# Elementos traza en el sistema planta-suelo: implicaciones para la ecología de especies leñosas y la restauración de zonas degradadas

**Memoria presentada para optar al grado de Doctora por la Universidad de Sevilla**

**María Teresa Domínguez Núñez**

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CSIC
Consejo Superior de Investigaciones Científicas

**Teodoro Marañón Arana, Investigador Científico del Consejo Superior de Investigaciones Científicas (CSIC)**

**José Manuel Murillo Carpio, Investigador Científico del Consejo Superior de Investigaciones Científicas (CSIC)**

**HACEN CONSTAR:**

Que el trabajo descrito en la presente memoria, titulado: "**Elementos traza en el sistema planta-suelo: implicaciones para la ecología de especies leñosas y la restauración de zonas degradadas**" ha sido realizado bajo su dirección por Dña. María Teresa Domínguez Núñez en el Instituto de Recursos Naturales y Agrobiología de Sevilla, CSIC, y reúne todos los requisitos necesarios para su aprobación como Tesis Doctoral.

Sevilla, 20 de Julio de 2009

Dr. Teodoro Marañón Arana

Dr. José Manuel Murillo Carpio

**Mª Cruz Díaz Antunez Barradas, Profesora Titular de la Universidad de Sevilla**

**HACE CONSTAR:**

Que el trabajo descrito en la presente memoria, titulado: "**Elementos traza en el sistema planta-suelo: implicaciones para la ecología de especies leñosas y la restauración de zonas degradadas**" ha sido realizado bajo su tutela por Dña. María Teresa Domínguez Núñez dentro del Programa de Doctorado de Biología de la Conservación, del Departemento de Biología Vegetal y Ecología de la Universidad de Sevilla.

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Dr. Mª Cruz Díaz Antunes Barradas

*A mis padres,*

*Pepe y Teresa*

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## Resumen

Durante el último siglo, los ciclos biogeoquímicos de los elementos traza (ET) han sufrido importantes alteraciones debido a las actividades humanas. La contaminación del suelo por este tipo de elementos puede tener distintos efectos sobre el funcionamiento de los ecosistemas terrestres, y dificultar el establecimiento de la cubierta vegetal en las zonas contaminadas. El conocimiento de estos efectos en ecosistemas terrestres mediterráneos es aún escaso, a pesar de que las zonas contaminadas por ET son bastante frecuentes, particularmente en el Sur de España. La presente Tesis Doctoral pretende contribuir en el conocimiento de la dinámica de los ET y sus efectos en especies forestales mediterráneas, incidiendo en algunos de los aspectos más relevantes para la restauración de la cubierta vegetal en zonas degradadas. Para ello se han combinado estudios en condiciones de campo y experimentos bajo condiciones controladas en invernadero. Los estudios de campo se desarrollaron en el Corredor Verde del Guadiamar (Sevilla), zona contaminada por un vertido minero en 1998, tras el cual se implementó un proyecto de restauración a gran escala.

Como primer objetivo, se estudiaron los patrones de acumulación de ET en la biomasa aérea de la comunidad de especies leñosas del Corredor Verde del Guadiamar, a lo largo de un gradiente de contaminación y sobre un rango amplio de propiedades del suelo. A pesar de las altas concentraciones de ET en el suelo, la transferencia de ET a las hojas de las especies leñosas estudiadas fue baja, y estuvo escasamente influenciada por los factores edáficos. La excepción fue el álamo blanco (*Populus alba*), para el cual las concentraciones foliares de cadmio (Cd) y cinc (Zn) alcanzaron los 3 y 410 mg $kg^{-1}$, respectivamente. Estos niveles están por encima del rango normal de concentraciones en plantas superiores y, en el caso del Cd, podrían llegar a ser tóxicos para los herbívoros.

En segundo lugar, se analizó el riesgo de transferencia de ET a través del consumo de pastizales por parte del ganado, como primer paso para evaluar la viabilidad del pastoreo selectivo como medida de control de los herbazales en zonas reforestadas. Para ello se analizó la composición florística y composición química de muestras de pastizales durante las estaciones de otoño y primavera en un gradiente de contaminación. Asimismo se analizaron muestras de pelos y heces de caballos que pastan en la zona, como posibles indicadores de la ingesta de ET. La composición florística influyó en las concentraciones de ET del pastizal: pastizales con predominio de gramíneas mostraron menores concentraciones de ET. A pesar de las diferencias estacionales en las concentraciones de ET en los pastizales (inferiores durante la primavera, debido al efecto de dilución de las

concentraciones por la mayor biomasa), la ingesta potencial de ET por el ganado fue tolerable en términos generales. Se comprobó además (mediante análisis de las heces), que se produce una excreta preferencial de elementos no esenciales, como arsénico, plomo, cadmio y talio, respecto de los esenciales para el ganado (cobre y cinc).

En tercer lugar, se estudió la influencia de la contaminación del suelo en el estado nutricional de las principales especies de árboles del Corredor Verde: acebuche (*Olea europaea* var. *sylvestris*), álamo blanco y encina (*Quercus ilex* subsp. *ballota*). Se observaron algunas deficiencias nutricionales, sobre todo de fósforo, en las especies estudiadas. Estas deficiencias de fósforo fueron más evidentes para el acebuche, y estuvieron acentuadas bajo condiciones de acidez, frecuentes en la zona de estudio debido a la oxidación de restos de pirita del vertido minero. En estos suelos ácidos, la asimilación de otros nutrientes como magnesio y azufre en el álamo blanco aumentó, posiblemente como respuesta a la liberación de cationes del complejo de cambio en estos suelos ácidos y a la alta disponibilidad de sulfatos.

En cuarto lugar, se estudiaron los factores edáficos que condicionan la biodisponibilidad de ET en los suelos reforestados, y la respuesta foliar de la encina, una de las especies más utilizadas en la reforestación de zonas degradadas, a cambios en los niveles de biodisponibilidad. El pH del suelo fue el factor de mayor influencia en la disponibilidad de cadmio, cobre, plomo y cinc, mientras que otros factores como el contenido en materia orgánica, la textura o la capacidad de intercambio catiónico tuvieron escasa influencia. Cadmio fue el elemento potencialmente más lábil, aunque en condiciones de campo fue escasamente translocado desde la raíz a las hojas de la encina. Se demostró experimentalmente la alta capacidad que presenta la encina para retener Cd en las raíces finas, a partir de plantas sometidas a tratamientos con diferentes niveles de Cd.

Finalmente, se analizó la respuesta ecofisiológica de plántulas de encina a exposiciones altas de Cd y talio (Tl), dos elementos traza no esenciales y potencialmente muy móviles en el sistema suelo-planta. Aunque a altas dosis ambos elementos provocaron efectos adversos sobre las plantas, los mecanismos responsables de estos efectos fueron distintos. Para el Cd, la tolerancia de las plántulas de encina fue relativamente buena; el fotosistema II permaneció relativamente inalterado, aunque las tasas de fotosíntesis neta disminuyeron, probablemente por una inhibición en la fijación del carbono inducida por el Cd. El Tl, sin embargo, provocó efectos más severos, incluso letales en las plántulas, con una fuerte inhibición del fotosistema II, disminución de las tasas de asimilación y conductancia estomática. Los patrones de transporte y acumulación de los dos elementos en la planta fueron bastante distintos: para el Cd existen mecanismos efectivos de retención a nivel de raíz, mientras que el Tl es retenido en menor medida en la raíz y ampliamente transportado a las hojas donde, a juzgar por la respuesta tóxica de las plantas, no hay mecanismos efectivos de tolerancia.

