 1.0). FAO and ISRIC.

Belyaeva, O.N., Haynes, R.J., Birukova, O.A., 2005. Barley yield and soil microbial and enzyme activities as affected by contamination of two soils with lead, zinc or copper. Biol. Fertil. Soils 41, 85-94.

Borneman, J., Martin, R.J., 2000. PCR primers that amplify fungal rRNA genes from environmental samples. Appl. Environ. Microbiol. 66(10), 4356-4360.

Brandt, K.K., Frandsen, R.J.N., Holm, P.E., Nybroe, O., 2010. Development of pollution-induced community tolerance is linked to structural and functional resilience of a soil bacterial community following a five-year field exposure to copper. Soil Biol. Biochem. 42, 748-757.

Brookes, P.C., 1995. The use of microbial parameters in monitoring soil pollution by heavy metals. Biol. Fertil. Soils 19(4), 269-279.

Burns, R.G., Pukite, A.H., McLaren, A.D., 1972. Concerning the location and the persistence of soil urease. Soil Sci. Soc. Am. Proc. 36, 308-311.

Chander, K., Brookes, P.C., 1991. Is the dehydrogenase assay invalid as a method to estimate microbial activity in copper contaminated soils? Soil Biol. Biochem. 23, 909-915.

Chander, K., Dyckmans, J., 2001. Different sources of heavy metals and their long-term effects on soil microbial properties. Biol. Fertil. Soils 34, 241-247.

de Boer, W., Folman, L.B., Summerbell, R.C., Boddy, L., 2005. Living in a fungal world: impact of fungi on soil bacterial niche development. FEMS Microbiol. Rev. 29(4), 795-811.

Dick, R.P., Breakwell, D.P., Turco, R.F., 1996. Soil enzyme activities and biodiversity measurements as integrative microbiological indicators. In: Doran JW, Jones AJ (eds) Methods for assessing soil quality. Special publication N°49. Soil Sci. Soc. Am., Madison, WI, USA, pp. 247-271.

Directive 86/278/EEC of 12 June 1986 on the protection of the environment, and in particular of the soil, when sewage sludge is used in agriculture.

FAO, 2006. World reference base for soil resources. A framework for international classification, correlation and communication. FAO, Roma.

Gadd, G.M., 2007. Geomycology: biogeochemical transformations of rocks, minerals, metals and radionuclides by fungi, bioweathering and bioremediation. Mycol. Res. 111, 3-49.

Gangneux, C., Akpa-Vinceslas, M., Sauvage, H., Desaire, S., Houot, S., Laval, K., 2011. Fungal, bacterial and plant dsDNA contributions to soil total DNA extracted from silty soils under different farming practices: Relationships with chloroform-labile carbon. Soil Biol. Biochem. 43, 431-437.

Gianfreda, L., Rao, M.A., Violante, A., 1995. Formation and activity of urease-tannate complexes affected by aluminium, iron, and manganese. Soil Sci. Soc. Am. J. 59(3), 805-810.

Hinojosa, M.B., Carreira, J.A., García-Ruíz, R., Dick, R.P., 2004. Soil moisture pre-treatment effects on enzyme activities as indicators of heavy metal-contaminated and reclaimed soils. Soil Biol. Biochem. 36, 1559-1568.

IGME (Instituto Geológico y Minero de España), 1990. Mapa geológico de España nº 535. Escala 1:50.000 (Algete), Madrid.

ISRIC (International Soil Reference and Information Center), 2002. Procedures for Soil Analysis, 3th ed. International Soil Reference and Information Center, Wageningen.

Jiménez Ballesta, R., Conde Bueno, P., Martín Rubí, J.A., García Giménez, R., 2010. Pedo-geochemical baseline content levels and soil quality reference values of trace elements in soils from the Mediterranean (Castilla La Mancha, Spain). Eur. J. Geosci. 2(4), 441-454.

Kızılkaya, R., Aşkın, T., Bayraklı, B., Sağlam, M., 2004. Microbiological characteristics of soils contaminated with heavy metals. Eur. J. Soil Biol. 40, 95-102.

Krull, E.J., Baldock, J., Skjemstad, J., 2001. Soil texture effects on decomposition and soil carbon storage. Net Ecosystem Exchange CRC Workshop Proc., pp. 103-110.

Kunito, T., Oyaizu, H., Matsumoto, S., 1998. Ecology of soil heavy metal-resistant bacteria and perspective of bioremediation of heavy metal-contaminated soils. Recent Research Dev. Agric. Biol. Chem. 2, 185-206.

Kunito, T., Senoo, K., Saeki, K., Oyaizu, H., Matsumoto, S., 1999. Usefulness of the sensitivity-resistance index to estimate the toxicity of copper on bacteria in copper-contaminated soils. Ecotoxic. Environ. Saf. 44, 182-189.

Lafuente, A.L., González, C., Quintana, J.R., Vázquez, A., Romero, A., 2008. Mobility of heavy metals in poorly developed carbonate soils in the Mediterranean region. Geoderma 145, 238-244.

Lindsay, W.L., Norvell, W.A., 1978. Development of a DTPA soil test for zinc, iron, manganese and copper. Soil Sci. Soc. Am. J.42, 421-428.

Marchesi, J.R., Sato, T., Weightman, A.J., Martin, T.A., Fry, J.C., Hiom, S.J., Wade, W.G., 1998. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for Bacterial 16S rRNA. Appl. Environ. Microbiol. 64(2), 795-799.

Martínez-Iñigo, M.J., Pérez-Sanz, A., Ortiz, I., Alonso, J., Alarcón, R., García, P., Lobo, M.C., 2009. Bulk soil and rhizosphere bacterial community PCR–DGGE profiles and b-galactosidase activity as indicators of biological quality in soils contaminated by heavy metals and cultivated with *Silene vulgaris* (Moench) Garcke. Chemosphere 75, 1376-1381.

Marzadori, C., Francioso, O., Ciavatta, C., Gessa, C., 2000. Influence of the content of heavy metals and molecular weight of humic acids fractions on the activity and stability of urease. Soil Biol. Biochem. 32, 1893-1898.

McLaughlin, M.J., 2001. Aging of metals in soils changes bioavailability. Fact Sheet Environ. Risk Assess. 4, 1-6.

Mico, C., Recatalá, L., Peris, M., Sánchez, J., 2006. Assessing heavy metal sources in agricultural soils of an European Mediterranean area by multivariate analysis. Chemosphere 65, 863-872.

Moreno, J.L., Bastida, F., Ros, M., Hernández, T., García, C., 2009. Soil organic carbon buffers heavy metal contamination on semiarid soils: Effects of different metal threshold levels on soil microbial activity. Eur. J. Soil Biol. 45, 220-228.

Moreno Merino, L., 1998. Estudio de la influencia del suelo sobre la composición de las aguas subterráneas a través de la solución del suelo. Modelo en Fluvisoles calcáricos. PhD thesis, Universidad Complutense de Madrid.

Muyzer, G., de Waal, E.C., Uitierlinden, A.G., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59, 695-700.

Nannipieri, P., Greco, S., Ceccanti, B., 1990. Ecological significance of the biological activity in soil. In: Bollag J-M., Stotzky G. (Eds.). Soil Biochem., vol. 6. Marcel Dekker, New York, pp. 293-355.

Obbard, J.P., 2001. Ecotoxicological assessment of heavy metals in sewage sludge amended soils. Appl. Geochem. 16, 1405-1411.

Olander, L.P., Vitousek, P.M., 2000. Regulation of soil phospahatase and chitinase activity by N and P availability. Biogeochem. 49, 175-190.

Pascual, J.A., Moreno, J.L., Hernández, T., García, C., 2002. Persistence of immobilised and total urease and phosphatase activities in a soil amended with organic wastes. Bioresour. Technol. 82, 73-78.

Peris, M., Micó, C., Recatalá, L., Sánchez, R., Sánchez, J., 2007. Heavy metal contents in horticultural crops of a representative area of the European Mediterranean region. Sci. Total Environ. 378, 42-48.

Piotrowska, A., Iamarino, G., Rao, M.A., Gianfreda, L., 2006. Short-term effects of olive mill waste water (OMW) on chemical and biochemical properties of a semiarid Mediterranean soil. Soil Biol. Biochem. 38(3), 600-610.

Plassard, F., Winiarski, T., Petit-Ramel, M., 2000. Retention and distribution of three heavy metals in a carbonated soil: comparison between batch and unsaturated column studies. J. Contam. Hydrol. 42, 99-111.

Renella, G., Ortigoza, A.L.R., Landi, L., Nannipieri, P., 2003. Additive effects of copper and zinc on cadmium toxicity on phosphatase activities and ATP content of soil as estimated by the ecological dose (ED50). Soil Biol. Biochem. 35, 1203-1210.

Renella, G., Landi, L., Nannipieri, P., 2004. Degradation of low molecular weight organic acids complexed with heavy metals in soil. Geoderma 122, 311-315.

Renella, G., Mench, M., Landi, L., Nannipieri, P., 2005. Microbial activity and hydrolase synthesis in long-term Cd-contaminated soils. Soil Biol. Biochem. 37, 133-139.

Rivas-Martínez, S., 1987. Memoria del mapa de series de vegetación de España. 1: 400.000. ICONA. Madrid.

Roca-Perez, L., Gil, C., Cervera, M.L., Gonzálvez, A., Ramos-Miras, J., Pons, V., Bech, J., Boluda, R., 2010. Selenium and heavy metals content in some Mediterranean soils. J. Geochem. Explor. 107, 110-116.

Rousk, J., Bååth, E., Brookes, P.C., Lauber, C.L., Lozupone, C., Caporaso, J.G., Knight, R., Fierer, N., 2010. Soil bacterial and fungal communities across a pH gradient in an arable soil. Int. Soc. Microb. Ecol. J. 4(10), 1340-1351.

Rovira, P., Vallejo, V.R., 2000. Evaluating thermal and acid hydrolysis methods as indicators of soil organic matter quality. Commun. Soil Sci. Plant Anal. 31, 81-100.

Sayyad, G., Afyuni, M., Mousavi, S.F., Abbaspour, K.C., Richards, B.K., Schulin, R., 2010. Transport of Cd, Cu, Pb and Zn in a calcareous soil under wheat and safflower cultivation - A column study. Geoderma 154, 311-320.

Schaefer, R., 1963. Dehydrogenase activity as a measurement of the global biological activity of soil. Annales de l'Institut Pasteur 105, 326-331.

Sinsabaugh, R.L., Reynolds, H., Long, T.M., 2000. Rapid assay for amidohydrolase (urease) activity in environmental samples. Soil Biol. Biochem. 32(14), 2095-2097.

SISS (Società Italiana della Scienza del Suolo), 1985. Metodi Normalizzati di Analisi del Suolo. Edagricole, Bologna.

Speir, T.W., Ross, D.J., Feltham, C.W., Orchard, V.A., Yeates, G., 1992. Assessment of the feasibility of using CCA (copper, chromium and arsenic)-treated and boric acid-treated sawdust as soil amendments. II. Soil biochemical and biological properties. Plant Soil 142, 249-258.

Tabatabai, M.A., Bremner, J.M., 1970. Arylsulfatase activity in soils. Soil Sci. Soc. Am. Proc. 34, 225-229.

Tabatabai, M.A., 1994. Soil enzymes. In: Weaver, R.W., Angle, J.S., Bottomley, P.S. (Eds.), Methods of Soil Analysis: Microbial and Biochemical Properties. Part 2. ASA, Madison, America, pp. 775-833.

Trasar-Cepeda, C., Leirós, M.C., Seoane, S., Gil-Sotres, F., 2008. Biochemical properties of soils under crop rotation. Appl. Soil Ecol. 39, 133-143.

Vig, K., Megharaj, M., Sethunathan, N., Naidu, R., 2003. Bioavailability and toxicity of cadmium to microorganisms and their activities in soil: a review. Adv. Environ. Res. 8, 121-135.

6. Discusión

Discusión

Los suelos agrícolas periurbanos del sureste de la Comunidad Autónoma de Madrid están sometidos en la actualidad a una intensa presión derivada de la creciente industrialización y urbanización. Los metales pesados son los contaminantes inorgánicos más tóxicos que se encuentran en los suelos, siendo algunos de ellos tóxicos incluso a muy bajas concentraciones (Siegel, 2002). En un trabajo paralelo a la presente Tesis Doctoral, la Red de Investigación CARESOIL (P2009/AMB-1648), a la que pertenezco, ha llevado a cabo estudios de patrones espaciales de distribución de metales pesados –Cd, Cu, Pb y Zn– en un rectángulo de 30 × 35 km (1.050 km^2) en el sureste del área metropolitana de Madrid, en el cual 125 emplazamientos fueron muestreados (Vázquez de la Cueva *et al.*, enviado).

Los primeros resultados mostraron que las concentraciones de estos metales aumentan en dirección sureste-noroeste, es decir, mostrando un gradiente de concentraciones metálicas que incrementa con la proximidad a la ciudad de Madrid, la principal fuente de contaminación. No obstante, los valores medios alcanzados para

los cuatro metales estudiados fueron inferiores a los obtenidos en otras áreas periurbanas de ciudades con una mayor historia industrial (Frangi y Richard, 1997; Imperato *et al.*, 2003; Kelly *et al.*, 1996; Manta *et al.*, 2002). Debido a que la contaminación en Madrid tiene lugar desde hace relativamente poco tiempo, el enriquecimiento en metales pesados en el suelo es más concordante al encontrado en ciudades de países en desarrollo, con una industrialización más reciente (Hu *et al.*, 2006; Xia *et al.*, 2011; Yang *et al.*, 2009).

En la presente Tesis, y como parte de objetivo principal de la Red CARESOIL, hemos seleccionado como zona de estudio un conjunto de Fluvisoles calcáricos (n = 10) de uso agrícola de la finca experimental "El Encín", emplazada en el sureste de Madrid (Alcalá de Henares), y gestionada por el Instituto Madrileño de Investigación y Desarrollo Rural, Agrario y Alimentario –IMIDRA– (Fig. 1). Esta población forma parte de un eje periurbano que combina la actividad agrícola y los principales usos del territorio residencial, empresarial e industrial de la Comunidad de Madrid, siendo un escenario representativo de las áreas metropolitanas europeas donde el recurso *suelo* está siendo afectado (Steinitz *et al.*, 2011). En este contexto, el estudio de la posible degradación de los Fluvisoles calcáricos, típicos de esta zona, es particularmente relevante debido a su alto valor agronómico y económico.

Fig 1. Área de estudio.