## Abstract

During the last century, biogeochemical cycles of trace elements (TE) have been much altered by human activities. Soil contamination may have different effects on the functioning of terrestrial ecosystems, which may hamper the establishment of vegetation in contaminated areas. Despite TE contaminated areas are very frequent in Southern Spain, the knowledge of the effects of this type of contamination on Mediterranean terrestrial ecosystems is still scarce. This Thesis aims to contribute to the knowledge of the dynamics and effects of TE on Mediterranean forest species, focussing on some of the most relevant factors for the restoration of degraded sites. For that purpose, different field and greenhouse studies were conducted. Field studies were conducted in the Guadiamar Green Corredor (Sevilla), where soils were contaminated by a mine in 1998.

Firstly, we studied the patterns of TE accumulation in the aboveground biomass of the woody plant community in the Guadiamar Green Corridor, along a pollution gradient and over a broad range of soil conditions. In spite of the high TE total concentrations in the soils, the transfer of these elements to the leaves of the woody plant species was low, and was scarcely influenced by edaphic conditions. The exception was white poplar (*Populus alba*), which showed foliar concentrations of Cd and Zn up to 3 and 410 mg $kg^{-1}$, respectively. These values were above the normal ranges for higher plants, and, in the case of Cd, could be toxic for herbivores.

Secondly, we analyzed the risk of TE transfer through pasture consumption by grazing, in order to assess the feasibility of selective grazing as a way of weed control. Floristic composition and chemical composition of pastures were analyzed during different seasons along a pollution gradient. Likewise, horse hair and excrement samples were analyzed, as possible indicators of TE ingestion. Floristic composition influenced the TE concentrations of the pastures: those pastures dominated by graminoid species showed the lowest TE concentrations. There were some seasonal differences in the accumulation of TE, which was lower during the spring season due to a dilution effect provoked by the higher biomass. However, the potential ingestion of TE by grazing animals was tolerable in all the seasons. Faeces analyses revealed that non-essential elements, such as arsenic, cadmium, lead and thallium were preferentially excreted, in comparison to those essential elements (copper and zinc).

Thirdly, we studied the influence of soil contamination on the nutritional status of the most

important tree species in the area: wild olive tree (*Olea europaea* var. *sylvestris*), white poplar and Holm oak *(Quercus ilex* subsp. *ballota*). Some nutritional deficiencies, mainly of phosphorus, were observed in these species. The highest phosphorus deficiencies were observed for wild olive tree, and they were enhanced in acidic soils, which are frequent in the study area due to the oxidation of the pyrite from the mine spill. In these acidic soils, the uptake of some other nutrients, such as magnesium and sulphur, by white poplar increased, possibly due to the release of the cations from the exchange sites and due to a high sulphate availability under such acidic conditions.

Soil factors influencing TE bioavailability in the remediated soils were also studied. The response of the foliar chemistry of Holm oak to changes in the bioavailable levels was also analyzed. Soil pH had the highest influence on the bioavailability of cadmium, copper, lead and zinc, while other soil factors such as texture, organic matter content, and cation exchange capacity had almost no influence on TE bioavailability. Cadmium showed the highest potential mobility in soils, although under field conditions it was scarcely translocated into the oak leaves. Under controlled conditions in a greenhouse experiment, we found that this plant species has a high capacity for Cd retention at the root level, especially at fine roots.

Finally, we studied the ecophysiological response of Holm oak seedlings to high exposures to Cd and Tl. These are two non-essential trace elements, with a potentially high mobility and toxicity in the soil-plant subsystem. Although high exposures to both elements provoked detrimental effects on the plants, the underlying mechanisms were different for each element. For Cd, the seedlings showed a relatively high tolerance; photosystem II was almost unaffected, although net photosynthesis rates decreased, probably due to an inhibition of carbon fixation. In contrast, Tl provoked severe effects on the photosystems, as well as a decrease in stomatal conductance and net assimilation rates. The patterns of accumulation of both elements were also very different: for Cd, there are effective mechanisms of exclusion at the root level, while Tl was scarcely retained at roots and widely transported into leaves, where it may produce severe toxic effects.

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# Introducción

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# Introducción general

La intensificación de las actividades humanas durante el último siglo está provocando importantes alteraciones biogeoquímicas en los ecosistemas (Schlesinger, 1997). Estas alteraciones constituyen uno de los más importantes componentes del cambio global, y centran una buena parte de la atención de la comunidad científica en la actualidad. La contaminación del aire, el suelo o el agua es uno de los principales factores responsables de estas alteraciones biogeoquímicas, y se ha relacionado con importantes problemas ambientales de las últimas décadas, como el decaimiento forestal (Likens et al., 1996; Schulze, 1989; Taylor et al., 1994) y la pérdida de biodiversidad (IPCC, 2001; Sala et al., 2001).

Los elementos traza (metales pesados y algunos metaloides, como el arsénico) constituyen un importante grupo de contaminantes. Estos elementos son constituyentes minoritarios de los seres vivos, y en condiciones normales sus concentraciones son relativamente bajas; por ejemplo, en las plantas (por definición) sus concentraciones son inferiores a 1 g $kg^{-1}$ (Bargagli, 1998). Sin embargo, distintas actividades humanas han alterado los ciclos biogeoquímicos de estos elementos, de manera que las entradas antropogénicas de elementos traza (ET) a los ecosistemas a través de la atmósfera, el agua o el suelo han aumentado sustancialmente a escala global durante el último siglo (Nriagu y Pacyna, 1988; Nriagu, 1996). Así lo demuestran diversos estudios que analizan sedimentos de lagos (Battarbee et al., 1985), turberas (Espi et al., 1997), depósitos en los hielos del Ártico (Boutron et al., 1991) o bandas anuales de corales (Guzman y Jarvis, 1996). La evolución de la composición química de la vegetación terrestre en algunas regiones también refleja este aumento en los niveles globales de disponibilidad de ET (Herpin et al., 1997; Peñuelas y Filella, 2002).

Actividades como la industria, la agricultura, el transporte o el tratamiento de residuos urbanos son fuentes de contaminación por ET. Sin embargo, la minería es la responsable en mayor medida de la movilización de los ET de la litosfera, hacia condiciones de mayor disponibilidad para los seres vivos. Hacia el año 2000, las estimas de producción anual de residuos contaminados con ET derivados de la minería oscilaban entre las 10.000 y las 600.000 toneladas a nivel mundial (Warhurst y Noronha, 2000). Así, los ET son algunos de los contaminantes más extendidos, a la vez que persistentes y difíciles de eliminar (Dickinson, 2000). En Europa, la Agencia Europea del Medio Ambiente identificó en el año 2001 1,5 millones de enclaves contaminados, en su mayoría por ET, 4.902 de ellos en España (Belluck et al., 2006). El alcance económico de estas cifras es bastante elevado: se ha estimado que el coste de la recuperación de suelos contaminados se sitúa entre los 101.000 y los 1,25 $10^6$ € $ha^{-1}$ (Berti y Cunningham, 2000). En consecuencia, el tratamiento de la superficie contaminada en un país como el Reino Unido (unas 100.000 ha) representaría un coste de entre el 0,4 y el 5,7 % de su producto interior bruto para el año 2008 (Dickinson, 2000). Debido a la magnitud ambiental y económica de la contaminación por ET, resulta muy interesante conocer la dinámica y los efectos de estos elementos en distintos compartimentos de los ecosistemas, así como las posibles implicaciones para la restauración de zonas contaminadas.

**Dinámica de los elementos traza en el sistema suelo-planta**

Los niveles de ET en el suelo pueden tener un origen litológico, como consecuencia de la meteorización de la roca madre. Así, algunos suelos en zonas de serpentina pueden tener de manera natural concentraciones relativamente altas de Ni, Cr, Co o Zn (Doherty et al., 2008; Caillaud et al., 2009). Sin embargo, las entradas más importantes al suelo se derivan de actividades humanas, mediante deposición atmosférica, aplicación o vertido directo al suelo. En bosques templados del norte de Europa y América, la deposición atmosférica de metales procedentes de las zonas industriales y de la quema de combustibles fue uno de los factores responsables del decaimiento forestal generalizado observado desde la segunda mitad del siglo XX, potenciado por la acidificación de los suelos como consecuencia de la lluvia ácida (Godbold y Hüttermann, 1985; Hütterman et al., 1999). En torno a zonas urbanas e industriales, la deposición atmosférica sigue siendo actualmente una importante entrada de metales en zonas forestales (Cao et al., 2008; Killönen et al., 2009). Las masas forestales actúan como captadores de los aerosoles y las partículas en suspensión (deposición seca), mientras que los metales disueltos en la precipitación (deposición húmeda) pueden trasladarse hasta zonas rurales más distantes de los focos de emisión (Avila et al., 2003; Hovmand et al., 2008).