En el presente estudio, tal y como se esperaba, el contenido total de Cd, Cu, Pb y Zn en los suelos fue similar al obtenido por otros autores en suelos agrícolas del área mediterránea (Acosta *et al.*, 2011; Jiménez Ballesta *et al.*, 2010; Micó *et al.*, 2006; Peris *et al.*, 2007; Ramos-Miras *et al.*, 2011) y en ningún caso se excedieron los valores límite de metales pesados en suelos propuestos por la Directiva Europea vigente

(Directiva 86/278/EEC). Sin embargo, basándonos en la premisa de que las muestras de suelo seleccionadas presentan un riesgo potencial de contaminación debido a su ubicación, se adicionó una mezcla de Cd, Cu, Pb y Zn con el objetivo de simular la situación que se produciría en un suelo contaminado multielemental. Con este fin, se seleccionaron dos niveles de contaminación metálica dentro de los límites propuestos por la Directiva 86/278/CEE: el nivel Tt1, que corresponde a los valores límite de concentración de metales pesados en suelos (3 mg kg^{-1} Cd + 140 mg kg^{-1} Cu + 300 mg kg^{-1} Pb + 300 mg kg^{-1} Zn) y el nivel Tt2, que corresponde a las concentraciones semi-máximas permitidas en lodos de depuradora (20 mg kg^{-1} Cd + 875 mg kg^{-1} Cu + 600 mg kg^{-1} Pb + 2000 mg kg^{-1} Zn).

Empleando estos niveles de contaminación, se llevó a cabo un estudio de sorción donde se confirmó que la capacidad de sorción metálica de estos suelos es muy elevada, siendo 64-92 % en el caso del Cd y 94-100 % en el caso de Cu, Pb y Zn (Apartados 3.1 y 3.3). A partir de estos datos analíticos, se calcularon los Coeficientes de Distribución (Kd). Altos valores de Kd indican una elevada sorción en la fase sólida del suelo, mientras que bajos valores de Kd muestran elevadas concentraciones de metales en la solución (Anderson y Christensen, 1988). Este coeficiente mostró que el Pb presentaba la mayor sorción, siendo ésta más elevada en suelos con mayores contenidos en arcilla y carbonato, lo que sugirió que estos constituyentes juegan un papel clave en el control de la sorción metálica en estos suelos.

Sin embargo, una elevada sorción no necesariamente implica una alta retención y los constituyentes implicados en la sorción metálica no siempre se corresponden con aquellos que controlan la desorción. Por ello, nos planteamos que el siguiente paso a seguir sería ahondar en el estudio del papel que estos constituyentes ejercen en la desorción metálica. Con este fin, y basándonos en la bibliografía, se decidió utilizar un conjunto de métodos de extracción química simple de metales, que *a priori* nos permitiría estimar las fracciones móviles y potencialmente móviles de los metales en los suelos (Gupta *et al.*, 1996).

No obstante, a la hora de llevar a cabo estos estudios en condiciones de laboratorio, tuvimos que tener en cuenta que la contaminación tuvo lugar mediante la adición de sales metálicas solubles. Este hecho podría sobrestimar la fracción metálica móvil o disponible (Fig. 2), ya que ésta es máxima inmediatamente después de la

entrada de los metales en el suelo (Gray *et al.*, 1998; Lock y Janssen, 2003; McLaughlin, 2001). Transcurrido un tiempo de contacto, los iones metálicos se redistribuyen desde fracciones de unión más débil –solubles en agua y fracciones de intercambio– a fracciones en las que se establecen uniones de mayor fuerza –carbonatada, óxido de hierro (Fe) y manganeso (Mn), orgánica y residual–, disminuyendo, por tanto, su disponibilidad (Han y Banin, 1999; Jalali y Khanlari, 2008; Lu *et al.*, 2005). Este fenómeno se conoce como efecto *aging* y se reconoce como un factor crítico cuando se llevan a cabo estudios de disponibilidad de metales (McLaughlin, 2001).

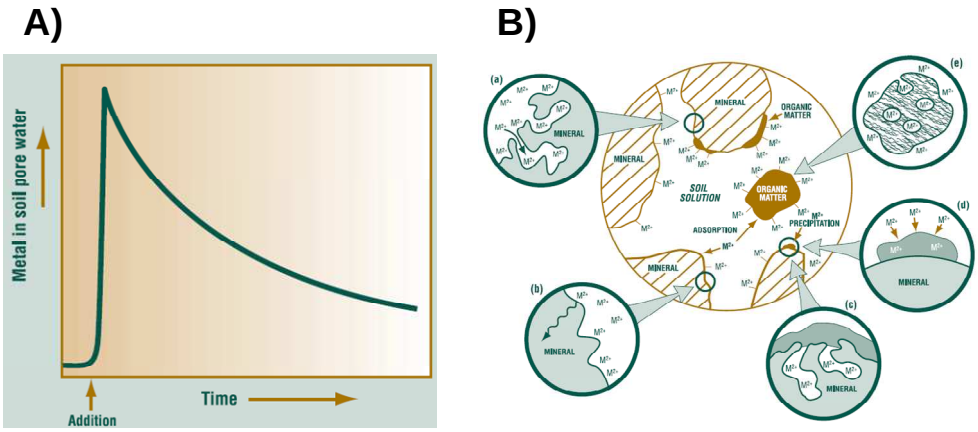

Fig 2. Evolución de la concentración metálica en la solución del suelo con el tiempo de contacto (A) y procesos de adsorción de metales y *aging* en los suelos (B): difusión en superficie en los poros (a), difusión en estado sólido (b), oclusión a través de mecanismos de precipitación (c), precipitación de nuevas fases sólidas (d) y oclusión en la materia orgánica (e) (McLaughlin, 2001).

Por ello, cada muestra de suelo se mezcló con la solución metálica correspondiente (nivel Tt1 o Tt2) y se mantuvo en contacto durante un periodo de 12 meses, a temperatura ambiente sin cobertura ni drenaje. Paralelamente, se añadió agua destilada a muestras de suelo que fueron consideradas como control. Durante el periodo de contacto, los suelos se secaron al aire, se mezclaron y se re-humectaron con agua destilada en ciclos de, aproximadamente, dos semanas. A diferentes intervalos de tiempo (1 día, 1, 3, 6 y 12 meses), se tomaron sub-muestras en las que se realizaron extracciones químicas simples de metales. En el primer Bloque de

Resultados, concerniente a los Apartados 3.1, 3.2 y 3.3, se recogen todos los análisis efectuados relativos a las extracciones químicas de los metales.

El Apartado 3.1 se centra en el estudio de las propiedades edáficas que controlan los patrones de extractabilidad de los metales seleccionados en el nivel de baja contaminación (Tt1) al cabo de 12 meses de contacto. Para ello, se emplearon tres extractantes químicos, $NaNO_3$, LMWOA (mezcla de ácidos orgánicos de bajo peso molecular) y DTPA.

En el Apartado 3.2, se estudiaron las tendencias temporales de las concentraciones metálicas extraídas, con $NaNO_3$ y DTPA, a lo largo de un periodo de 12 meses de incubación. Se hizo especial hincapié en el papel jugado por la dosis metálica aplicada (Tt1 o Tt2) y por las fracciones edáficas.

El Apartado 3.3 complementó el estudio realizado en el Apartado 3.1, incluyendo un mayor número de métodos de extracción simple y los dos niveles de contaminación. La fracción metálica móvil fue estimada con los métodos $CaCl_2$ 0,01M, $MgCl_2$ 1M, $NaNO_3$ 0,1M, y NH_4NO_3 1M y la potencialmente móvil con DTPA 5mM, EDTA 0,05M, HNO_3 0,5M, HAc 0,11M, LMWOA 10mM y NH_4Ac 1M. Los datos analíticos derivados de las extracciones químicas fueron discutidos en función de las propiedades físico-químicas de los suelos, datos mineralógicos de los suelos control y contaminados, y porcentajes de las diferentes especies químicas metálicas en los extractos realizados, predichos por el programa Visual Minteq.

La primera evaluación que se llevó a cabo fue el estudio de las tendencias temporales de las concentraciones extraídas de los metales con $NaNO_3$ y DTPA. En el análisis de los resultados (Apartados 3.1 y 3.2), se observó que los porcentajes de extracción de los metales con $NaNO_3$, en general, disminuyeron con el tiempo de contacto del experimento de incubación, atribuido a una redistribución metálica, lo que implicaría una menor concentración de los metales en las posiciones de intercambio. Los resultados mostraron que la fracción carbonatada fue el factor principal que controló las tendencias temporales de la concentración de los metales con este método. Así, el papel ejercido por esta fracción fue más importante, que el de la dosis metálica, en determinar las tendencias temporales de la extractabilidad de Cd y Cu, lo que condujo a que las concentraciones metálicas de ambos niveles tendiesen a igualarse a los 12 meses de contacto. A pesar de que el nivel de

contaminación fue determinante en el caso de las tendencias temporales de Zn extraído con $NaNO_3$, la fracción carbonatada más fina (caliza activa, CA) mostró jugar un papel más importante. Las bajas concentraciones de Pb extraídas, atribuido a procesos de precipitación, no nos permitieron estudiar las tendencias temporales de este metal. En el caso de la movilidad metálica potencial, estimada por el método del DTPA, las concentraciones de los metales extraídos no alcanzaron el equilibrio dentro del tiempo de incubación, destacando que el nivel de contaminación fue el factor más importante en el control de las tendencias temporales. Sin embargo, la acción combinada de las fracciones carbonatada, orgánica, óxido de hierro y arcilla determinaron las diferentes tendencias temporales observadas, para cada metal, en ambos niveles.

Por otra parte, los resultados pusieron de manifiesto que procesos competitivos podrían estar afectando a estas tendencias, por lo que futuras investigaciones en esta dirección serían de gran interés. Ya que los procesos de *aging* han mostrado jugar un papel clave en la extractabilidad de los metales en estos suelos, se ha corroborado que, antes de llevar a cabo estudios de disponibilidad metálica en condiciones de laboratorio, se debe mantener la mezcla metálica en contacto con el suelo durante un tiempo determinado (Zapusek y Lestan, 2009). No obstante, tal y como se discute en el Apartado 3.2, este tiempo fue diferente en función de varios factores, como son la dosis metálica, la naturaleza del metal y las propiedades de los suelos.

Paralelamente, se analizó la eficiencia de los métodos de extracción empleados. En el análisis de los resultados reflejado en los Apartados 3.1, 3.2 y 3.3, se observó que el mayor porcentaje de metales extraído fue con extractantes complejantes y ácidos, siendo el menor con sales neutras. No obstante, es en el Apartado 3.3 donde se hace un análisis más exhaustivo de la eficiencia de extracción de los métodos empleados en las muestras de suelo que permanecieron 12 meses de contacto con las mezclas metálicas. Los bajos porcentajes de metales extraídos con sales neutras fueron atribuidos al hecho de que el rango de pH de estos suelos (~ 8,1-8,7) favorece uniones fuertes e irreversibles de los metales con la materia orgánica (MO) y los minerales de carga variable mediante procesos de adsorción específica. Dentro de las sales neutras, es una excepción el método del $MgCl_2$ 1M, con el cual se cuantificaron

concentraciones metálicas muy elevadas. Este hecho pudo ser debido a: i) su mayor concentración, ii) el efecto combinado de la acción complejante del cloruro y el desplazamiento de metal del complejo de cambio por Mg^{2+} (Meers *et al.*, 2007), y iii) una leve disolución del carbonato (Gleyzes *et al.*, 2002). Los cálculos termodinámicos del programa Visual Minteq fueron empleados para determinar la especiación de los metales en los extractos, que depende de la composición química y del pH de las soluciones (Ettler *et al.*, 2007; Pérez-Esteban *et al.*, 2013).

En el caso de los extractos con HNO_3, se extrajeron concentraciones metálicas muy elevadas, predominando la proporción de ión metálico libre, atribuido bien a la disolución de alguno de los componentes del suelo, bien al desplazamiento de los metales del complejo de cambio por el exceso de H^+ (Vidal *et al.*, 2004).

La alta proporción de metales extraídos con EDTA (60-82%) se atribuyó a su elevada constante de estabilidad de quelación (Meers *et al.*, 2007). Además, el pH de los suelos favoreció una elevada formación de complejos solubles, ~ 100 %, en los extractos de EDTA y DTPA (Me-EDTA^{-3} y Me-DTPA^{-2}). No obstante, este hecho podría dar lugar a que la extracción metálica fuese menor en aquellos suelos con un alto contenido en materia orgánica debido a procesos de re-adsorción en dicha fracción (Ettler *et al.*, 2007; Peters, 1999). Esto podría estar corroborado por las correlaciones significativas negativas obtenidas en todos los casos entre los metales extraídos con EDTA y el contenido en MO (Apartado 3.3).

En el caso de las extracciones con HAc y NH_4Ac, la especiación estuvo dominada por complejos acetato, encontrando mayor proporción de metal libre conforme decrece el valor de pH de los extractos. En las extracciones metálicas con la mezcla LMWOA, la mayor constante de quelación del ácido cítrico condujo a que la especie predominante fuese el Me-citrato$^-$ ($\leq$ 97 %). No obstante, las menores constantes de estabilidad de los complejos de Cd y Pb con citrato, dieron lugar a que se produjese un mayor porcentaje de extracción de Cu y Zn (Barton y Abadía, 2006). En definitiva, los elevados porcentajes de extracción metálica con métodos de extracción de mayor fuerza mostraron que existe una significativa fracción potencialmente disponible de metales pesados en estos suelos (Gupta *et al.*, 1996); indicando que, a pesar de su elevada capacidad de sorción, la retención no sería del todo eficaz. Los resultados, por tanto, implican que se podría producir una

removilización metálica ante un cambio en las condiciones del medio (Plassard *et al.*, 2000; Sayyad *et al.*, 2010). Este hecho entrañaría un elevado riesgo de contaminación de otros compartimentos del ecosistema y la posible entrada de contaminantes en la cadena trófica, y pone de manifiesto la vulnerabilidad de estos suelos ante una acumulación metálica (Batjes, 2000).

Por ello, cabe preguntarse cuáles son los constituyentes y propiedades de estos suelos que están controlando la extractabilidad de los metales con estos procedimientos químicos y que, por tanto, podrían, bien favorecer, bien minimizar, una posible removilización de los metales. En este sentido, tal y como concluimos en los Apartados 3.1, 3.2 y 3.3, el contenido en carbonato cálcico equivalente (CCE), por sí solo, no explica los patrones de extractabilidad de los metales. De este modo, observamos que cada metal, añadido como mezcla multielemental, mostraba diferentes patrones y que éstos difirieron, en algunos casos, en función del nivel de contaminación estudiado.

Cuando se investigó el comportamiento del Cd, se observó que la extractabilidad de este metal, con los métodos de extracción relacionados tanto con las fracciones móviles, como con las potencialmente móviles, estuvo controlada por varios constituyentes del suelo pertenecientes a la fracción carbonatada, como son el CCE, la CA, el calcio (Ca) y magnesio (Mg) total, la calcita y la dolomita– (Apartados 3.1 y 3.3). La importancia de esta fracción edáfica en el control de la extractabilidad de Cd también se puso de manifiesto en el estudio recogido en el Apartado 3.2. En este estudio se observó que esta fracción determinó la tendencia temporal de la extractabilidad de Cd con $NaNO_3$, disminuyendo ésta drásticamente durante el primer mes de contacto, y alcanzando el equilibrio a partir de entonces, tanto en el nivel Tt1 (en todos los casos) como en el nivel Tt2 (en suelos con mayor contenido en carbonato).