Los entornos de las zonas mineras reciben asimismo cantidades relativamente altas de ET por escorrentía o vertido directo al suelo. En España, zonas como las cuencas de los ríos Tinto y Odiel, en Huelva (Cambrollé et al., 2008), el distrito minero de Cartagena-La Unión y Mar Menor, en Murcia (Conesa y Jiménez-Cárceles, 2007), o la cuenca del río Guadiamar en Sevilla (Cabrera et al., 2008) tienen suelos muy enriquecidos en ET, como consecuencia de la actividad minera.

Una vez en el suelo, la dinámica que siguen los ET puede ser bastante compleja. De la cantidad total de un elemento traza en el suelo se pueden distinguir fracciones con distinta movilidad, de las cuales la fracción biodisponible, que puede ser más fácilmente absorbida por las plantas, es la que presenta mayor interés. La biodisponibilidad de un ET para una especie vegetal depende de la propia naturaleza del elemento, de múltiples factores del suelo, así como de la propia especie vegetal que se estudia, ya que las plantas pueden modificar las condiciones de la rizosfera de distintas formas, accediendo a fracciones de ET potencialmente poco disponibles (McGrath et al., 1997). Así, la biodisponibilidad real de un ET no siempre coincide con la fracción más fácilmente extraíble por métodos químicos (mediante, por ejemplo, sales neutras o agentes quelantes).

En general, se considera que los factores más importantes para la disponibilidad de los ET en los suelos son: el pH, el contenido en materia orgánica, la capacidad de intercambio catiónico y contenido en arcillas, y el potencial redox (Greger, 1999). Los elementos catiónicos (p. ej., Cd, Cu, Cr, Pb y Zn) se movilizan en condiciones de acidez, y son retenidos por las cargas negativas de las arcillas y la materia orgánica. No ocurre lo mismo con otros elementos que se presentan en forma aniónica, como el As, cuya movilidad es normalmente mayor en condiciones de alto pH (Adriano, 2001). Los óxidos de Fe y Al también pueden jugar un papel muy importante en la retención de algunos ET en el suelo (Naidu, 2001). La influencia de cada uno de estos factores en la movilidad depende del elemento. Cobre, Cr y Pb presentan una alta tendencia a formar complejos con las sustancias húmicas, de manera que suelen acumularse en los horizontes B del suelo (Bergkvist et al., 1989; McBride, 1989). Cadmio, Ni y Zn tienen menos afinidad por los ácidos orgánicos y son más dependientes de las variaciones del pH; así, pueden ser lixiviados más fácilmente por debajo de la zona de enraizamiento en condiciones de acidez (Bergkvist et al., 1989; Egiarte et al., 2008).

El movimiento de ET desde la solución del suelo hacia el interior de la raíz normalmente es un proceso pasivo, no metabólico, de difusión o flujo masa (Marschner, 1995). Estos procesos que no son específicos pueden estar influenciados por los niveles de transpiración o la cantidad externa de nutrientes, con los que los ET establecen relaciones competitivas por los lugares de intercambio en la rizosfera. Una vez en el interior de la raíz, los ET pueden ser transportados hacia el xilema por vía apoplástica o simplástica, interviniendo en este último caso (vía simplástica) los mecanismos de difusión (transporte pasivo) o de transporte activo con regulación metabólica, necesaria para la absorción de nutrientes esenciales para la planta, como puede ser el caso de algunos ET que tienen carácter de micronutriente (Kahle, 1993).

La asociación con hongos micorrícicos puede tener una gran influencia en la absorción de ET por la raíz. Los mecanismos por los que las micorrizas pueden prevenir, aunque no siempre, la absorción de metales por la planta son diversos y bastante especie-específicos, e incluyen la retención en las paredes celulares del micelio, la formación de polifosfatos o complejos con ácidos orgánicos y péptidos (Godbold et al., 1998; Jentschke y Godbold, 2000). En el caso del As, la forma más común de absorción de la planta (arsenato) es un ión análogo al fosfato, y la asociación con micorrizas en algunos casos puede aumentar la absorción de As por la raíz (Meharg y Hartley-Whitaker, 2002).

Una vez en el xilema, los ET son transportados en forma de complejos con ácidos orgánicos y aminoácidos, y en menor medida, como iones libres (Greger, 1999). La translocación a las distintas partes aéreas depende mucho del elemento y la especie en cuestión. Incluso para una misma especie, distintos genotipos pueden tener distintas tasas de translocación y acumulación en la biomasa aérea (Pulford y Watson, 2003). En general, al tener mucha afinidad por las pectinas de las paredes celulares, una buena parte de los ET que entran en la planta queda retenida en el sistema radical (Kahle, 1993; Brunner et al., 2008). Elementos como Pb, Cr, y Cu tienen mayor afinidad por las pectinas y son ampliamente retenidos en las raíces; otros elementos, como Cd, Ni y Zn son más fácilmente transferibles a las hojas (Turner y Dickinson, 1993; Unterbrunner et al., 2007). En las especies leñosas, la corteza y la madera pueden actuar en algunos casos como importantes depósitos de ET (Nabais et al., 2001; Satake, 2001). En estos tejidos metabólicamente poco activos, los ET pueden permanecer inactivos y se acumulan durante amplios periodos de tiempo, reciclándose de manera más lenta que los ET contenidos en la hojarasca, debido a las menores tasas de descomposición de la madera (Pulford y Watson, 2003).

La cantidad de ET que vuelve al suelo reciclándose a través de la vegetación depende, por tanto, de los patrones de translocación y acumulación de cada especie. En general, el ciclado de elementos como Pb y Cr es muy bajo, y la mayor parte de las cantidades de estos elementos acumuladas en los horizontes superficiales de los suelos proceden del vertido directo o la deposición atmosférica (Bergvist et al., 1989; Hovmand et al., 2009), aunque en el algunos casos la descomposición de las raíces finas puede suponer un importante aporte de Cr y Pb al suelo (Johnson et al., 2003). Cadmio y Zn presentan las mayores tasas de reciclado a través de los aportes de hojarasca (Bergvist et al., 1989; Starr et al., 2003; Johnson et al., 2003). Algunas especies de árboles, como las que pertenecen a la familia salicácea (*Populus* y *Salix*) presentan altas tasas de acumulación de Cd y Zn en sus hojas (Robinson et al., 2000; Madejón et al., 2004). Para estas especies, el retorno de metales al suelo debido a la descomposición de la hojarasca puede provocar la acumulación y el aumento de los niveles biodisponibles de estos elementos en los horizontes más superficiales del suelo (Mertens et al., 2007; Vandecasteele et al., 2008).

Un esquema simplificado de la dinámica de los ET en el sistema suelo-planta de las especies leñosas se muestra en la Fig. 1.1.

**Efectos de los elementos traza en el sistema suelo-planta**

Algunos ET como Cu, Zn o Fe son esenciales para las plantas (micronutrientes) y deben ser absorbidos en pequeñas cantidades. Otros ET, como Pb, Cd y As, no tienen función biológica conocida, y superados ciertos umbrales de tolerancia pueden provocar una respuesta tóxica, incluso letal, en la planta o los microorganismos del suelo. Cantidades excesivas de un ET traza esencial también pueden provocar toxicidad. Los posibles efectos negativos de la presencia de ET en un sistema dependen, por tanto, del tipo de elemento y su función biológica, y de sus niveles de biodisponibilidad.

Los mecanismos por los que causan dicha toxicidad son muy diversos, y pueden manifestarse a nivel molecular, subcelular o celular (revisiones en Påhlsson, 1989; Sanitá di Topi y Gabbrielli, 1999). De manera muy simplificada, la repuesta tóxica se debe al estrés oxidativo que producen los cationes metálicos, a inhibiciones enzimáticas y/o a inhibiciones en la división y elongación celular.

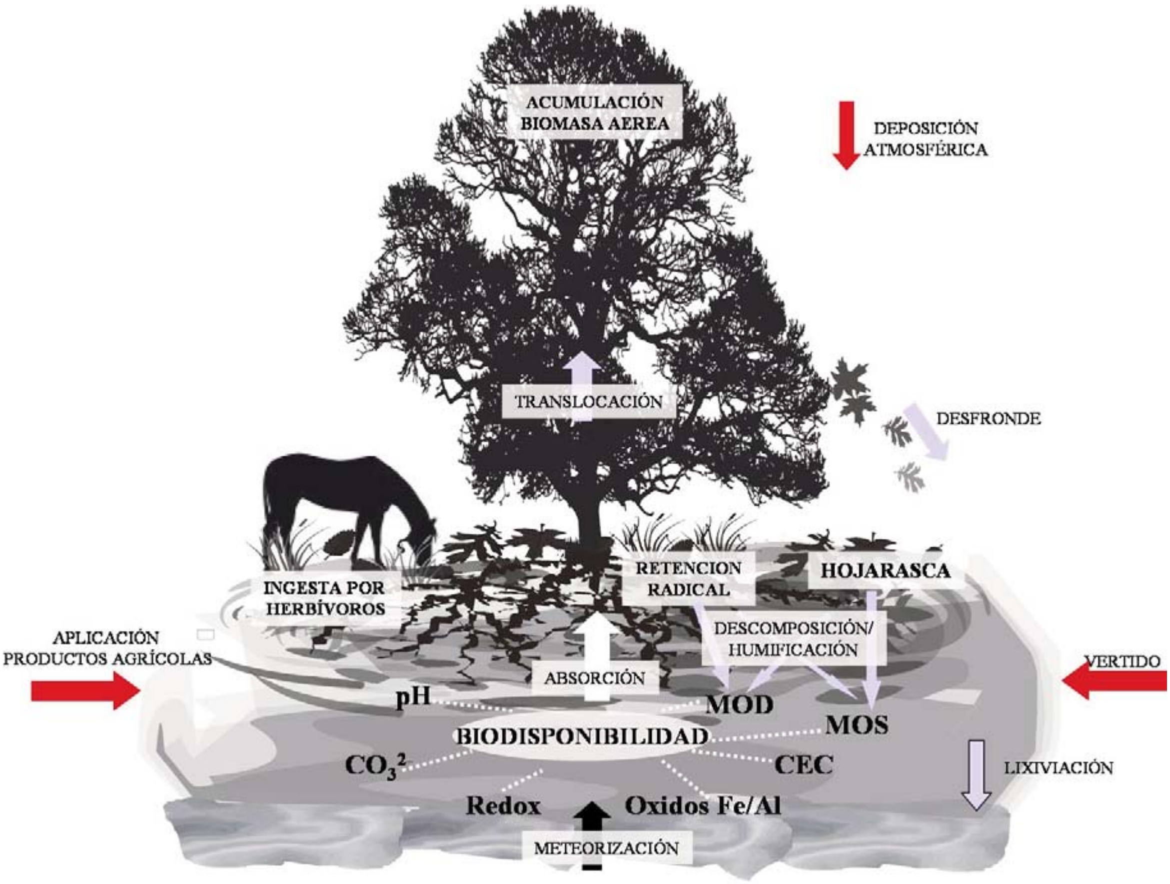


**Fig. 1.1.** Esquema de la dinámica de los elementos traza en el sistema suelo-planta.

Como la mayor parte de la contaminación queda retenida en los horizontes más superficiales del suelo, son las comunidades de microorganismos las que pueden verse más afectadas por la presencia de ET. Muchos estudios han demostrado que la exposición a ET altera las comunidades de microorganismos del suelo, produciendo normalmente una disminución en la diversidad y la biomasa microbiana (Baath, 1989; Naidu et al., 2001), incluyendo las especies de hongos formadores de micorrizas y el grado de asociación de estos hongos con las raíces de las plantas leñosas (Ruotsalainen et al., 2007; Zarei et al., 2008). Como consecuencia de estos cambios, muchos procesos microbianos del suelo se ven alterados. Las respuestas más comunes incluyen una disminución de la respiración basal del suelo (Cotrufo et al., 1995; Valsecchi et al., 1995), de las tasas de nitrificación (Naidu et al., 2001), y de los niveles de algunas actividades enzimáticas (Mench et al, 2006; Hinojosa et al., 2008; Renella et al., 2008; Moreno et al., 2009). Así, los procesos de mineralización de la materia orgánica pueden ralentizarse en zonas forestales contaminadas (Helmisaari et al., 1995; Nieminen y Helmisaari, 1996). Las tasas de descomposición de la hojarasca están muy influenciadas por la composición química de las hojas, y por tanto pueden verse especialmente afectadas por altos niveles de ET en la hojarasca (Cotrufo et al., 1995; Breymeyer et al., 2007; Johnson y Hale, 2004).

A nivel de la raíz, el primer signo de toxicidad es la inhibición del crecimiento radical. Numerosos estudios con especies de árboles han mostrado que la exposición a ET provoca una inhibición de la elongación de la raíz o de la producción de raíces finas, disminuyendo en general la biomasa del sistema radical (Arduini et al., 1994; Godbold y Hütterman, 1985; Reichmann et al., 2001; Lunackova et al., 2003). Esto puede limitar la capacidad de exploración de agua y nutrientes por la planta. Además, algunos ET pueden competir con  los nutrientes por los sitios de intercambio en la matriz del suelo y la rizosfera; por ejemplo, se establecen relaciones competitivas entre As y P (Lambkin y Alloway, 2003), Tl y K (Kwan y Smith, 1991), o Cd y Ca (Perfus-Barbeoch, et al., 2002). Por ello, frecuentemente los árboles que crecen en zonas forestales contaminadas muestran algún tipo de deficiencia nutricional (Reimann et al., 2001; Mankovska et al., 2004; Jamnickà et al., 2007).

A nivel de hoja, los ET pueden interferir en múltiples procesos y componentes implicados en la fotosíntesis, como la síntesis de clorofilas (Sheoran et al., 1990; Ouzonidou, 1995), el transporte de electrones del fotosistema II (Clijsters y Van Assche, 1985; Prasad y Strzalka, 1999), o la actividad de algunas enzimas, como la Rubisco (Van Assche y Clijsters, 1990). El intercambio gaseoso también puede verse alterado, especialmente por el Cd, que en algunas especies induce al cierre estomático (Poschenrieder y Barceló, 1999). Además, la detoxificación de los ET que se encuentran libres en el la célula puede tener un alto coste metabólico, por lo que las plantas que crecen en condiciones de estrés presentan normalmente mayores tasas de respiración, y menores tasas de asimilación neta y crecimiento (Baker, 1987; Lösch y Kölh, 1999).

En definitiva, los ET pueden interferir en múltiples procesos que tienen lugar en el sistema suelo-planta, con importantes repercusiones para el ciclo de nutrientes del ecosistema, y el crecimiento y establecimiento de las especies leñosas.