Con el fin de detectar una posible asociación del Cd a la fracción carbonatada, se seleccionó una muestra de suelo de elevada proporción en CCE, contaminada con el nivel Tt2, al principio y al final del experimento de incubación, y se caracterizó por microscopía electrónica de transmisión –TEM– usando espectroscopía de energía dispersiva –EDS– (Fig. 3). Esta técnica espectroscópica permite detectar la distribución elemental en las muestras, por lo que, además de los metales de estudio –

Cd, Cu, Pb y Zn–, se consideraron otros elementos químicos –Ca, Mg, silicio (Si), Fe, carbono (C) y aluminio (Al)– representativos de las diferentes fracciones del suelo (Sayen *et al.*, 2009).

Sin embargo, la técnica mostró ciertas limitaciones, que impidieron que, finalmente, se incluyese en los Capítulos de Resultados presentados en este trabajo de Tesis. Por un lado, la preparación de la muestra requiere el empleo de una resina con base carbonada, por lo que no se pudieron estudiar las asociaciones de los metales con la fracción orgánica del suelo, y la detección del carbonato hubo de hacerse de forma indirecta mediante el análisis de la distribución de Ca y Mg. Por otro lado, debido al límite de cuantificación del equipo, se requieren tiempos de exposición demasiado largos.

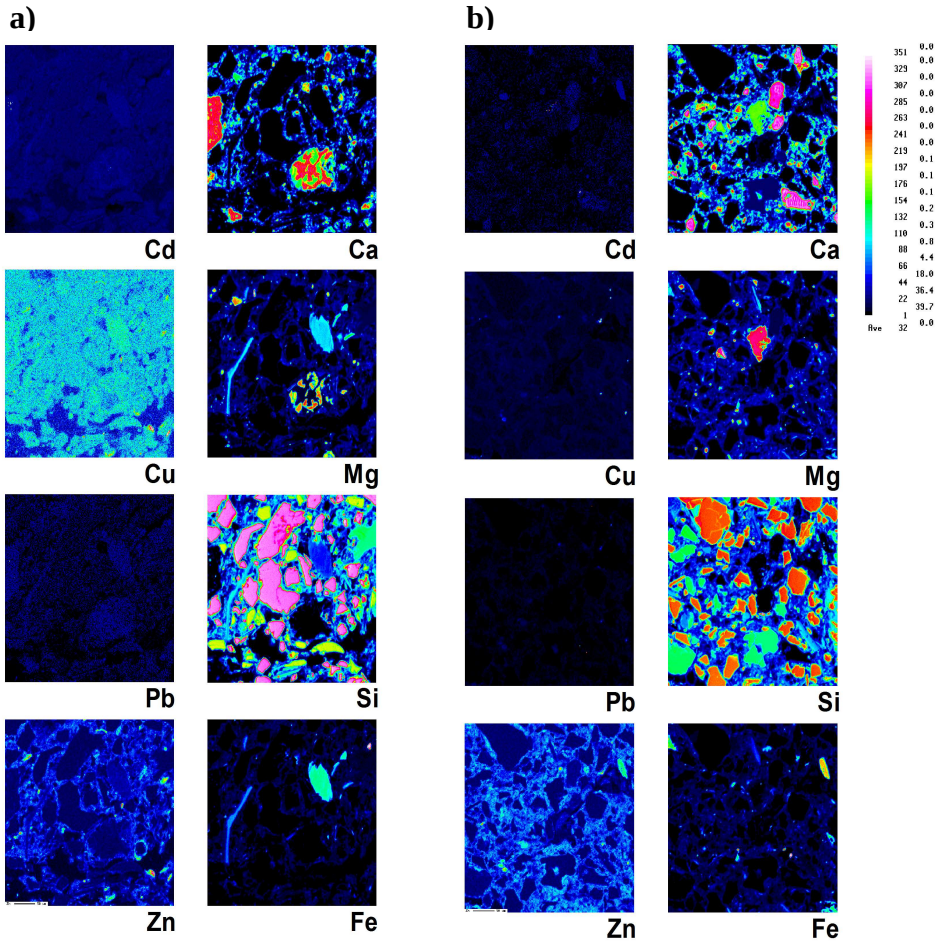

Fig 3. Análisis de microscopía electrónica de transmisión –TEM– usando espectroscopía de energía dispersiva –EDS– en la muestra de suelo M1 en 1 semana (a) y a los 12 meses (b) de tiempo de contacto en el nivel Tt2. La nomenclatura de las muestras corresponde a la de los Apartados 3.1, 3.2 y 3.3.

De las figuras mostradas se deduce que el tiempo de exposición empleado no fue suficiente para detectar apropiadamente la distribución de los metales objeto de estudio, Cd, Cu, Pb y Zn (Fig. 3). Además, también supuso un impedimento, para la correcta interpretación de los resultados, el hecho de que los metales presentasen una distribución relativamente homogénea. No obstante, ciertas asociaciones observadas entre elementos merecen ser comentadas. En primer lugar, en las imágenes se observó que aquellas regiones con mayor proporción de Cd coinciden con aquellas con mayor proporción de Ca, mostrando la fuerte asociación entre estos dos elementos, tanto al principio del experimento de incubación como al final, lo que podría indicar una asociación de Cd con la fracción carbonatada. También se observaron resultados interesantes en el caso del Zn, como se discutirá más adelante.

A la vista de esta situación, se optó por llevar a cabo una investigación mineralógica empleando Difracción de Rayos X –DRX– (Apartado 3.3). Con el empleo de esta técnica, no se detectó la formación de carbonato de Cd cristalizado, lo que nos hizo suponer que el proceso que podría estar controlando la extractabilidad de este metal fuese el de fijación, mediante la difusión de Cd en los defectos de los cristales o los poros de la caliza (Buekers *et al.*, 2007). No obstante, teniendo en cuenta que las concentraciones de Cd añadidas estaban por debajo del límite de cuantificación del equipo de DRX, no descartamos la opción de que una fracción del Cd añadido se encontrase precipitada en la superficie de los minerales. Sin embargo, se debe tener en cuenta que es posible que, en el nivel Tt2, las posiciones de adsorción específica estén saturadas, quedando una fracción de Cd en posiciones de unión más débil, como las posiciones de intercambio catiónico. Esta hipótesis podría estar apoyada por las correlaciones positivas obtenidas con el Cd extraído con $MgCl_2$, tanto con la Capacidad de Intercambio Catiónico (CIC), como con el contenido en arcilla (Apartado 3.3).

Finalmente, a pesar de que la fracción carbonatada mostró ser clave en el control de la extractabilidad de Cd, cabe señalar que también se puso de manifiesto que otros constituyentes del suelo, como la MO y los óxidos de Fe cristalino (Krishnamurti y Naidu, 2003; Prokop *et al.*, 2003), también podrían jugar un papel clave en la regulación de los procesos de sorción-desorción de Cd en estos suelos,

principalmente cuando éste se encuentra a elevadas concentraciones como en el nivel Tt2 de este estudio.

El estudio de los constituyentes que controlan los patrones de extractabilidad de Cu fue más complejo. Al igual que en el caso del Cd, la extractabilidad de Cu con $NaNO_3$ disminuyó con el tiempo de contacto del experimento, indicando una posible redistribución de este metal con el tiempo en las diferentes fracciones del suelo, disminuyendo la concentración de Cu en posiciones de cambio (Sayen *et al.*, 2009). La velocidad de esta redistribución fue mayor en aquellos suelos con mayor proporción de CCE, durante el primer mes en el nivel Tt1, aunque se prolongó hasta los 6 meses en el nivel Tt2 para aquellos suelos con bajas proporciones de CCE (Apartado 3.2.). Estos resultados indicarían *a priori* que la fracción carbonatada controlaría los procesos de sorción-desorción de Cu. Paralelamente, se observó que en el nivel Tt1, la extractabilidad de Cu con LMWOA estaba negativamente relacionada con el contenido en MO, a las 24 horas de contacto, y con el de CCE, a los 12 meses. Esto nos hizo pensar que sería posible que MO y carbonato compitan en el proceso de retención de Cu a lo largo del experimento de incubación (Apartado 3.1). En este sentido, la fracción recalcitrante de la MO (RP) fue el parámetro que pareció determinar las dinámicas temporales de la concentración de Cu extraído con DTPA, a pesar de que a los 12 meses de contacto fue la fracción limo la que explicó la desorción de Cu con este método.

Con el fin de aclarar esta cuestión, se llevó a cabo un estudio más exhaustivo en el Apartado 3.3. En éste, observamos que la extractabilidad de Cu en el nivel Tt1 podría estar controlada, en parte, por la fracción carbonatada, a través de procesos de adsorción, principalmente en la fracción más fina, la CA, descartándose un posible fenómeno de precipitación, al no encontrar evidencias de la formación de carbonato de Cu mediante DRX. Del mismo modo, se observó que otros constituyentes de la fracción mineral más fina del suelo < 2 μm, como óxidos de Fe y filosilicatos, también ejercían un importante papel en la disminución de la extractabilidad de Cu a bajas dosis, principalmente con DTPA. En este contexto, la presencia de Fe podría aumentar la capacidad de adsorción de minerales de menor superficie específica como la mica y la ilita (Sipos *et al.*, 2008). Por otra parte, concluimos que el tiempo de contacto del experimento de incubación podría haber favorecido la redistribución de

Cu a las fracciones orgánica y óxido de Fe, quedando una parte significativa del Cu añadido inmovilizado en estas fracciones y disminuyendo, por tanto, su disponibilidad (Buekers *et al.*, 2007; Jalali y Khanlari, 2008). En este sentido, la formación de asociaciones organominerales, probablemente de tipo Fe-humato (Besnard *et al.*, 2001; Sayen y Guillon, 2010; Sipos *et al.*, 2008), favorecidas por el grado de humificación de la MO de estos suelos (relación C/N ~ 11), podría condicionar la menor desorción de este metal en ambos niveles Tt1 y Tt2.

Por todo ello, pudimos concluir que los coloides del suelo, tanto de naturaleza inorgánica como orgánica, están controlando la extractabilidad de Cu en estos suelos y, por tanto, su disponibilidad. Estos resultados ponen de manifiesto la posible vulnerabilidad de estos suelos, ya que un aumento en la mineralización de la MO (muy común en el clima mediterráneo) podría afectar a la capacidad de almacenamiento, movilidad y disponibilidad de Cu, conllevando un riesgo de contaminación ambiental (Hernández-Soriano y Jiménez-López, 2012; Martínez *et al.*, 2003).

La elevada sorción de Pb, mostrada por los Kd calculados, se vio también reflejada en la baja extractabilidad de este metal, obtenida con métodos de extracción química de menor fuerza, como las sales neutras (Apartados 3.1, 3.2 y 3.3). La baja extractabilidad de Pb se produjo desde el principio del experimento, mostrando que la redistribución de este metal, en las diferentes fracciones del suelo, pudo tener lugar en un tiempo, probablemente, anterior a las 24 horas de contacto (Jalali y Khanlari, 2008).

Con el fin de dilucidar si la rápida sorción, y elevada retención, se debía a que una proporción de Pb se encontraba en la fracción carbonatada, se llevó a cabo un procedimiento de extracción secuencial (BCR; European Community Bureau of Reference), al principio y al final del experimento de incubación, en las muestras contaminadas en el nivel Tt2 (Fig. 4). Estos análisis se efectuaron durante una estancia científica realizada en la División de Química del Departamento de Tecnología del Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas –CIEMAT–

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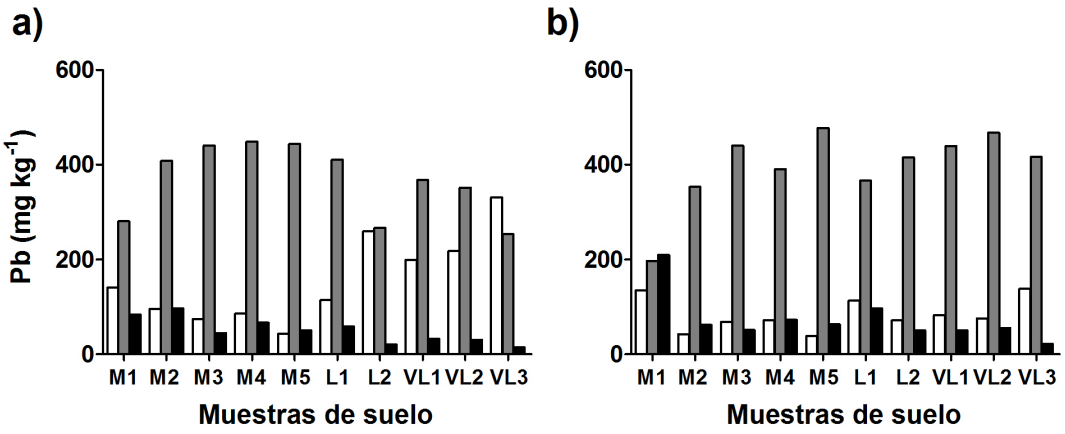

Fig 4. Concentraciones de Pb en las diferentes etapas de la extracción secuencial BCR en las muestras de suelo contaminadas en el nivel Tt2 después de una semana (a) y 12 meses (b) de tiempo de contacto. La nomenclatura de las muestras corresponde a la de los Apartados 3.1, 3.2 y 3.3.

Sin embargo, los resultados obtenidos no fueron concluyentes, pues se observó que el valor de pH de los extractos, correspondientes a las distintas etapas, se incrementaba en torno a 2 unidades, implicando una menor desorción de Pb en la primera etapa (soluble en ácido) y un aumento en las sucesivas (reducible y oxidable), debido, principalmente, a una parcial disolución del carbonato (Cappuyns *et al.*, 2007; Sulkowski e Hirner, 2006). Por ello, no se han empleado estos datos en los Capítulos de Resultados del presente trabajo de Tesis y, actualmente, se está trabajando en la elaboración de un protocolo modificado del BCR original, más apropiado para suelos calcáreos, en colaboración con el CIEMAT.

Finalmente, en el estudio concerniente al Apartado 3.3, optamos por emplear la técnica de DRX, por medio de la cual pudimos detectar la formación de una fase intermedia de carbonato de óxido de plomo de Pb ($4PbCO_3 \cdot 3PbO$; JCPDS No. 00-017-0732), principalmente a elevadas concentraciones de Pb añadidas (nivel Tt2), en aquellos suelos que presentaban un contenido moderado de CCE (Fig. 5).