**Implicaciones para la restauración de zonas degradadas**

Debido a sus potenciales efectos negativos, la acumulación de ciertos niveles de ET en un sistema es indeseable, y en determinadas circunstancias es necesaria la intervención para eliminarlos o atenuar sus efectos. Las técnicas de descontaminación de suelos que incluyen la excavación y/o el tratamiento químico del suelo son normalmente costosas, agresivas con el suelo, y generalmente poco aplicables a áreas extensas. En las últimas dos décadas se ha potenciado el uso de plantas para la remediación de suelos contaminados (fitorremediación). Estas técnicas son a priori más económicas que los tratamientos químicos o mecánicos del suelo, y pueden proporcionan otras ventajas como la protección del suelo contra la erosión y la mejora estética de la zona contaminada. Durante los últimos años el uso de especies de árboles se perfila como una alternativa viable para el control de la contaminación en zonas relativamente extensas (Dickinson, 2000; Pulford y Dickinson, 2006). La reforestación de zonas degradadas es particularmente importante en la cuenca mediterránea, para la cual los distintos escenarios de cambio global prevén un aumento de los riesgos de erosión y desertificación (Moreno, 2005).

La reforestación de zonas contaminadas puede ser un objetivo difícil de alcanzar. Además de los posibles efectos negativos de los ET, concurren comúnmente otros factores como el exceso de radiación, falta de nutrientes o estructura del suelo muy alterada, especialmente en zonas de influencia minera (Tordoff et al., 2000; Wong, 2003). Las especies seleccionadas deben ser capaces de tolerar las altas concentraciones disponibles de ET en el suelo que se pretende reforestar, así como otros estreses ambientales que se presentan simultáneamente.

En la mayoría de especies de leñosas, la tolerancia a ET parece estar más relacionada con la capacidad de respuesta fenotípica de las plantas que con la presencia de mecanismos fisiológicos específicos con base genética (Dickinson et al., 1991; Turner y Dickinson, 1993). En cualquier caso, las especies leñosas pueden diferir en el grado de tolerancia a los ET, como se ha observado en algunas zonas forestales contaminadas, donde se han producido cambios en la composición específica y abundancia relativa de las especies (Chernenkova y Kuperman, 1999; Saleema et al., 2001). La fase de plántula es generalmente más sensible a la presencia de ET en el suelo. La inhibición del crecimiento del sistema radical puede repercutir negativamente en la emergencia de las plántulas y en la capacidad de la planta para explorar agua y nutrientes, pudiendo influir en la resistencia de la plántula a la sequía estival. El estrés causado por la sequía es una de las principales causa de mortalidad durante los primeros años en las plantaciones de especies leñosas mediterráneas (Rey-Benayas, 1998; Pausas et al., 2004; Villar-Salvador et al., 2004). Algunos estudios han mostrado que las tasas de evapotranspiración en plantaciones jóvenes y la eficiencia en el uso del agua de las plantas pueden disminuir como consecuencia de los efectos de los ET a nivel de raíz (Menon et al., 2005; Menon et al., 2007). Las deficiencias nutricionales también pueden condicionar el crecimiento de las especies leñosas en ambientes mediterráneos, cuyos suelos son usualmente pobres en fósforo y nitrógeno (Sardans et al., 2004; Romanyà y Vallejo, 2004). Estas deficiencias pueden potenciarse en zonas contaminadas, tal como se expuso anteriormente.

La tolerancia a los ET puede tener su coste ecofisiológico, en términos de un menor crecimiento de los individuos. El coste de la tolerancia a un estrés ambiental en ocasiones repercute en otros procesos, como la reproducción, de manera que en situaciones de estrés por ET algunas plantas producen menor cantidad de propágulos (Cox, 1988a, b). En otras especies, sin embargo, la contaminación se asocia con un incremento de la reproducción sexual frente al crecimiento vegetativo, lo cual se interpreta como un mecanismo para incrementar las posibilidades de la progenie de evitar las condiciones locales de estrés químico, mediante la colonización de zonas no contaminadas (Zvereva y Kozlov, 2005; Zverev et al., 2008). Los costes de tolerar el estrés químico, ya sea en términos de reproducción o de crecimiento, unido a las posibles dificultades para la germinación y el establecimiento de plántulas, pueden resultar en un lento establecimiento de la cubierta vegetal leñosa en zonas contaminadas por ET.

Los patrones de acumulación de ET en las especies forestales empleadas tienen mucha importancia en la restauración de zonas contaminadas. En algunos casos se persigue la extracción de los ET del suelo mediante las plantas, para lo cual es deseable que las plantas tengan unas altas tasas de absorción de metales por la raíz y de translocación a partes aéreas cosechables (método de fitoextracción), además de elevada capacidad para generar biomasa. Sin embargo, esto conlleva la acumulación de contaminantes en la biomasa aérea, lo cual puede aumentar el riesgo de bioacumulación de los ET a través de la red trófica. Por ello, esta estrategia no es recomendable en zonas extensas o que se quieren proteger para la fauna, donde no se controla la producción de biomasa, y donde las plantas reforestadas pueden interaccionar con los herbívoros o el ganado. Por el contrario, en estos casos es preferible que las plantas contribuyan a la estabilización de los contaminantes del suelo, manteniendo unos niveles bajos de disponibilidad en el suelo y una baja translocación hacia la biomasa aérea (método de fitoestabilización). Las especies leñosas mediterráneas pueden tener un alto potencial de fitoestabilización: el sistema radical de estas especies es normalmente muy amplio, y sobre un rango de especies estudiadas, la retención de metales y otros ET como el As en la raíz parece ser elevada (Arduini et al., 1994; Fuentes et al., 2007; Moreno-Jimenez et al., 2008). La asociación con micorrizas puede potenciar estos procesos de retención radical (Galli et al., 1993; van Tichelen et al., 2001).

La posible acumulación de ET en la vegetación puede limitar la capacidad de usos de la zona contaminada, en particular en lo referente al pastoreo y la actividad cinegética. El riesgo real que supone la acumulación de determinados ET en la vegetación para otros componentes del ecosistema, particularmente para los herbívoros, es difícil de evaluar, debido a las grandes diferencias interespecíficas que pueden existir en la translocación suelo-planta de ET, a las diferencias estacionales en los patrones de acumulación en la plantas, y a que los tipos de dieta varían ampliamente entre distintas especies de herbívoros y están sujetos a variaciones estacionales en la disponibilidad de alimento. Si existen grandes diferencias en las concentraciones de ET en las especies vegetales consumidas, incluso pequeñas variaciones en la dieta pueden resultar en riesgos de ingestión de ET muy diferentes. Algunos estudios han mostrado una alta bioacumulación de ET tóxicos en especies de herbívoros en hábitats donde algunas especies vegetales acumuladoras, como *Salix* spp., son frecuentes (Wren et al., 1994). En bosques boreales, las especies de *Populus* y *Salix* son consumidas preferentemente por los grandes herbívoros durante ciertos períodos, en los que llegan a ingerir hasta 7 mg de Cd al día (Brekken y Steinnes, 2004). En general, las especies animales suelen poseer mayores mecanismos de regulación de los niveles internos de ET esenciales, como Cu, Fe, Mn y Zn, que de elementos no esenciales, como Cd (Hunter et al., 1987a, b; González et al., 2008). Algunos estudios muestran que éste último elemento tiene una alta movilidad en el sistema suelo-planta-invertebrado-mamífero (Hunter et al., 1987b).

En sistemas sometidos a un alto grado de estrés ambiental las interacciones entre plantas pueden jugar un papel muy importante en los patrones de reclutamiento de las especies leñosas. El balance entre las relaciones de competencia y facilitación puede ser bastante complejo, dependiendo de las propiedades de cada sistema, cambiando a distintas escalas o siendo especie-específico (Holgrem et al., 1997; Dormann et al., 2002; Gómez-Aparicio et al., 2005a; Maestre et al., 2005). En situaciones de alto estrés abiótico, las relaciones de facilitación suelen potenciarse frente a las de competencia (Brooker y Callaghan, 1998; Callaway et al., 2002; Cook, 2002;). Así, en ciertos ambientes mediterráneos, la facilitación por matorral u otras especies perennes puede favorecer el establecimiento de las especies leñosas, debido principalmente a la modificación de las condiciones microclimáticas (Maestre et al., 2001; Gómez-Aparicio et al., 2005b), pero también a una mejora de las condiciones físicas, químicas y biológicas del suelo bajo la cubierta del matorral (Tirado y Pugnaire, 2003; Armas y Pugnaire, 2005; Cortina y Maestre, 2005; Goberna et al., 2007). Este efecto nodriza se ha propuesto como técnica aplicable a la restauración ecológica en algunos sistemas mediterráneos (Maestre et al., 2001; Castro et al., 2004; Gómez-Aparicio et al., 2004).

El grado de estrés químico derivado de los ET también puede mostrar cierta heterogeneidad espacial, influido por la distribución espacial de la vegetación. Los pocos estudios que analizan facilitación y competencia en situaciones de estrés por ET señalan un balance positivo hacia la facilitación en el reclutamiento de plántulas. Si bien los principales mecanismos de facilitación se relacionan con el amortiguamiento de las condiciones microclimáticas, que pueden ser especialmente desfavorables en zonas contaminadas, la presencia del matorral o árbol adulto puede disminuir la exposición de las plántulas a los ET, ya sea por amortiguación de la deposición atmosférica o por un aumento en los niveles de materia orgánica del suelo, que disminuye la biodisponibilidad de ET (Ginoccio et al., 2004; Eränen y Kozlov, 2007; Zvereva y Kozlov, 2007).

En definitiva, la dinámica de los ET en el sistema suelo-planta puede ser bastante compleja e influir en múltiples procesos a nivel individual (por ejemplo, establecimiento radical y crecimiento) o ecosistémico (ciclo de nutrientes, bioacumulación a través de la red trófica), con importantes implicaciones para la restauración de las zonas degradadas. Estos aspectos están poco estudiados en sistemas mediterráneos. Si bien en los últimos años se ha generado bastante información sobre la monitorización de la acumulación de ET en árboles y arbustos mediterráneos (Alfani et al., 2000; Aboal et al., 2004; Madejón et al., 2004, 2006; Rossini-Oliva y Mingorance, 2006), aún existe escaso conocimiento sobre las posibles consecuencias biogeoquímicas y ecológicas de los ET en especies forestales mediterráneas. La presente Tesis Doctoral pretende contribuir en este tema, incidiendo en algunos de los aspectos más relevantes para la restauración de la cubierta vegetal en zonas degradadas. En el siguiente capítulo se exponen los principales objetivos de la Tesis y su estructuración.

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# Capítulo 2

# Objetivos

# Capítulo 2

## Objetivos y estructuración de la Tesis

El objetivo general de la tesis es estudiar la dinámica de los elementos traza en el sistema suelo-planta en zonas reforestadas, así como la influencia de estos elementos en algunos procesos de especies leñosas mediterráneas, como la adquisición de nutrientes, las tasas de crecimiento y asimilación de carbono, y crecimiento de plántulas. Para ello se han combinado estudios en condiciones de campo y bajo condiciones controladas en invernadero.

Los estudios de campo se han desarrollado en el Corredor Verde del Guadiamar (Sevilla). Esta zona fue afectada por un vertido minero en 1998 que contaminó el suelo con distintos elementos traza, y posteriormente se implementó un proyecto de restauración a gran escala que incluyó la aplicación de enmiendas y la reforestación con especies leñosas (ver **Capítulo 3**). Así, el Corredor Verde es una zona idónea para analizar la respuesta de las especies leñosas a la contaminación por elementos traza, y evaluar las implicaciones de la contaminación para la restauración de zonas degradadas.

En el **Capítulo 4** se describe la transferencia de elementos traza del suelo a la biomasa aérea de la comunidad de leñosas del Corredor Verde del Guadiamar (tanto en individuos adultos como en plantones reforestados) en un rango de condiciones de suelo (propiedades generales y nivel de contaminación). Las preguntas que se plantean son las siguientes: ¿cuáles son los patrones de acumulación de ET en las especies leñosas mediterráneas? ¿Cuál es el riesgo de acumulación y posible transferencia a la red trófica? En función de estos patrones, ¿cuáles son las especies más adecuadas para reducir los flujos de elementos traza en el sistema suelo-planta de una zona a restaurar? Este capítulo se complementa con el **Capítulo 5**, donde se aborda el riesgo de transferencia de ET a través del consumo de pastizales por parte del ganado. Con ello se pretende evaluar la viabilidad del uso del ganado como forma de control de los herbazales en la zona reforestada. El control de las herbáceas es un aspecto muy importante en la gestión de la zona, debido al actual riesgo de incendio y a la posible competencia que puede suponer para los plantones el desarrollo de los herbazales. Para ello se analizó la composición florística y composición química de muestras de pastizales durante las estaciones de otoño y primavera a lo largo de seis zonas en un gradiente de contaminación; asimismo se analizaron muestras de pelos y heces de caballos que pastan en la zona, como posibles indicadores de la ingesta de ET.

En el **Capítulo 6** se analiza la influencia de las condiciones del suelo (entre ellas, el nivel de contaminación por elementos traza) en el estado nutricional (contenido de nutrientes y clorofila) de las tres especies de árboles dominantes en la zona: acebuche (*Olea europaea* var. *sylvestris*), álamo blanco (*Populus alba*) y encina (*Quercus ilex* subsp. *ballota*). Con ello se pretende responder a las siguientes preguntas: ¿presentan los árboles algún desorden nutricional, al crecer en estos suelos alterados? ¿Cuál es la importancia relativa de la contaminación en la absorción de nutrientes, en comparación con otros factores de suelo como el pH, la textura o la disponibilidad de nutrientes? ¿Qué tipo de relaciones (sinérgicas, antagónicas o neutras) existen entre los elementos esenciales y los elementos traza a nivel de hoja?

En el **Capítulo 7** se analizan en condiciones de campo los patrones de biodisponibilidad de ET en el suelo y los factores edáficos que la determinan. Se analiza con mayor detalle la respuesta foliar de la encina a cambios en la disponibilidad de elementos traza; dado que el Cadmio (Cd) es uno de los elementos potencialmente más disponibles, se analiza el proceso de absorción y acumulación de Cd en plántulas de encina, bajo condiciones controladas, y se evalúa la tolerancia de esta especie a exposiciones de alta disponibilidad de Cd. Las preguntas específicas son las siguientes: ¿cómo afectan distintos factores del suelo a la disponibilidad de Cd y otros metales en la zona restaurada? ¿Cuál es la relación entre el Cd disponible en el suelo y las concentraciones de Cd en las hojas de encina? ¿Cómo es el proceso de traslocación de Cd desde el sustrato a las raíces y las hojas? ¿Es capaz la encina de retener y tolerar altos niveles de Cd en sus raíces? Teniendo en cuenta los resultados obtenidos, ¿cuál es el potencial de esta especie para la fitoestabilización de suelos contaminados con Cd?

En el **Capítulo 8** se analiza en detalle la respuesta ecofisiológica de plántulas de encina a distintas exposiciones de Cd y Tl, en condiciones controladas en invernadero. Se trata de dos elementos potencialmente muy móviles en el sistema suelo-planta, y para los que hay poco conocimiento sobre su toxicidad y efectos ecofisiológicos en plantas leñosas mediterráneas, especialmente en el caso del Tl. Se estudiaron las tasas de crecimiento, asimilación de carbono, patrones de fluorescencia de clorofilas y composición química de las plántulas, con el objetivo de comparar los efectos de estos dos elementos en las plántulas y los posibles mecanismos de tolerancia.