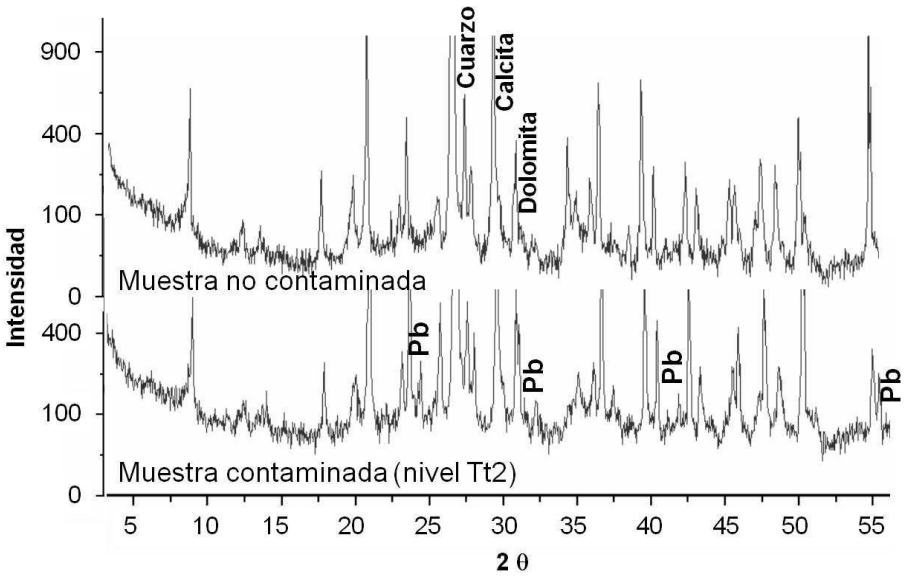

Fig 5. Difractogramas de Rayos X de la fracción tierra fina del suelo (muestra de suelo M1, no contaminada y contaminada en el nivel Tt2). Pb = carbonato de óxido de plomo ($4PbCO_3 \cdot 3PbO$). La nomenclatura de la muestra corresponde a la de los Apartados 3.1, 3.2 y 3.3.

A pesar de la evidencia de la precipitación de Pb en la fracción carbonatada, los constituyentes relacionados con esta fracción –CCE y CA– no mostraron correlaciones negativas significativas con el Pb extraído con ninguno de los extractantes empleados. En su lugar, se obtuvieron correlaciones positivas significativas entre estos constituyentes y el Pb extraído con HNO_3 y HAc en el nivel Tt1, atribuido a la disolución parcial del carbonato, liberando el Pb a la solución (Vidal *et al.*, 2004). En este sentido, fue la proporción relativa de dolomita (< 2 mm) la que mostró, en ambos niveles (Tt1 y Tt2), correlaciones positivas significativas con estos extractantes.

Curiosamente, el único parámetro del suelo con el que encontramos relaciones negativas fue el contenido en Mg total del suelo, correlacionándose con el contenido de Pb extraído con sales neutras, DTPA y LMWOA. Estos resultados nos llevaron a la conclusión de que probablemente se hubiese producido una nucleación de microprecipitados de Pb en la superficie de minerales de arcilla ricos en Mg, como la dolomita (Rangel-Porras *et al.*, 2010). Por todo ello, concluimos que la fase dolomítica

podría controlar la extractabilidad de Pb, poniendo de manifiesto que la movilidad potencial de este metal dependería, en cierta medida, del ciclo del C inorgánico. No obstante, otros constituyentes del suelo, como la MO y/o los óxidos de Fe, podrían estar implicados en la retención de Pb (Clemente *et al.*, 2006). Sin embargo, no se obtuvieron correlaciones significativas con el resto de parámetros edáficos medidos en este estudio, probablemente debido a que los constituyentes implicados en los procesos de sorción no siempre se corresponden con los implicados en los de desorción.

En el estudio tanto de la sorción como de la extractabilidad de Zn, se observó que la distribución de tamaño de partícula fue el principal factor explicativo en ambos niveles de contaminación metálica. Tal y como mostramos en el Apartado 3.1, la mayor sorción de Zn en el nivel Tt1 se produjo en suelos con mayores proporciones de fracción mineral fina, así como con los mayores valores de CIC (Jalali y Khanlari, 2008). A este nivel, la disminución de la extractabilidad de Zn con $NaNO_3$ se produjo durante el primer mes de contacto, indicando una rápida distribución de este metal (Apartado 3.2). En el nivel Tt2, el tiempo requerido para la concentración de Zn extraído con $NaNO_3$ en alcanzar el equilibrio fue superior a los 6 meses de contacto en aquellos suelos con un bajo contenido en CA. Sin embargo, a los 12 meses de tiempo de contacto, no se detectó la presencia de carbonato de Zn mediante DRX (Apartado 3.3).

Paralelamente, se observó que la extractabilidad de Zn con LMWOA y con DTPA incrementaba durante el primer mes de tiempo de contacto en ambos niveles, siendo este comportamiento más acusado en suelos con menor proporción de arcilla (Apartados 3.1 y 3.2). De hecho, observamos que los valores de concentración de Zn extraído, al final del experimento de incubación, con prácticamente todos los métodos de extracción empleados, se correlacionaron significativa y negativamente con los contenidos de arcilla, limo y Mg total, indicando, probablemente, procesos de adsorción específica en constituyentes de la fracción mineral fina. En el análisis de distribución elemental mediante TEM-EDS, se observó que la distribución de Zn estuvo fuertemente asociada a la fracción silicatada del suelo, obteniéndose mayores concentraciones de este metal alrededor de las partículas silicatadas, así como asociaciones con Fe y Mg (Fig. 3), revelando un proceso de adsorción sobre estas

partículas. Estos resultados nos sugirieron que los minerales del suelo con una mayor capacidad de adsorción, probablemente ricos en Mg, son los principales responsables del control de las fracciones, tanto móviles como potencialmente móviles, de Zn en estos suelos (Apartado 3.3).

Por todo ello, se deduce que los patrones de extractabilidad de los metales en estos suelos están controlados, en mayor o menor medida, por el contenido y composición de diferentes fracciones del suelo: carbonatada, orgánica, óxido de Fe y arcilla. A partir de estos resultados, nos hicimos varias preguntas. En primer lugar, nos planteamos si en estos suelos, que presentan una elevada capacidad de sorción metálica, la biodisponibilidad sería reducida, con lo que se produciría una baja toxicidad. En segundo lugar, nos preguntamos si, en el caso de existir una significativa fracción biodisponible de los metales, ésta estaría controlada por los mismos constituyentes del suelo que controlan la disponibilidad estimada con métodos de extracción química.

Para poder responder a estas preguntas, se llevó a cabo un experimento de bioensayo con *Lactuca sativa* L. –variedad romana–, en los suelos, controles y contaminados (Tt1 y Tt2), después de 6 meses de incubación (Apartado 4.1). La biodisponibilidad de los metales se evaluó teniendo en cuenta la concentración metálica en las hojas de las plantas de lechuga. En este estudio, pudimos comprobar que se producía una elevada fitotoxicidad en la plantas cultivadas en los suelos contaminados, puesta de manifiesto por la significativa disminución de la biomasa aérea (Fig. 6) y la alteración de la absorción de nutrientes esenciales, como Ca y potasio (K).

Fig 6. Biomasa de las plantas de *Lactuca sativa* L. en los suelos no contaminados (A) y en los contaminados en el nivel Tt1 (B) y en el nivel Tt2 (C).

Además, se observó que las concentraciones cuantificadas de los cuatro metales estudiados en las partes comestibles de las plantas estaban dentro del rango considerado como excesivo o tóxico (Kabata-Pendias y Pendias, 2001), estando el Cd y el Pb por encima de los valores máximos permitidos por la legislación Europea actual para hortalizas de hoja (Reglamento Nº 466/2001). Estos resultados nos indicaron *a priori* que la retención metálica no ha sido del todo eficaz en estos suelos, tal y como habíamos observado anteriormente al estudiar la extractabilidad de los metales. No obstante, se observó un amplio rango de variación en la bioacumulación metálica entre los diferentes suelos, lo que puso de manifiesto que los constituyentes edáficos desempeñaban un papel clave en la minimización de la transferencia de estos metales a las hojas de las plantas.

Por ello, el siguiente paso planteado fue el estudio de la relación entre los constituyentes del suelo y la biodisponibilidad de los metales. Para llevar a cabo este estudio, optamos por realizar tanto un análisis de correlación de Pearson, como un análisis multivariante de redundancia –RDA–, a partir de los cuales se evaluaron los patrones de biodisponibilidad de los metales en las hojas en función de los parámetros físico-químicos de los suelos.

En este estudio, el análisis de correlación de Pearson mostró que las texturas gruesas favorecían la absorción de los metales en ambos niveles (para el Cu y el Pb), o en el nivel Tt2 (para el Cd y el Zn). No obstante, en el caso del Cd, el principal factor explicativo en el nivel Tt2 fue la fracción carbonatada del suelo, estando relacionada negativamente, tal y como mostraron los diagramas de ordenación del análisis de RDA. Por ello, a pesar de que las concentraciones cuantificadas de Cd en las hojas estuvieron por encima de los límites permitidos, el carbonato del suelo podría ejercer un papel clave en minimizar la biodisponibilidad de este metal (Recatalá *et al.*, 2010), tal y como habíamos observado en el estudio de la extractabilidad de Cd (Apartados 3.1, 3.2 y 3.3). Por otra parte, las elevadas concentraciones de Ca en la solución, típicas de estos suelos, podrían conllevar a un proceso competitivo entre el Ca y el Cd por los sitios de adsorción y/o absorción en las plantas. Esto podría explicar el ligero incremento detectado en la cantidad de Ca en las hojas de las plantas, así como en la solución del suelo, en los suelos contaminados.

En el caso del Cu y el Pb, el contenido en CCE mostró relaciones positivas con el contenido de estos metales en las hojas de las plantas cultivadas en los suelos contaminados. A pesar de que las plantas podrían aumentar el contenido soluble de Cu y Pb mediante disolución parcial de los carbonatos (Sayyad *et al.*, 2010), esta relación positiva con la fracción carbonatada, opuesta a la obtenida para el Cd, se atribuyó a procesos competitivos por la unión a la superficie de la raíz entre el Cd y estos metales. De hecho, el análisis RDA puso de manifiesto la acción ejercida por otros constituyentes edáficos, como la MO, principalmente la fracción recalcitrante, y los óxidos de Fe. Tal y como se había discutido en los Apartados 3.1, 3.2 y 3.3, la formación de asociaciones organominerales con los metales, favorecida por el rango de pH de los suelos, especialmente en presencia de Fe, podría ser un factor relevante en la disminución de la biodisponibilidad de metales como el Cu y Pb, con elevada afinidad por la MO (Besnard *et al.*, 2001; Martínez *et al.*, 2010; Sipos *et al.*, 2008; Zheljazkov *et al.*, 2006).

De forma paralela, aunque los compuestos húmicos parecieron controlar la biodisponibilidad de estos metales, también se observó que la biodisponibilidad de los metales en las hojas podría estar favorecida en suelos donde hubiese mayores proporciones de fracción orgánica lábil. Aunque este hecho podría ser, simplemente, un artefacto, en oposición a la fracción recalcitrante, también podría ser debido al hecho de que el material orgánico lábil puede promover la absorción de los metales y/o su translocación a las partes aéreas a través de los procesos de quelación (Clemente *et al.*, 2006). De esta forma, es posible que la fracción orgánica desempeñe un papel dual, en función de su naturaleza, minimizando la transferencia de los metales, la fracción recalcitrante, y favoreciendo la absorción metálica, la fracción lábil.

No obstante, a pesar de las claras evidencias del papel que ejercen estos constituyentes en los patrones de biodisponibilidad de los metales, se hizo evidente la limitación que suponía, para la interpretación de los resultados, no disponer de los valores de bioacumulación metálica en las raíces de las plantas. Por ello, se llevó a cabo un posterior experimento de bioensayo en el que se cuantificaron los contenidos de los metales tanto en las hojas como en las raíces de las plantas cultivas en los suelos control y contaminados (Tt1 y Tt2) a los 12 meses de incubación (Apartado

4.2). En este nuevo trabajo, se contó con dos variedades de *Lactuca sativa* L. –romana e iceberg–. En el estudio realizado observamos que se producía, igualmente, un desequilibrio en la absorción de nutrientes esenciales, así como una elevada inhibición de la producción de biomasa, tanto aérea como radicular, siendo ésta más pronunciada en el caso de la variedad iceberg (Fig. 7).

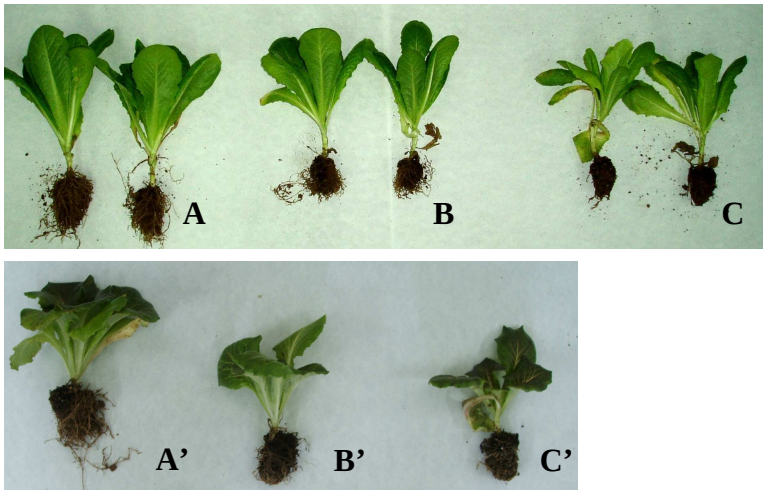

Fig 7. Biomasa de las plantas de *Lactuca sativa* L. en los suelos no contaminados (A y A') y en los contaminados en el nivel Tt1 (B y B') y en el nivel Tt2 (C y C'), para las variedades romana (A, B y C) e iceberg (A', B' y C').

Al igual que en el Apartado 4.1, se observó una elevada bioacumulación metálica. Paralelamente, las concentraciones cuantificadas en raíces fueron superiores a las detectadas en hojas, indicando una importante restricción del transporte metálico interno. Sin embargo, esto no impidió que se produjese una elevada translocación metálica, principalmente, en el caso del Zn. Cabe resaltar, no obstante, que, a pesar de que las concentraciones de Cd añadidas a los suelos fueron considerablemente más bajas, el porcentaje de transferencia de este metal a las partes comestibles de las plantas fue muy elevada, siendo mayor en el caso de la lechuga de la variedad romana.