# Capítulo 3

# Área de estudio

# Capítulo 3

# Área de estudio

Los estudios de campo realizados durante la presente Tesis Doctoral se han desarrollado en el Corredor Verde del Guadiamar (Sevilla). El río Guadiamar es el último afluente del río Guadalquivir por su margen derecha, con nacimiento en la Sierra de los Gallos. La cuenca del Guadiamar abarca unos 1300 $km^2$ entre las provincias de Sevilla y Huelva y constituye el sistema de conexión natural entre Sierra Morena y los ecosistemas de Doñana (Fig. 3.1). Hasta hace poco tiempo era el principal subsistema hidrológico que inundaba las marismas de Doñana, y aún constituye uno de los complejos fluviales apenas sin regular del sistema hidrográfico andaluz. Gran parte de los tramos medios y bajos de la cuenca se vieron afectados por el accidente minero de Aznalcóllar (Sevilla) en 1998, que supuso la contaminación de los suelos con elementos traza y propició un proyecto de restauración a gran escala, el Corredor Verde del Guadiamar.

La zona hoy conocida como Corredor Verde del Guadiamar comprende nueve municipios de la provincia de Sevilla, y está integrada en la Red de Espacios Naturales Protegidos de Andalucía (RENPA) bajo la figura de Paisaje Protegido. El clima de la zona es mediterráneo semi-árido, con inviernos suaves y veranos cálidos y secos. La temperatura media anual de es 19 ºC (máxima mínima mensual de 9 ºC en enero, máxima de 27 ºC en julio). La precipitación anual media es de 484 mm y la evapotranspiración potencial es de 1139 mm (estación meteorológica del IRNAS-CSIC, Coria del Río, periodo 1971-2004).

En las zonas situadas al norte del Corredor Verde del Guadiamar, los tipos de roca predominantes son esquistos y pizarras, y los suelos son ligeramente ácidos. En las zonas centrales del Corredor Verde, los suelos se desarrollan predominantemente sobre calizas y calcarenitas, y son suelos margosos neutros y básicos. Finalmente, en los tramos más hacia el sur de la cuenca, cercanos a las marismas de Doñana, los suelos son arcillosos y ricos en carbonatos. Descripciones más completas sobre las características geomorfológicas de la cuenca del Corredor Verde y sus tipos de suelo pueden consultarse en Borja et al. (2008), Cabrera et al. (1999), Clemente et al. (2000) y Nagel et al. (2003).


**Fig. 3.1**. Situación del Corredor Verde del Guadiamar, en el contexto de la Red de Espacios Naturales Protegidos de Andalucía (RENPA).

El accidente minero de Aznalcóllar tuvo lugar el 25 de abril de 1998, cuando se produjo la rotura de uno de los muros de la balsa de decantación de la mina de los Frailes, vertiendo su contenido al río Agrio, afluente del Guadiamar. La balsa, antes de ser utilizada en la mina de los Frailes, fue utilizada durante 16 años en la mina de Aznalcóllar (1979-1995), período en el que se trataron unos 43 millones de toneladas de sulfuros polimetálicos (López-Pamo et al., 1999). A través de la brecha de la balsa se vertieron unos 2 $hm^3$ de lodos y 3-4 $hm^3$ de aguas ácidas, que inundaron las cuencas de los ríos Agrio y Guadiamar, 60 km aguas debajo de la balsa, hasta la zona de Entremuros, límite del Parque Nacional de Doñana. El espesor de la capa de lodos depositada en el terreno dependió de la distancia a la balsa y de la elevación con respecto al nivel del río, oscilando entre menos de 2 cm y más de 1,5 m. En total, fueron afectadas 4.286 ha, 2.710 de ellas cubiertas por lodos y el resto afectadas por las aguas ácidas. Comprendían, en su mayoría, zonas agrícolas y pastizales. El yacimiento de los Frailes, situado en el Cinturón Pirítico Ibérico, estaba formado fundamentalmente por sulfuros polimetálicos de Fe, Cu, Pb, Zn y As (pirita, calcopirita, galena, esfalerita y arsenopitita). Los lodos tenían, por tanto, una composición muy parecida a la del yacimiento, y presentaban arsenopirita, esfalerita, galena, silicatos, cuarzo y yeso. Un análisis detallado de las características del accidente, composición de los lodos y aguas ácidas, así como sus primeros efectos sobre los suelos, aguas, acuíferos, aire y cultivos puede consultarse en Grimalt et al. (1999).

Durante los primeros meses posteriores al accidente tuvo lugar la retirada de los lodos depositados y la capa superficial de suelo contaminada. El análisis de los suelos contaminados, realizados incluso antes de la retirada de los lodos, pusieron en evidencia que los niveles de numerosos elementos traza de los horizontes superficiales (0-15 cm) estaban muy por encima de los rangos normales de suelos no contaminados de la cuenca, en particular As, Cd, Cu, Pb, Sb, Tl y Zn (Cabrera et al., 1999; López-Pamo et al., 1999). El incremento medio de estos elementos con respecto a suelos no afectados fue del 70 % para el As, 45 % para el Zn, 50 % para el Cu y 23 % para el Pb (López-Pamo et al., 1999).

Una vez concluidas las tareas de limpieza, y paralelamente a la adquisición de los terrenos afectados por la Junta de Andalucía, se procedió a la adición de enmiendas al suelo. Se añadieron enmiendas orgánicas y productos ricos en carbonatos, para inmovilizar los elementos traza y mejorar la fertilidad del suelo. La espuma de azucarera fue una de las enmiendas más empleadas, con contenidos entre 70 % y 80 % de $CaCO_3$ (pH 9), y concentraciones de N, P y K de hasta 9,8, 5,1 y 5,3 g $kg^{-1}$, respectivamente (Madejón et al., 2006). Se aplicaron dosis entre 3 y 50 t $ha^{-1}$, dependiendo del grado de contaminación de los suelos (Arenas et al., 2008).

Inmediatamente después se procedió a la revegetación de los terrenos con especies leñosas mediterráneas. Para ello se diseñaron distintos marcos de plantación, teniendo en cuenta, a gran escala, la heterogeneidad de la zona afectada. Los marcos diseñados se englobaron en tres grupos: bosque de ribera, bosque mediterráneo (implantado en las terrazas aluviales) y vegetación de transición hacia la marisma, con un total de siete marcos de plantación distintos (Tabla 3.1). En ellos, las especies arbóreas se disponían de forma regular en distintas líneas, y se iban alternando distintas manchas monoespecíficas de arbustos. En total, se emplearon 26 especies leñosas autóctonas (**Tabla 3.2**). Las especies de árboles más abundantes fueron la encina (*Quercus ilex* subp. *ballota*), el acebuche (*Olea europaea* var. *sylvestris*) y el algarrobo (*Ceratonia siliqua*), en las zonas más alejadas del río, y el álamo blanco (*Populus alba*), el sauce (*Salix atrocinerea*) y el fresno (*Fraxinus angustifolia*) en las riberas del Guadiamar. Hacia las marismas el taraje (*Tamarix africana*) fue muy utilizado.

El éxito de las reforestaciones ha sido irregular, dependiendo de las características de cada zona en particular y de la especie utilizada. Durante los primeros cinco años, la supervivencia de plantones de árboles fue muy baja en las zonas cercanas a la mina, en comparación con las zonas centrales y próximas a la marisma (Domínguez et al., 2009). En estas terrazas aluviales, las especies arbóreas tuvieron una supervivencia menor que las de matorral. La encina y el algarrobo presentaron las menores tasas de supervivencia, en torno al 20 y el 30 %, respectivamente. Durante el año 2004, que fue especialmente seco, se produjeron grandes mortandades de estas dos especies. El acebuche fue la especie arbórea con mejores resultados en las zonas de terrazas. Actualmente, 8-9 años después de haber sido plantadas, las especies arbustivas son las que presentan mayor porte en las terrazas aluviales, especialmente la retama, el romero, el lentisco, así como el acebuche, con una superficie cubierta por la copa de 9,1, 6,9, 5,0, y 4,6 $m^2$, respectivamente (Tabla 3.3). Las especies de ribera alcanzaron porcentajes de supervivencia y crecimiento razonablemente altos, sobre todo el álamo blanco. Los ejemplares de esta especie alcanzaron en algunas zonas de ribera alturas superiores a los 3 metros, con diámetros de tronco en torno a los 15 cm y una superficie de copa superior a los 20 $m^2$, cinco años después de ser plantados (Domínguez et al., 2009).