Por otra parte, se observaron correlaciones significativas entre el contenido de los metales, tanto en las hojas como en la raíces de las plantas. En las plantas de lechuga cultivadas en los suelos en el nivel Tt2, tanto en la variedad romana como en la iceberg, se encontraron correlaciones positivas entre, prácticamente, todos los

metales acumulados en hojas y raíces, indicando una posible saturación de los mecanismos de regulación de las plantas a concentraciones metálicas elevadas. Sin embargo, en el nivel Tt1, el patrón observado fue más complejo. A este nivel, se encontraron correlaciones positivas en el contenido en hojas entre Cd y Zn, por un lado, y entre Cu y Pb, por el otro, tanto en la variedad romana como en la iceberg. En un sistema multi-componente, se producen procesos de competencia por la unión de los iones metálicos a la superficie de la raíz; y cuando metales, como Cu y Pb, se unen fuertemente al material orgánico, otros, como Cd y Zn, podrían acceder con mayor facilidad a los sitios de transporte en la membrana celular de las raíces, favoreciendo su posterior absorción y translocación a las hojas (Kalis, 2006). Además, interacciones entre el Cd y el Zn podrían estar favoreciendo estos procesos sinérgicos (Podar y Ramsey, 2005; Zorrig *et al.*, 2012). Ahora bien, este patrón sólo se vio reflejado en las hojas, pues en la biodisponibilidad de los metales en las raíces, las correlaciones obtenidas fueron entre Cd y Pb, y entre Cu y Zn. El diferente patrón podría deberse a una sobrestimación de la concentración metálica absorbida por las raíces, ya que, en este trabajo, el metal apoplástico –unido a las paredes celulares de la raíz– no fue eliminado (Cattani *et al.*, 2006). De hecho, se ha observado que la proporción de Pb adsorbido podría ser mayor que la del absorbido, ya sea por procesos de precipitación en las raíces, como fosfato o carbonato de Pb, o bien por una falta de transportadores específicos para este metal (Kalis, 2006).

Cabe resaltar, no obstante, que la relación entre la concentración metálica en la solución y la adsorbida en las raíces (Etapa II, Fig. 8) no se ha investigado suficientemente y, por lo tanto, todavía se discute sobre los métodos a utilizar, en general basados en extracciones con EDTA o $CaCl_2$, y sobre cómo interpretar los resultados. En este sentido, una concentración demasiado elevada de EDTA podría modificar la permeabilidad de membrana y, a su vez, resulta complejo distinguir si la proporción de metales extraídos tiene su origen en la adsorción a las raíces o también en las bio-películas de bacterias y algas que crecen en las mismas (Kalis, 2006). Por ello, nos planteamos como perspectiva futura profundizar en este aspecto.

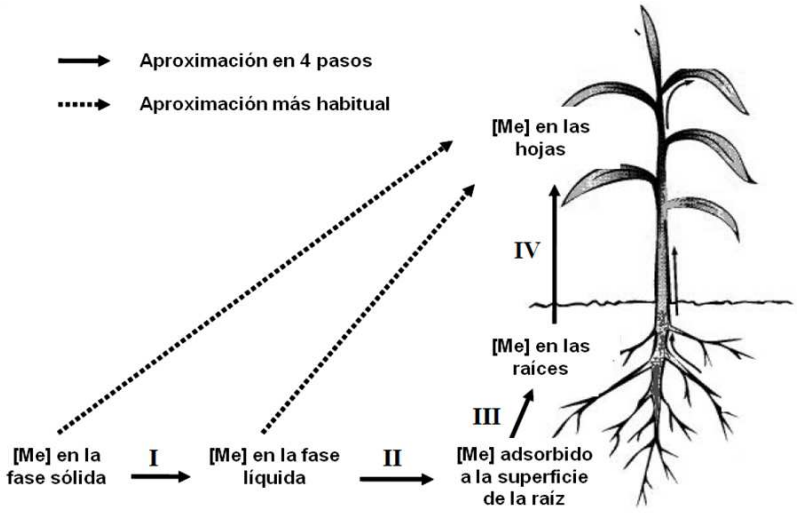

Fig 8. Esquema general de las fases de absorción de metales por las plantas (adaptación de Kalis, 2006).

Como continuación a los resultados obtenidos en el trabajo referente al Apartado 4.1, en este estudio (Apartado 4.2) se hizo más hincapié en la discusión de los constituyentes del suelo que controlan los patrones de biodisponibilidad de los metales en las raíces de las plantas de lechuga. En primer lugar, cabe destacar que, a pesar de que las dos variedades estudiadas se vieron afectadas de forma diferente ante la contaminación metálica, los patrones de biodisponibilidad de los metales fueron equivalentes para ambas.

En el estudio de la biodisponibilidad de Cd, tanto en hojas como en raíces, se obtuvieron relaciones negativas con varios constituyentes de la fracción carbonatada (CCE, calcita y dolomita), mostrando que esta fracción es el principal factor explicativo de los patrones de biodisponibilidad de este metal, tal y como habíamos obtenido anteriormente en el estudio de la extractabilidad de Cd (Apartados 3.1, 3.2 y 3.3) y en el de su biodisponibilidad en hojas (Apartado 4.1). Sin embargo, la fracción carbonatada no explicó los patrones de biodisponibilidad de Pb, a pesar de haber demostrado la precipitación de este metal en esta fracción (Apartado 3.3). En su lugar, tal y como hemos mencionado en el Apartado 4.1, para la biodisponibilidad en las hojas, se obtuvieron relaciones negativas entre la fracción orgánica y el contenido de Cu y Pb en las raíces de las plantas (Chiu *et al.*, 2006; Recatalá *et al.*, 2011; Wang *et al.*, 2010). Esto podría apoyar la hipótesis planteada en cuanto a la competencia por la

adsorción metálica en la raíz, proceso en el cual ante un mayor contenido en material orgánico en los suelos, donde el Cu y el Pb podrían estar retenidos, se vería favorecida la adsorción de otros metales, como Cd y Zn, y, por tanto, su absorción (Fernández *et al.*, 2005; Kalis *et al.*, 2006; Qian *et al.*, 2012). Sin embargo, en este estudio se confirmó el papel dual ejercido por la fracción orgánica en el control de los patrones de biodisponibilidad de Cu y Pb, dependiendo de su composición, del órgano de la panta estudiado y del metal. En este sentido, la fracción orgánica lábil favoreció una mayor translocación de Cu y Pb a las partes aéreas de las plantas de lechuga a ambos niveles de contaminación (Inaba y Takenaka, 2005; Kidd, 2009; Liao *et al.*, 2006).

En cuanto a las raíces, se obtuvieron relaciones negativas entre la fracción recalcitrante y el contenido de Pb en el nivel Tt1, sugiriendo una fuerte retención del Pb en los ácidos húmicos (Clemente *et al.*, 2006; Zeng *et al.*, 2011). No obstante, en el caso del Cu, se observó un patrón opuesto. Así, la biodisponibilidad de Cu en las raíces estuvo negativamente relacionada con el contenido total de MO, principalmente con la fracción lábil, y, en consecuencia, positivamente con la recalcitrante. Esto se atribuyó al hecho de que a pesar de que la MO lábil facilita la movilidad del Cu en los suelos, a través de la formación de complejos orgánicos, estos complejos podrían no estar disponibles en la absorción por las raíces de las plantas (Cattani *et al.*, 2006).

En nivel Tt2, tanto el Cu como el Pb mostraron el mismo patrón, estando sus valores de concentración en raíces positivamente relacionados con la fracción orgánica lábil. Este diferente resultado, en el nivel de elevada contaminación metálica, podría ser debido a procesos competitivos entre el Cu y el Pb para unirse a la MO, resultando en un aumento de la biodisponibilidad de ambos metales cuando éstos están en concentraciones más altas (Zheljazkov *et al.*, 2006).

Otra posible explicación para la relación positiva con la fracción recalcitrante y negativa, por tanto, con la lábil, podría ser la posible descomposición de la MO por los microorganismos del suelo, liberando los metales que, posteriormente, serían fácilmente absorbidos por las plantas (Wang *et al.*, 2004). Paralelamente, la bioacumulación de Zn en las raíces fue mayor en aquellos suelos con menor proporción de fracción mineral fina, en ambos niveles de contaminación (Covelo *et al.*, 2008), indicando que procesos de adsorción específica en esta fracción

controlarían su biodisponibilidad, lo que se corresponde con los resultados obtenidos anteriormente en el estudio de la extractabilidad (Apartados 3.1, 3.2 y 3.3).

Por todo ello, a partir de los experimentos de bioensayo con *Lactuca sativa* L., se podría deducir que se produce una significativa fitotoxicidad y bioacumulación metálica tanto en las hojas como en las raíces, de ambas variedades romana e iceberg. En este sentido, la acción combinada del contenido y composición de diferentes fracciones del suelo (carbonatada, orgánica, óxido de Fe y arcilla) fue un factor clave en la minimización de los procesos de absorción de los metales.

Una vez que llegamos a estas conclusiones, nos planteamos si alguno de los métodos de extracción empleados (Apartados 3.1, 3.2 y 3.3) podría simular los patrones de biodisponibilidad de los metales (Apartados 4.1 y 4.2).

En el caso del Cd y del Zn, los métodos de extracción que fueron adecuados para simular los patrones de biodisponibilidad en las raíces también lo fueron en las hojas. Sin embargo, en el caso del Cu y el Pb, se obtuvieron diferentes resultados para las hojas y para las raíces, así como para los dos niveles de contaminación. De hecho, en el nivel Tt1 prácticamente no se obtuvieron correlaciones significativas y en el nivel Tt2 fueron escasas (en el caso del Pb) o nulas (en el caso del Cu). Estas observaciones, además de corroborar lo obtenido previamente al estudiar los patrones de biodisponibilidad metálica, sugirieron que el transporte interno de Cd y Zn probablemente se deba a procesos de difusión, mientras que en el caso del Cu y el Pb se podría producir, bien una importante restricción en el transporte interno desde las raíces hacia las partes aéreas (Dahmani-Muller *et al.*, 2001), bien una fuerte adsorción en la pared celular de las raíces favorecida por el elevado pH (Chaignon *et al.*, 2003).

Por otra parte, como era de esperar, el mayor número de correlaciones entre la biodisponibilidad metálica en las hojas y las concentraciones de los metales extraídos, en general, se obtuvo con procedimientos basados en sales neutras, mostrando que estos métodos podrían ser adecuados con fines ecotoxicológicos en la evaluación del riesgo de entrada de metales en la cadena trófica (Menzies *et al.*, 2007).

No obstante, cabe resaltar que se obtuvieron numerosas correlaciones significativas, entre el contenido metálico en hojas y raíces, con métodos que estiman las fracciones potencialmente móviles de los metales, como HAc, NH_4Ac, DTPA y LMWOA. En el ambiente de la rizosfera, los ácidos orgánicos, liberados por las raíces

de las plantas y los microorganismos, juegan un papel clave en la adsorción metálica por las raíces, pudiendo promover la absorción y su posterior translocación desde las raíces hacia las partes aéreas (Feng *et al.*, 2005; Han *et al.*, 2005; Liao *et al.*, 2006; Pinto *et al.*, 2004; Wang *et al.*, 2004; Zorrig *et al.*, 2010). Ya que la biodisponibilidad de los metales en el suelo es el resultado de la interacción raíz-suelo-microorganismo, la idoneidad de estos procedimientos podría ser atribuida al hecho de que estos métodos de extracción simularían más apropiadamente estos procesos. Esto podría estar apoyado por las relaciones positivas observadas entre los patrones de biodisponibilidad de los metales en las hojas y la fracción orgánica lábil del suelo, que incluye ácidos orgánicos, hidratos de C y proteínas.

En último lugar, los métodos de extracción de mayor fuerza, como EDTA y HNO_3, mostraron no ser apropiados en la simulación de la biodisponibilidad de los metales a la plantas de lechuga, probablemente porque pueden extraer metales de fracciones del suelo no lábiles (Feng *et al.*, 2005). Sin embargo, estos métodos podrían ser adecuados en predicciones de riesgo potencial a más largo plazo.

A la vista de las claras evidencias del papel que ejercen tanto el contenido como la composición de las fracciones carbonatada, orgánica, óxido y arcilla en los patrones de biodisponibilidad de los metales para las plantas en estos suelos, nos planteamos si estas fracciones también explicarían los patrones de alteración de la actividad microbiana del suelo ante una contaminación metálica. Este estudio nos pareció particularmente relevante, puesto que teniendo presente que la MO desempeña un papel fundamental, tanto como precursor para la síntesis enzimática como en la estabilización física, el bajo contenido de este constituyente en estos suelos podría limitar la respuesta de las poblaciones microbianas ante el estrés resultante de la contaminación por metales pesados (Moreno *et al.*, 2009).

Por todo ello, se llevó a cabo una evaluación de la alteración de un conjunto de parámetros bioquímicos y microbiológicos en estos suelos a los 12 meses de tiempo de contacto del experimento de incubación (Apartado 5.1). Estos análisis se llevaron a cabo en el Departamento de Physicochimie et Toxicologie des Sols d'Agrosystèmes Contaminés –PESSAC– del Institut National de la Recherche Agronomique –INRA– en Francia, a lo largo de una estancia científica financiada por la Red CARESOIL.

Con el objetivo de estudiar la alteración bioquímica de los suelos ante la contaminación metálica, se evaluaron las actividades extracelulares fosfatasa (PHOS), ureasa (URE), β-galactosidasa (BGAL) y arilsulfatasa (ARYS), puesto que estas actividades están implicadas en los principales ciclos biogeoquímicos de los nutrientes de los suelos –fósforo, nitrógeno, carbono y azufre– (Fig. 9).

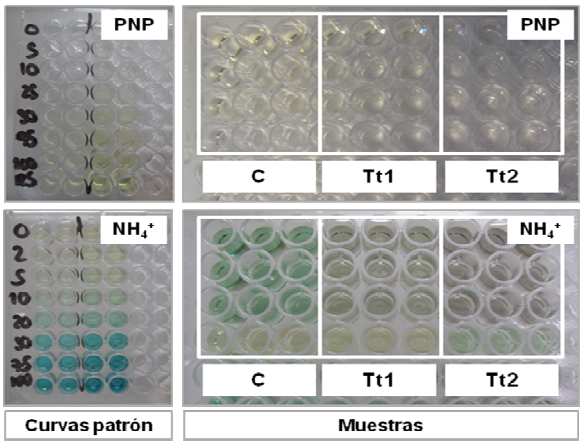

Fig 9. Medida de las actividades PHOS, BGAL y ARYS (cuantificación de PNP, p-nitrofenol, a 405 nm) y URE (cuantificación de NH_4^+, a 610 nm) en los suelos no contaminados (C) y en los contaminados en el nivel Tt1 y en el nivel Tt2.

Como era de esperar, todas las actividades enzimáticas disminuyeron con la contaminación metálica, mostrando la siguiente secuencia de porcentajes de inhibición: PHOS (30-60%) < URE (1-98%) ≤ ARYS (38-90%) ≤ BGAL (30-94%) en el nivel Tt1 y PHOS (41-64%) < ARYS (83-97%) < URE (85-100%) < BGAL (89-100%) en el nivel Tt2.

De la secuencia de inhibición se podrían derivar varias observaciones (Apartado 5.1). Lo primero que cabe observar es el diferente grado de inhibición entre las actividades enzimáticas medidas, lo que podría atribuirse al posible desarrollo de microorganismos resistentes a los metales, que liberarían enzimas más resistentes a las elevadas concentraciones metálicas (Kunito *et al.*, 1998, 1999). En este sentido, la actividad enzimática PHOS fue la que presentó una menor inhibición y URE, BGAL y ARYS las que se vieron más inhibidas. Estos resultados son consistentes con los

obtenidos por otros autores, que propusieron las actividades URE, BGAL y ARYS como bio-indicadores sensibles de los efectos derivados de la degradación del suelo, causada por una contaminación metálica, en suelos con pHs ligeramente alcalinos (Hinojosa *et al.*, 2004; Belyaeva *et al.*, 2005; Martínez-Iñigo *et al.*, 2009). En segundo lugar, en algunos casos se alcanzaron valores de inhibición próximos al 100 %, indicando que el centro activo de las enzimas podría estar inactivado por los metales, impidiendo, por ello, llevar a cabo su acción hidrolítica. Para finalizar, se observó que en el nivel Tt1, para cada actividad, existe una gran amplitud en el rango de inhibición, lo que indicaría que los constituyentes edáficos están jugando un papel muy importante en minimizar este proceso. En el nivel Tt2, sin embargo, el elevado porcentaje de inhibición, así como la uniformidad de los valores entre los distintos suelos, no nos permitieron establecer una clara discriminación entre las propiedades edáficas.

El mayor porcentaje de inhibición de las actividades URE, BGAL y ARYS en el nivel Tt1 se produjo en un grupo de suelos que presentan un conjunto de características comunes. Por un lado, presentan una menor proporción de fracción mineral fina, orgánica recalcitrante y óxido de Fe cristalino. El menor contenido de estas fracciones podría limitar la estabilización física de las enzimas en el medio, a través de la formación de complejos humus-hidrolasa y/o arcilla-humus-hidrolasa, que protegerían a las enzimas frente a la actividad de las proteasas (Burns *et al.*, 1972; Gianfreda *et al.*, 1995). Del mismo modo, el establecimiento de asociaciones organominerales con los metales aumentaría la retención metálica (Besnard *et al.*, 2001; Renella *et al.*, 2004), mitigando la inhibición enzimática y/o de los organismos que las producen. Estos resultados indicaron, por tanto, que estos componentes edáficos, son esenciales para la persistencia de estas actividades enzimáticas, frente a la contaminación por metales pesados en estos suelos calcáreos del área mediterránea, a pesar del bajo contenido de MO que presentan.

Paralelamente, cabe señalar que, en aquellos suelos con mayores proporciones de cationes divalentes, Ca^{2+} y Mg^{2+}, en la solución del suelo la actividad URE mostró un porcentaje de inhibición considerablemente menor, ~ 90 veces. Estos cationes proporcionan puentes entre coloides cargados negativamente, como los grupos funcionales de naturaleza orgánica y las superficies de las arcillas (Krull *et al.*, 2001), lo

que podría favorecer la estabilización de los complejos arcilla-humus-hidrolasa, mencionados anteriormente.

En el caso de la inhibición de la actividad PHOS, se observó que ésta se producía en porcentajes similares en ambos niveles de contaminación y que esta inhibición era considerablemente menor, lo que sugiere que esta enzima, o los organismos que la producen, es menos sensible a la mezcla metálica empleada. El porcentaje de inhibición de la actividad PHOS fue ligeramente menor en suelos con mayor contenido en MO, lo que favorecería su persistencia (Pascual *et al.*, 2002). Sin embargo, fue el contenido en fósforo total, el parámetro que mostró correlaciones negativas significativas con esta actividad, atribuido a mecanismos de regulación de la producción de enzimas, por medio de procesos de retroalimentación negativa (Olander y Vitousek, 2000).

La elevada inhibición enzimática observada nos indicó que la contaminación metálica había producido una fuerte alteración de procesos biológicos clave del suelo, como son la mineralización de nitrógeno o la degradación de celulosa. No obstante, las enzimas extracelulares son generalmente dependientes del estado del organismo (activo, inactivo o muerto), existiendo un desfase temporal entre la muerte celular y la disminución de la actividad enzimática (Brookes, 1995).

Con el fin de dilucidar si la disminución de la actividad enzimática se debía en mayor medida a la inhibición de las enzimas, o bien a la alteración de la comunidad microbiana que las sintetiza, el siguiente paso planteado fue evaluar la alteración de la actividad deshidrogenasa (DHA), la cual se considera un buen indicador de la actividad microbiana global (Obbard, 2001). Los resultados mostraron una fuerte inhibición de la actividad DHA (~100%), lo que *a priori* sugeriría una disminución en el tamaño de la población microbiana del suelo (Fig. 10).

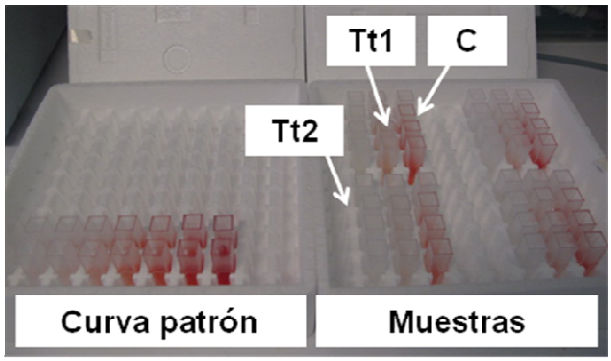

Fig 10. Medida de la actividad DHA (cuantificación de 1,3,5-trifenyl formazan, a 485 nm) en los suelos no contaminados (C) y en los contaminados en el nivel Tt1 y en el nivel Tt2.

Sin embargo, ya que aceptores de electrones, como los nitratos o los ácidos húmicos, pueden causar una sobreestimación de la actividad DHA, y metales, como el Cu, pueden inhibir el desarrollo de color del formazán (Chander y Brookes, 1991), decidimos no tomar estos resultados como definitivos. Así, se optó por estimar la biomasa microbiana mediante la cuantificación del ADN total extraído de las muestras de suelo (Fig. 11).

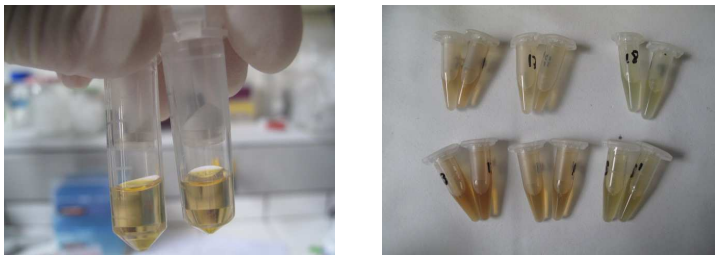

Fig 11. Extractos de ADN del suelo empleando un *Fast DNA kit* para suelos (*MP Biomedical*).

A partir de los valores obtenidos, se llevó a cabo un análisis de correlación de Pearson entre la concentración de ADN total y los valores de la actividad DHA, obteniéndose únicamente correlaciones significativas en los suelos control, lo que mostró que los resultados derivados de la cuantificación de la actividad DHA en los suelos contaminados no eran concluyentes. Por ello, decidimos evaluar únicamente la

alteración de la concentración de ADN. En este sentido, se observó que la concentración de ADN total disminuía en las muestras contaminadas, en torno a un 50 %, revelando una elevada perturbación de la comunidad microbiana, la principal fuente de enzimas. Con el objetivo de dilucidar qué población estaba siendo más afectada, se cuantificó la concentración de ADN bacteriano y fúngico mediante PCRs cuantitativas en tiempo real.

En primer lugar, observamos que en los suelos no contaminados, el ADN fúngico representó entre el 3 y el 17 % del ADN total y el bacteriano entre el 62 y el 100 %, mostrando que la población fúngica es minoritaria en estos suelos. Curiosamente, se observó la misma proporción de ADN fúngico en las muestras de suelo contaminadas. Sin embargo, la elevada disminución de la concentración de ADN bacteriano, hasta un 20 % en el nivel Tt1 y hasta un 50 % en el Tt2, indicó una mayor sensibilidad de la comunidad bacteriana frente a la contaminación metálica (Vig *et al.*, 2003). La comunidad fúngica del suelo suele presentar una mayor capacidad de adaptación en condiciones de estrés (Chander y Dyckmans, 2001), probablemente debido a mecanismos de adaptación intrínsecos, que pueden estar acompañados por la exclusión de cepas sensibles a los metales, lo que implicaría una alteración en la estructura de la comunidad, aunque no necesariamente un diferente tamaño de la población (Baldrian, 2003; Gadd, 2007). Además, el menor pH de los suelos contaminados, como resultado de la adición de las sales metálicas, podría haber favorecido la proliferación de la comunidad fúngica que, generalmente, presenta un crecimiento óptimo dentro de un rango de pH más amplio que la comunidad bacteriana (Rousk *et al.*, 2010).

Por otra parte, observamos que no se obtuvieron correlaciones significativas entre las actividades enzimáticas extracelulares medidas y la concentración de ADN total, bacteriano y fúngico, corroborando que las enzimas no están asociadas con microorganismos activos y que, por lo tanto, deben ser estabilizadas por los componentes del suelo (Trasar-Cepeda *et al.*, 2008). Sin embargo, la menor disminución de la concentración de ADN bacteriano, al igual que en el caso de las actividades enzimáticas, se produjo en suelos con mayor proporción de MO, arcilla y/o CCE, mostrando que estos componentes juegan un papel clave en la supervivencia y persistencia de la comunidad bacteriana, mediante estabilización física

y/o reducción de la disponibilidad de los metales (Martínez-Iñigo *et al.*, 2009; Moreno *et al.*, 2009).

Para finalizar, cabe resaltar que la relación ADN fúngico/bacteriano aumentó en algunas de las muestras de suelo contaminadas, principalmente en el nivel Tt2, atribuido a la extinción de especies carentes de suficiente tolerancia al estrés y a la proliferación de especies resistentes (Brandt *et al.*, 2010). En cualquier caso, la alteración de este equilibrio implicaría una perturbación en el sistema, lo que podría indicar una alteración de procesos clave como la descomposición de la MO recalcitrante, en la que hongos y bacterias participan mediante interacciones complejas, tanto competitivas como mutualistas (de Boer *et al.*, 2005). Además, ya que la disminución de la biomasa bacteriana fue mucho mayor que la de la biomasa total y fúngica, pensamos que se ha podido favorecer la proliferación de organismos oportunistas, lo que podría plantearse como perspectiva futura de trabajo.

Por todo ello, podríamos deducir de este Apartado 5.1, que la alteración de las actividades enzimáticas fue mucho mayor que la disminución de la biomasa total, fúngica y bacteriana. Dentro del conjunto de parámetros medidos, las actividades URE, BGAL y ARYS, así como la concentración de ADN bacteriano, han mostrado ser bio-indicadores apropiados de la contaminación metálica empleada en estos suelos calcáreos de uso agrícola del área mediterránea.

Bibliografía

Acosta, J.A., Faz, A., Martínez-Martínez, S., Arocena, J.M., 2011. Enrichment of metals in soils subjected to different land uses in a typical Mediterranean environment (Murcia City, southeast Spain). Appl. Geochem. 26, 405-414.

Anderson, P. R., Christensen, T. H., 1988. Distribution coefficients of Cd, Co, Ni, and Zn in soils. J. Soil Sci. 39, 15-22.

Barton, L., Abadía, J., 2006. Iron nutrition in plants and rhizospheric microorganisms. Springer, Dordrecht.

Batjes, N.H., 2000. Soil Vulnerability to Diffuse Pollution in Central and Eastern Europe SOVEUR Project (Version 1.0). FAO and ISRIC.

Belyaeva, O.N., Haynes, R.J., Birukova, O.A., 2005. Barley yield and soil microbial and enzyme activities as affected by contamination of two soils with lead, zinc or copper. Biol. Fertil. Soils 41, 85-94.

Besnard, E., Chenu, C., Robert, M., 2001. Influence of organic amendments on copper distribution among particle-size and density fractions in Champagne vineyard soils. Environ. Pollut. 112, 329-337.

Brandt, K.K., Frandsen, R.J.N., Holm, P.E., Nybroe, O., 2010. Development of pollution-induced community tolerance is linked to structural and functional resilience of a soil bacterial community following a five-year field exposure to copper. Soil Biol. Biochem. 42, 748-757.

Brookes, P.C., 1995. The use of microbial parameters in monitoring soil pollution by heavy metals. Biol. Fertil. Soils 19(4), 269-279.

Buekers, J., Van Laer, L., Amery, F., Van Buggenhout, S., Maes, A., Smolders, E., 2007. Role of soil constituents in fixation of soluble Zn, Cu, Ni and Cd added to soils. Eur. J. Soil Sci. 58, 1514-1524.

Burns, R.G., Pukite, A.H., McLaren, A.D., 1972. Concerning the location and the persistence of soil urease. Soil Sci. Soc. Am. Proc. 36, 308-311.

Cappuyns, V., Swennen, R., Niclaes, M., 2007. Application of the BCR sequential extraction scheme to dredged pond sediments contaminated by Pb–Zn mining: A combined geochemical and mineralogical approach. J. Geochem. Explor. 93, 78-90.

Cattani, I., Fragoulis, G., Boccelli, R., Capri, E., 2006. Copper bioavailability in the rhizosphere of maize (Zea mays L.) grown in two Italian soils. Chemosphere 64, 1972-1979.

Chaignon, V., Sanchez-Neira, I., Herrmann, P., Jaillard, B., Hinsinger, P., 2003. Copper bioavailability and extractability as related to chemical properties of contaminated soils from a vine-growing area. Environ. Pollut. 123, 229-238.

Chander, K., Brookes, P.C., 1991. Is the dehydrogenase assay invalid as a method to estimate microbial activity in copper contaminated soils? Soil Biol. Biochem. 23, 909-915.

Chander, K., Dyckmans, J., 2001. Different sources of heavy metals and their long-term effects on soil microbial properties. Biol. Fertil. Soils 34, 241-247.

Chiu, K.K., Ye, Z.H., Wong, M.H., 2006. Growth of *Vetiveria zizanioides* and *Phragmities australis* on Pb/Zn and Cu mine tailings amended with manure compost and sewage sludge: A greenhouse study. Bioresour. Technol. 97, 158-170.

Clemente, R., Escolar, A., Bernal, M.P., 2006. Heavy metals fractionation and organic matter mineralization in contaminated calcareous soil amended with organic materials. Bioresour. Technol. 97, 1894-1901.

Covelo, E.F., Matías, J.M., Vega, F.A., Reigosa, M.J., Andrade, M.L., 2008. A tree regression analysis of factors determining the sorption and retention of heavy metals by soil. Geoderma 147, 75–85.

Dahmani-Muller, H., van Oort, F., Balabane, M., 2001. Metal extraction by Arabidopsis halleri grown on an unpolluted soil amended with various metal-bearing solids: a pot experiment. Environ. Pollut. 114, 77-84.

de Boer, W., Folman, L.B., Summerbell, R.C., Boddy, L., 2005. Living in a fungal world: impact of fungi on soil bacterial niche development. FEMS Microbiol. Rev. 29(4), 795-811.

Directiva 86/278/EEC de 12 Junio de 1986 relativa a la protección del medio ambiente y, en particular, de los suelos, en la utilización de los lodos de depuradora en agricultura.

Ettler, V., Mihaljevič, M., Šebek, O., Grygar, T., 2007. Assessment of single extractions for the determination of mobile forms of metals in highly polluted

soils and sediments - Analytical and thermodynamic approaches. Anal. Chim. Acta 602(1), 131-140.

Feng, M., Shan, X., Zhang, S., Wen, B., 2005. A comparison of the rhizosphere-based method with DTPA, EDTA, $CaCl_2$, and $NaNO_3$ extraction methods for prediction of bioavailability of metals in soil to barley. Environ. Pollut. 137, 231-240.

Fernández, M.D., Cagigal, E., Vega, M.M., Urzelai, A., Babín, M., Pro, J., Tarazona, J.V., 2005. Ecological risk assessment of contaminated soils through direct toxicity assessment. Ecotoxicol. Environ. Saf. 62, 174-184.

Frangi, J.P., Richard, D., 1997. Heavy metal soil pollution cartography in northern France. Sci. Total Environ. 205, 71-79.

Gadd, G.M., 2007. Geomycology: biogeochemical transformations of rocks, minerals, metals and radionuclides by fungi, bioweathering and bioremediation. Mycol. Res. 111, 3-49.

Gangneux, C., Akpa-Vinceslas, M., Sauvage, H., Desaire, S., Houot, S., Laval, K., 2011. Fungal, bacterial and plant dsDNA contributions to soil total DNA extracted from silty soils under different farming practices: Relationships with chloroform-labile carbon. Soil Biol. Biochem. 43, 431-437.

Gianfreda, L., Rao, M.A., Violante, A., 1995. Formation and activity of urease-tannate complexes affected by aluminium, iron, and manganese. Soil Sci. Soc. Am. J. 59(3), 805-810.

Gleyzes, C., Tellier, S., Astruc, M., 2002. Fractionation studies of trace elements in contaminated soils and sediments: a review of sequential extraction procedures. Trends. Anal. Chem. 21(6+7), 451-467.

Gray, C.W., McLaren, R.G., Roberts, A.H.C., Condron, L.M., 1998. Sorption and desorption of cadmium from some New Zealand soils: effect of pH and contact time. Aust. J. Soil Res. 36(2), 199-216.

Gupta, S.K., Vollmer, M.K., Krebs, R., 1996. The importance of mobile, mobilisable and pseudo total heavy metal fractions in soil for three-level risk assessment and risk management. Sci. Total Environ. 178, 11-20.

Han, F.X., Banin, A., 1999. Long-term transformation and redistribution of potentially toxic heavy metals in arid-zone soils: II. Incubation at the field capacity moisture content. Water. Air. Soil Pollut. 114(3), 221-250.

Han, F., Shan, X.Q., Zhang, J., Xie, Y.N., Pei, Z.G., Zhang, S.Z., Zhu, Y.G., Wen, B., 2005. Organic acids promote the uptake of lanthanum by barley roots. New Phytol. 165, 481-492.

Hernandez-Soriano, M.C., Jimenez-Lopez, J.C., 2012. Effects of soil water content and organic matter addition on the speciation and bioavailability of heavy metals. Sci. Total Environ. 423, 55-61.

Hinojosa, M.B., Carreira, J.A., García-Ruíz, R., Dick, R.P., 2004. Soil moisture pre-treatment effects on enzyme activities as indicators of heavy metal-contaminated and reclaimed soils. Soil Biol. Biochem. 36, 1559-1568.

Hu, K.L., Zhang, F.R., Li, H., Huang, F., Li, B.G., 2006. Spatial Patterns of Soil Heavy Metals in Urban-Rural Transition Zone of Beijing. Pedosphere 16, 690-698.

Imperato, M., Adamo, P., Naimo, D., Arienzo, M., Stanzione, D., Violante, P., 2003. Spatial distribution of heavy metals in urban soils of Naples city (Italy). Environ. Pollut. 124, 247-256.

Inaba, S., Takenaka, C., 2005. Effects of dissolved organic matter on toxicity and bioavailability of copper for lettuce sprouts. Environ. Internat. 31, 603- 608.

Jalali, M., Khanlari, Z. V., 2008. Effect of aging process on the fractionation of heavy metals in some calcareous soils of Iran. Geoderma 143, 26-40.

Jiménez Ballesta, R., Conde Bueno, P., Martín Rubí, J.A., García Giménez, R., 2010. Pedo-geochemical baseline content levels and soil quality reference values of trace elements in soils from the Mediterranean (Castilla La Mancha, Spain). Eur. J. Geosci. 2(4), 441-454.

Kabata-Pendias, A., Pendias, H., 2001. Trace Elements in Soils and Plants, third ed. Boca Ratón, New York.

Kalis, E.J.J., 2006. Chemical speciation and bioavailability of heavy metals in soil and surface water. Ph.D. thesis Wageningen University, Wageningen, the Netherlands.

Kalis, E.J.J., Temminghoff, E.J.M., Weng, L., van Riemsdijk, W.H., 2006. Effects of humic acid and competing cations on metal uptake by *Lolium perenne*. Environ. Toxicol. Chem. 25(3), 702-711.

Kelly, J., Thornton, I., Simpson, P.R., 1996. Urban Geochemistry: A study of the influence of anthropogenic activity on the heavy metal content of soils in traditionally industrial and non industrial areas of Britain. Appl. Geochem. 11, 363-370.

Kidd, P., Barceló, J., Bernal, M.P., Navari-Izzo, F., Poschenrieder, C., Shilev, S., Clemente, R., Monterroso, C., 2009. Trace element behaviour at the root–soil interface: Implications in phytoremediation. Environ. Exp. Bot. 67, 243-259.

Krishnamurti, G.S.R., Naidu, R., 2003. Solid-solution equilibria of cadmium in soils. Geoderma 113, 17-30.

Krull, E.J., Baldock, J., Skjemstad, J., 2001. Soil texture effects on decomposition and soil carbon storage. Net Ecosystem Exchange CRC Workshop Proceedings, pp. 103-110.

Kunito, T., Oyaizu, H., Matsumoto, S., 1998. Ecology of soil heavy metal-resistant bacteria and perspective of bioremediation of heavy metal-contaminated soils. Recent Res. Dev. Agric. Biol. Chem. 2, 185-206.

Kunito, T., Senoo, K., Saeki, K., Oyaizu, H., Matsumoto, S., 1999. Usefulness of the sensitivity-resistance index to estimate the toxicity of copper on bacteria in copper-contaminated soils. Ecotoxicol. Environ. Saf. 44, 182-189.

Lafuente, A.L., González, C., Quintana, J.R., Vázquez, A., Romero, A., 2008. Mobility of heavy metals in poorly developed carbonate soils in the Mediterranean region. Geoderma 145, 238-244.

Liao, Y.C., Chang Chien, S.W., Wang, M.C., Shen, Y., Hung, P.L., Das, B., 2006. Effect of transpiration on Pb uptake by lettuce and on water soluble low molecular weight organic acids in rhizosphere. Chemosph. 65, 343-351.

Lock, K., Janssen, C.R., 2003. Influence of ageing on zinc bioavailability in soils. Environ. Pollut. 126, 371-374.

Lu, A., Zhang, S., Shan, X., 2005. Time effect on the fractionation of heavy metals in soils. Geoderma 125, 225-234.

Manta, D.S., Angelone, M., Bellanca, A., Neri, R., Sprovieri, M., 2002. Heavy metals in urban soils: a case study from the city of Palermo (Sicily), Italy. Sci. Total Environ. 300, 229-243.

Martínez, C.E., Jacobson, A.R., McBride, M.B., 2003. Aging and temperature effects on DOC and elemental release from a metal contaminated soil. Environ. Pollut. 122, 135-143.

Martinez, R.E., Sharma, P., Kappler, A., 2010. Surface binding site analysis of Ca^{2+}-homoionized clay-humic acid complexes. J. Coll. Interface Sci. 352, 526-534.

Martínez-Iñigo, M.J., Pérez-Sanz, A., Ortiz, I., Alonso, J., Alarcón, R., García, P., Lobo, M.C., 2009. Bulk soil and rhizosphere bacterial community PCR–DGGE profiles and b-galactosidase activity as indicators of biological quality in soils contaminated by heavy metals and cultivated with *Silene vulgaris* (Moench) Garcke. Chemosphere 75, 1376-1381.

McLaughlin, M.J., 2001. Aging of metals in soils changes bioavailability. Fact Sheet Environ. Risk Assess. 4, 1-6.

Meers, E., du Laing, G., Unamuno, V., Ruttens, A., Vangronsveld, J., Tack, F.M.G., Verloo, M.G., 2007. Comparison of cadmium extractability from soils by commonly used single extraction protocols. Geoderma 141, 247-259.

Meng, F., Zhang, J., Shi, Q.Q., Liu, M., 2008. Spatial structure and distribution of heavy metals in agricultural soils of peri-urban area in Pudong of Shanghai, China. Proceedings of SPIE 7145.

Menzies, N.W., Donn, M.J., Kopittke, P.M., 2007. Evaluation of extractants for estimation of the phytoavailable trace metals in soils. Environ. Pollut. 145, 121-130.

Micó, C., Recatalá, L., Peris, M., Sánchez, J., 2006. Assessing heavy metal sources in agricultural soils of an European Mediterranean area by multivariate analysis. Chemosphere 65, 863-872.

Moreno, J.L., Bastida, F., Ros, M., Hernández, T., García, C., 2009. Soil organic carbon buffers heavy metal contamination on semiarid soils: Effects of different metal threshold levels on soil microbial activity. Eur. J. Soil Biol. 45, 220-228.

Obbard, J.P., 2001. Ecotoxicological assessment of heavy metals in sewage sludge amended soils. Appl. Geochem. 16, 1405-1411.

Olander, L.P., Vitousek, P.M., 2000. Regulation of soil phospahatase and chitinase activity by N and P availability. Biogeochem. 49, 175-190.

Pascual, J.A., Moreno, J.L., Hernández, T., García, C., 2002. Persistence of immobilised and total urease and phosphatase activities in a soil amended with organic wastes. Bioresour. Technol. 82, 73-78.

Pérez-Esteban, J., Escolástico, C., Moliner, A., Masaguer, A., 2013. Chemical speciation and mobilization of copper and zinc in naturally contaminated mine soils with citric and tartaric acids. Chemosphere 90, 276-283.

Peris, M., Micó, C., Recatalá, L., Sánchez, R., Sánchez, J., 2007. Heavy metal contents in horticultural crops of a representative area of the European Mediterranean region. Sci. Total Environ. 378, 42-48.

Peters, R.W., 1999. Chelant extraction of heavy metals from contaminated soils. J. Hazard. Mater. 66, 151-210.

Pinto, A.P., Mota, A.M., de Varennes, A., Pinto, F.C., 2004. Influence of organic matter on the uptake of cadmium, zinc, copper and iron by sorghum plants. Sci. Total Environ. 326, 239-247.

Plassard, F., Winiarski, T., Petit-Ramel, M., 2000. Retention and distribution of three heavy metals in a carbonated soil: comparison between batch and unsaturated column studies. J. Contam. Hydrol. 42, 99-111.

Podar, D., Ramsey, M.H., 2005. Effect of alkaline pH and associated Zn on the concentration and total uptake of Cd by lettuce: comparison with predictions from the CLEA model. Sci. Total Environ. 347, 53-63.

Prokop, Z., Cupr, P., Zlevorova-Zlamalikova, V., Komarek, J., Dusek, L., Holoubek, I., 2003. Mobility, bioavailability, and toxic effects of cadmium in soil samples. Environ. Res. 91, 119-126.

Qian, Y., Gallaghe, F.J., Feng, H., Wu, M., 2012. A geochemical study of toxic metal translocation in an urban brownfield wetland. Environ. Pollut. 166, 23-30.

Ramos-Miras, J.J., Roca-Perez, L., Guzmán-Palomino, M., Boluda, R., Gil, C., 2011. Background levels and baseline values of available heavy metals in Mediterranean greenhouse soils (Spain). J. Geochem. Explor. 110, 186-192.

Rangel-Porras, G., García-Magno, J.B., González-Muñoz, M.P., 2010. Lead and cadmium immobilization on calcitic limestone materials. Desalin. 262, 1-10.

Recatalá, L., Sacristán, D., Arbelo, C., Sánchez, J., 2011. Can a Single and Unique Cu Soil Quality Standard be Valid for Different Mediterranean Agricultural Soils under an Accumulator Crop? Water Air Soil Pollut. 223(4), 1503-1517.

Recatalá, L., Sánchez, J., Arbelo, C., Sacristán, D., 2010. Testing the validity of a Cd soil quality standard in representative Mediterranean agricultural soils under an accumulator crop. Sci. Total Environ. 409, 9-18.

Reglamento (CE) nº 466/2001 de 8 de marzo de 2001 por el que se fija el contenido máximo de determinados contaminantes en los productos alimenticios.

Renella, G., Landi, L., Nannipieri, P., 2004. Degradation of low molecular weight organic acids complexed with heavy metals in soil. Geoderma 122, 311-315.

Rousk, J., Bååth, E., Brookes, P.C., Lauber, C.L., Lozupone, C., Caporaso, J.G., Knight, R., Fierer, N., 2010. Soil bacterial and fungal communities across a pH gradient in an arable soil. Intern. Soc. Microb. Ecol. J. 4(10), 1340-1351.

Sayen, S., Guillon, E., 2010. X-ray absorption spectroscopy study of Cu^{2+} geochemical partitioning in a vineyard soil. J. Colloid Interface Sci. 344, 611-615.

Sayen, S., Mallet, J., Guillon, E., 2009. Aging effect on the copper sorption on a vineyard soil: Column studies and SEM–EDS analysis. J. Colloid Interface Sci. 331, 47-54.

Sayyad, G., Afyuni, M., Mousavi, S.F., Abbaspour, K.C., Richards, B.K., Schulin, R., 2010. Transport of Cd, Cu, Pb and Zn in a calcareous soil under wheat and safflower cultivation - A column study. Geoderma 154, 311-320.

Siegel, F.R., 2002. Environmental Geochemistry of Potentially Toxic Heavy Metals, Springer-Verlag, Heidelberg.

Sipos, P., Németh, T., Kovács Kis, V., Mohai, I., 2008. Sorption of copper, zinc and lead on soil mineral phases. Chemosphere 73, 461-469.

Steinitz, C., del Pozo, C., Vargas-Moreno, J.C., Canfield, T., 2011. Futuros alternativos para los paisajes dinámicos del Corredor del Henares. Fundación Paisaje 2011.

Sulkowski, M., Hirner, A.V., 2006. Element fractionation by sequential extraction in a soil with high carbonate content. Applied Geochem. 21, 16-28.

Trasar-Cepeda, C., Leirós, M.C., Seoane, S., Gil-Sotres, F., 2008. Biochemical properties of soils under crop rotation. Appl. Soil Ecol. 39, 133-143.

Vidal, J., Pérez-Sirvent, C., Martínez-Sánchez, M.J., Navarro, M.C., 2004. Origin and behaviour of heavy metals in agricultural Calcaric Fluvisols in semiarid conditions. Geoderma 121, 257-270.

Vig, K., Megharaj, M., Sethunathan, N., Naidu, R., 2003. Bioavailability and toxicity of cadmium to microorganisms and their activities in soil: a review. Adv. Environ. Res. 8, 121-135.

Wang, X., Chen, X., Liu, S., Ge, X., 2010. Effect of molecular weight of dissolved organic matter on toxicity and bioavailability of copper to lettuce. J. Environ. Sci. 22(12), 1960-1965.

Wang, Z.W., Zhang, S.Z., Shan, X.Q., 2004. Effects of low-molecular-weight organic acids on uptake of lanthanum by barley roots. Plant Soil 261, 163-170.

Xia, X., Chen, X., Liu, R., Liu, H., 2011. Heavy metals in urban soils with various types of land use in Beijing, China. J Hazard Mater 186, 2043-2050.

Yang, Y., Zhang, F., Li, H., Jiang R., 2009. Accumulation of cadmium in the edible parts of six vegetable species grown in Cd-contaminated soils. J. Environ. Manag. 90, 1117-1122.

Zapusek, U., Lestan, D., 2009. Fractionation, mobility and bio-accessibility of Cu, Zn, Cd, Pb and Ni in aged artificial soil mixtures. Geoderma 154, 164-169.

Zeng, F., Ali, S., Zhang, H., Ouyang, Y., Qiu, B., Wu, F., Zhang, G., 2011. The influence of pH and organic matter content in paddy soil on heavy metal availability and their uptake by rice plants. Environ. Pollut. 159, 84-91.

Zheljazkov, V.D., Craker, L.E., Xing, B., 2006. Effects of Cd, Pb, and Cu on growth and essential oil contents in dill, peppermint, and basil. Environ. Exp. Bot. 58, 9-16.

Zorrig, W., Rouached, A., Shahzad, Z., Abdelly, C., Davidian, J., Berthomieu, P., 2010. Identification of three relationships linking cadmium accumulation to cadmium tolerance and zinc and citrate accumulation in lettuce. J. Plant Physiol. 167, 1239-1247.

Zorrig, W., Shahza, Z., Abdelly, C., Berthomieu, P., 2012. Calcium enhances cadmium tolerance and decreases cadmium accumulation in lettuce (*Lactuca sativa*). Afr. J. Biotechnol. 11(34), 8441-8448.

7. Conclusiones

Conclusiones

- La extractabilidad de los metales, estimada con el método del $NaNO_3$, disminuyó con el tiempo de contacto del experimento de incubación, excepto en el caso del Pb, mientras que con el método del DTPA los valores de concentración metálica extraídos nunca llegaron a alcanzar el equilibrio dentro del periodo estudiado. La fracción carbonatada determinó la tendencia temporal de la extractabilidad de Cd, Cu y Zn con $NaNO_3$, mostrando ser, en general, un factor explicativo más importante que la dosis metálica aplicada. Sin embargo, en el caso de la movilidad potencial de los metales, estimada con DTPA, fueron determinantes tanto la dosis metálica como la acción combinada de las fracciones carbonatada, orgánica, óxido de hierro y arcilla.

- Los elevados porcentajes de metales extraídos con métodos de extracción complejantes mostraron que existe una significativa fracción potencialmente disponible de metales pesados en estos suelos.

- La fracción carbonatada explicó los patrones de extractabilidad de Cd, aunque no se descartó la posible influencia de otros constituyentes edáficos. Los coloides del suelo, tanto de naturaleza inorgánica como orgánica, determinaron la extractabilidad de Cu. La distribución de tamaño de partícula mostró ser el principal factor explicativo de la extractabilidad de Zn. A pesar de que se obtuvieron evidencias de la precipitación de Pb en la fracción carbonatada, esta fracción no explicó los patrones de extractabilidad de este metal.

- La significativa disminución de la biomasa, tanto aérea como radicular, siendo ésta más pronunciada en el caso de la variedad iceberg, y la alteración de la absorción de nutrientes esenciales mostró que la mezcla metálica añadida resultó ser fitotóxica para las plantas de *Lactuca sativa* L., principalmente en el nivel de elevada contaminación.

- Las concentraciones metálicas cuantificadas en las raíces de *Lactuca sativa* L. fueron superiores a las detectadas en las hojas, lo que no impidió que se produjese una significativa translocación a las partes comestibles de las plantas, principalmente en el caso del Zn.

- Al igual que en el estudio de la extractabilidad metálica, la fracción carbonatada (en el caso del Cd) y la granulometría (en el caso del Zn) fueron los factores determinantes en el control de la biodisponibilidad de estos metales. La fracción orgánica fue el principal factor explicativo de los patrones de biodisponibilidad de Cu y Pb, ejerciendo un papel dual, en función de su composición. Mientras que la materia orgánica, tanto lábil como recalcitrante, disminuye la absorción de los metales, la fracción orgánica lábil promueve los procesos de translocación.

- Los métodos basados en sales neutras mostraron, en general, ser adecuados en la estimación de los patrones de biodisponibilidad metálica en las hojas de *Lactuca sativa* L., indicando que podrían ser empleados en la evaluación del riesgo de entrada de metales en la cadena trófica. La idoneidad de métodos, como DTPA y LMWOA, en la simulación de la biodisponibilidad metálica dependió del tejido de la planta, de la naturaleza del metal y del nivel de contaminación. Métodos de extracción de mayor fuerza, como EDTA y HNO_3, no fueron apropiados en la simulación de la biodisponibilidad de los metales.

- Las actividades ureasa, ß-galactosidasa y arilsulfatasa, así como la concentración de ADN bacteriano, mostraron ser bio-indicadores sensibles de los efectos derivados de la contaminación metálica.

- La elevada inhibición de las actividades enzimáticas medidas, así como el incremento de la relación ADN fúngico/bacteriano, indicaron que procesos biológicos clave del suelo podrían estar alterados.

- La persistencia de las actividades enzimáticas y la supervivencia de las poblaciones bacterianas estuvieron determinadas, principalmente en el nivel de baja contaminación, por las mismas fracciones del suelo que controlaron tanto la disponibilidad como la biodisponibilidad de los metales, como son la orgánica, la carbonatada, óxido y la arcilla.

En resumen, el alto porcentaje de extracción metálica, la elevada fitotoxicidad y bioacumulación metálica, así como la fuerte alteración de los parámetros bioquímicos y microbiológicos, ponen de manifiesto la vulnerabilidad de estos suelos. Los resultados obtenidos, por tanto, muestran que los suelos objeto de estudio se verían altamente afectados ante una creciente acumulación metálica en áreas agrícolas periurbanas. Teniendo en cuenta este escenario y que una mayor proporción de las fracciones orgánicas e inorgánicas (carbonatada, mineral fina y óxido de hierro) en los suelos podría minimizar este impacto, sería aconsejable que los límites individuales propuestos por la legislación europea actual fueran establecidos de acuerdo con las características edáficas y/o revisados cuando la contaminación se produce por una mezcla metálica. Desde esta perspectiva, se plantea una posible línea de investigación a desarrollar en futuros trabajos, con el fin de mejorar las bases para proponer estándares adecuados de calidad de acuerdo con las estrategias europeas para la protección de los suelos.

Conclusions

- The extractability of metals, estimated with the $NaNO_3$ method, decreased throughout the incubation time, except in the case of Pb. However, using the DTPA method, extractable metal concentration values never reached equilibrium within the period studied. Carbonate fraction determined the temporal trends of Cd, Cu and Zn extractability with $NaNO_3$, showing to be, in general, a more important factor than the metal dose applied. Nevertheless, in the case of the metal potential mobility, estimated with DTPA, both the contamination level and the joint action of carbonate, organic, Fe-oxide and clay fractions were determining.

- The high percentages of metals extracted with complexing extraction methods showed that there is a significant potential metal available fraction in these soils.

- Carbonate fraction explained Cd extractability patterns, although it was not excluded the potential influence of other soil fractions. Both inorganic and organic nature soil colloids determined the Cu extractability. Particle size distribution was found to be the main factor explaining the Zn extractability. Despite the evidence of Pb precipitation in the carbonate fraction, this fraction did not explain the extractability patterns of this metal.

- The significant decrease in both aerial and root biomasses, being more pronounced in the case of the iceberg variety, and the nutrient imbalance proved that the metallic mixture was phytotoxic to *Lactuca sativa* L., mainly at the high contamination level.

- Metal concentrations in the roots of *Lactuca sativa* L. were higher than those found in the leaves, which did not prevent the significant translocation to the edible parts of the plants, mainly in the case of Zn.

- As in the study of metal extractability, the carbonate fraction (in the case of Cd), and the particle-size distribution (in the case of Zn) were decisive in affecting bioavailability patterns of these metals. Organic fraction was the main factor explaining Cu and Pb bioavailability patterns, playing a dual role, depending on its composition. While organic matter, both labile and recalcitrant fractions, decreases metal absorption, labile organic fraction promotes translocation processes.

- Methods based on neutral salts showed, in general, to be suitable to estimate metal bioavailability patterns in leaves of *Lactuca sativa* L., indicating that may be used in assessing the risk of metals entering the food chain. The suitability of methods (such as DTPA and LMWOA) for simulating metal bioavailability depended on the plant tissue, the metal nature and the contamination level. Extraction methods with a stronger extraction capacity (such as EDTA and HNO_3) were not suitable.

- Urease, ß-galactosidase and arylsulfatase activities, as well as the concentration of bacterial DNA, were shown to be sensitive bio-indicators of the effects derived from metal contamination.

- The high inhibition of the enzyme activities measured, as well as the increase of the fungal/bacterial DNA ratio, indicated that key soil biological processes may be altered.

- The persistence of enzyme activities and the survival of bacterial and fungal populations were governed, mainly at the low contamination level, by soil fractions thereof that controlled both the availability and the bioavailability of metals, such as organic, carbonate, oxide and clay.

In summary, the high percentage of metal extraction, the elevated phytotoxicity and metal bioaccumulation and the significant alterations in the biochemical and microbiological parameters highlight the vulnerability of these soils. The results thus demonstrate that the soils in this study would be greatly impacted on the case of an increased accumulation of metals in periurban agricultural areas. Taking into account this scenario and that a greater proportion of organic and inorganic fractions (carbonate, fine mineral and Fe-oxide) in soils could minimize this impact, it would be recommended that the individual limits proposed by the current European legislation were established according to the characteristics of the soils, and/or revised when the contamination is produced by a combination of metals. This approach suggests a possible line to be developed in future research works in order to improve the basis for the proposal of adequate quality standards according to the European strategies for the protection of soils.

Conclusions

- L'extractabilité des éléments trace métalliques (ETM), estimée par la méthode du $NaNO_3$, a diminuée tout au long du temps de contact de l'essaie d'incubation, à exception du Pb, alors que les valeurs de concentration des ETM extraits avec la méthode du DTPA n'ont jamais atteint l'équilibre dans la période d'étude. La fraction carbonatée a déterminé la tendance temporelle d'extractabilité de Cd, Cu et Zn avec du $NaNO_3$, prouvant être, en général, un facteur explicatif plus important que la dose métallique appliquée. Pourtant, dans le cas de la mobilité potentielle des ETM, estimée par la méthode du DTPA, la dose métallique et l'action combinée des fractions carbonaté, organique, oxyde de fer et argile, ont montré être déterminants.

- Les forts pourcentages d'extraction des ETM avec des extractants complexants montrent qu'il existe une fraction significative des ETM potentiellement disponibles dans ces sols.

- La fraction carbonatée a expliqué les patrons d'extractibilité du Cd, bien que l'influence d'autres constituants du sol n'ait pas été exclue. Colloïdes du sol, de nature inorganique et organique, ont déterminé l'extractibilité du Cu. La distribution de taille des particules a fait preuve d'être le principal facteur explicatif d'extractabilité du Zn. Bien que le Pb ait été précipité dans la fraction carbonatée, cette fraction n'a pas expliqué l'extractibilité du Pb.

- La forte diminution de la biomasse, à la fois aérienne et racinaire, encore plus prononcée dans la variété iceberg, ainsi que l'altération de l'absorption des cations majeurs, ont montré que le mélange des ETM employé a été phytotoxique pour les plantes de *Lactuca sativa* L., principalement dans le niveau de contamination élevé.

- Les teneurs des ETM quantifiés dans les racines de *Lactuca sativa* L. étaient plus élevés que ceux trouvés dans les feuilles, ce qui n'a pas empêché que se produisais une translocation significative vers les parties comestibles des plantes, principalement dans le cas du Zn.

- De même que dans l'étude d'extractabilité, la fraction carbonatée (dans le cas du Cd) et la granulométrie (dans le cas du Zn) ont été les facteurs déterminants dans le contrôle de la biodisponibilité de ces ETM. La fraction organique a été le principal facteur explicatif des patrons de biodisponibilité du Cu et du Pb, jouant un double rôle, en fonction de sa composition. Alors que la matière organique, fractions labile et récalcitrant, diminue l'absorption des ETM, la fraction organique labile favorise les processus de translocation.

- Les méthodes basées sur des sels neutres ont montré, en général, être appropriées pour l'estimation des patrons de biodisponibilité métallique dans les feuilles de *Lactuca sativa* L., indiquant qu'ils peuvent être employées dans l'évaluation du risque d'entré des ETM dans la chaîne trophique. L'adéquation des méthodes, telles que le DTPA et le LMWOA, dans la simulation de la biodisponibilité des ETM a dépendu du tissu végétal, de la nature du métal et du niveau de contamination. Les méthodes d'extraction de plus grande force, telles que l'EDTA et le HNO_3, n'ont pas été propres pour simuler la biodisponibilité des ETM.

- Les activités uréase, ß-galactosidase et arylsulfatase, ainsi que la concentration de l'ADN bactérien, se sont avérés sensibles bio-indicateurs des effets de la contamination par des ETM.

- La forte inhibition des activités enzymatiques mesurées, ainsi que l'augmentation du ratio de l'ADN fongique/bactérien, ont indiqué que des processus biologiques clés du sol peuvent être perturbés.

- La persistance des activités enzymatiques et la survie des populations bactériennes ont été déterminées, principalement dans le niveau de contamination basse, par les fractions organique, carbonatée, l'oxyde et l'argile, les mêmes fractions du sol qui contrôlent à la fois la disponibilité et la biodisponibilité des ETM.

En résumé, le haut pourcentage d'extraction des ETM, la forte phytotoxicité et bioaccumulation métallique, ainsi que la perturbation des paramètres biochimiques et microbiologiques mettent en évidence la vulnérabilité de ces sols. Les résultats obtenus montrent donc que les sols d'étude seraient très affectés face à une croissante accumulation métallique dans des zones périurbaines agricoles. Compte tenu de ce scénario et qu'une proportion plus élevée des fractions organiques et inorganiques (carbonate, minérale fine et des oxydes de fer) pourrait minimiser cet impact, il serait conseillé que les limites individuelles proposées par la législation européenne actuelle étaient établis en conformité avec les caractéristiques du sol et/ou révisées en cas de contamination par un mélange des ETM. Au sein de cette perspective, nous proposons une possible ligne de recherche à développer dans des travaux futurs, afin d'améliorer les bases pour proposer des normes de qualité appropriées selon les stratégies européennes pour la protection des sols.