**Tabla 3.1**. Principales características de los marcos de plantación empleados en las reforestaciones del Corredor Verde del Guadiamar. Fuente: CMA (2004).

| | Tipo de marco | Densidad (pies/ha) | nº especies | tipos de manchas | nº total manchas | dist. (m) |
|---|---|---|---|---|---|---|
| Ribera | Ordinaria | 980 | 6 | 4 | 20 | 5 |
| | Extraordinaria | 830 | 10 | 7 | 30 | 7.5 |
| Bosque mediterráneo | Calcáreo | 725 | 11 | 7 | 29 | 25 |
| | Silíceo | 550 | 9 | 6 | 23 | 25 |
| Transición a marisma | Ribera ordinaria de transición | 670 | 4 | 2 | 17 | 47.5 |
| | Ribera extraordinaria de transición | 560 | 4 | 1 | 17 | 57.5 |
| | Bosque mediterráneo calcáreo de transición | 480 | 9 | 6 | 22 | 30 |

**Tabla 3.2**. Especies empleadas en la reforestación del Corredor Verde del Guadiamar en los distintos marcos de plantación. Tipos de marcos: ribera ordinaria (Rib Ord), ribera extraordinaria (Rib Ext), bosque mediterráneo calcáreo (BM Cal), bosque mediterráneo silíceo (BM Sil), ribera ordinaria transición a marisma (Tr Rib Ord), ribera extraordinaria transición a marisma (Tr Rib Ext), bosque mediterráneo calcáreo transición a marisma (Tr BM Cal). Fuente: CMA (2004).

| Especie | Rib Ord. | Rib Ext | BM Cal | BM Sil | Tr Rib Ord | Tr Rib Ext | Tr BM Cal |
|---|---|---|---|---|---|---|---|
| *Arbutus unedo* | | X | | | | | |
| *Celtis australis* | | X | | | | | |
| *Ceratonia siliqua* | | | X | | | | |
| *Chamaerops humilis* | | | X | | | | X |
| *Crataegus monogyna* | | X | | | | | |
| *Fraxinus angustifolia* | X | X | | | X | X | |
| *Lavandula stoechas* | | X | X | | | | |
| *Myrtus communis* | | X | X | X | | | X |
| *Nerium oleander* | X | | | | X | | |
| *Olea europaea* | | X | X | X | | X | X |
| *Phillyrea angustifolia* | | | X | X | | | X |
| *Pinus pinea* | | | X | X | | | X |
| *Pistacia lentiscus* | | | X | X | | | X |
| *Populus alba* | X | | | | | | |
| *Pyrus bourgeaeana* | | X | | | | | |
| *Quercus coccifera* | | | | X | | | X |
| *Quercus ilex subp. ballota* | | | X | | | | X |
| *Quercus suber* | | | | X | | | |
| *Retama sphaerocarpa* | | | X | | | X | |
| *Rhamnus oleoides* | | | | X | | | |
| *Rosa canina* | | X | | | | | |
| *Rosmarinus officinalis* | | | X | X | | | X |
| *Rubus ulmifolius* | X | | | | X | | |
| *Salix atrocinerea* | X | | | | | | |
| *Tamarix africana* | X | | | | X | X | |
| *Viburnum tinus* | | X | | | | | |
| Total especies | 26 | | | | | | |

**Tabla 3.3**. Características de las especies arbustivas plantadas en el Corredor Verde del Guadiamar (1999-2000), hacia el otoño de 2008. Valores de media ± desviación estándar (Rodríguez et al., 2009).

| Especie | N | Altura (cm) | Cubierta ($m^2$) |
|---|---|---|---|
| *Retama sphaerocarpa* | 9 | 356 ± 55 | 9.1 ± 6 |
| *Crataegus monogyna* | 6 | 339 ± 57 | 3.4 ± 1 |
| *Arbutus unedo* | 5 | 277 ± 62 | 3.4 ± 2 |
| *Olea europaea* | 15 | 228 ± 77 | 4.6 ± 4 |
| *Phillyrea angustifolia* | 12 | 209 ± 40 | 2.7 ± 1 |
| *Pistacia lentiscus* | 10 | 177 ± 40 | 5.0 ± 2 |
| *Rosmarinus officinalis* | 6 | 155 ± 37 | 6.9 ± 3 |
| *Myrtus communis* | 3 | 126 ± 23 | 3.0 ± 1 |
| *Cistus salvifolius* | 5 | 99 ± 21 | 1.0 ± 0.6 |

Durante todo el proceso de configuración del Corredor Verde, desde el accidente minero hasta la actualidad, se ha desarrollado una intensa actividad científica en torno a la dinámica de la contaminación del suelo y sus efectos sobre los ecosistemas de la zona. Durante los primeros años, esta actividad se centró en la monitorización de los elementos traza en los suelos, aguas superficiales y subterráneas, y en distintos grupos de seres vivos (programas PICOVER I y II; CMA, 2003). Posteriores programas de investigación han profundizado en el seguimiento de la restauración de la zona, incluyendo la recuperación de los procesos biogeoquímicos del suelo, el estudio de la diversidad de distintos grupos funcionales de seres vivos y la funcionalidad como corredor ecológico para distintas especies animales (programa SECOVER; CMA, 2008). Asimismo, el Corredor Verde ha servido como zona de experimentación en multitud de trabajos sobre técnicas de remediación de suelos contaminados por elementos traza (Walker et al., 2004; Clemente et al., 2006; Madejón et al., 2006; Vázquez et al., 2006; Pérez de Mora et al., 2007). Continuando la línea científica de los investigadores del IRNASE-CSIC desde los primeros momentos después del accidente (Cabrera et al. 1999, Murillo et al. 1999), esta Tesis ha contribuido al programa SECOVER de seguimiento de las reforestaciones (Capítulos 4 y 6), al convenio con la Junta de Andalucía sobre evaluación del pastoreo Capítulo 5), y con una aproximación experimental sobre la respuesta de las plantas a los elementos traza en condiciones controladas (Capítulos 7 y 8).

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**Foto 3.1.** Rotura de la balsa de decantación de la mina de los Frailes (Aznalcóllar, Sevilla), en abril de 1998. Foto: archivo de la Consejería de Medio Ambiente.

**Foto 3.2.** Vista aérea de la cuenca del Guadiamar, inundada por los lodos, en abril de 1998. Foto: archivo de la Consejería de Medio Ambiente.

**Foto 3.3.** Río Guadiamar, durante la primavera de 2005. Al fondo, vertedero de la mina de Aznalcóllar.

**Foto 3.4.** Vista de la vegetación de las terrazas aluviales del Corredor Verde: plantones de retama (en primer plano) y encina (dentro de tubos protectores). Primavera de 2006.

# Capítulo 4

# Capítulo 4. Acumulación de elementos traza en especies leñosas en la cuenca de río Guadiamar, SO de España: fitomanejo a gran escala de una zona contaminada

Este capítulo reproduce el siguiente manuscrito:

**Domínguez, M.T., Marañón, T., Murillo, J.M., Schulin, R., Robinson, B.H., 2008. Trace element accumulation in woody plants of the Guadiamar valley, SW Spain: a large scale phytomanagement case study. Environmental Pollution 152, 50-59.**

## Resumen

El fitomanejo de una zona contaminada consiste en la combinación del uso de la vegetación y la aplicación de enmiendas para reducir el riesgo ambiental derivado de la contaminación del suelo. En este trabajo, investigamos la distribución de elementos traza en los suelos y las plantas leñosas en una amplia zona, la cuenca del río Guadiamar (SO de España), siete años después de la contaminación de la zona por un vertido minero (ocurrido en 1998) y de la posterior implementación de un programa de fitomanejo. En las zonas afectadas por el vertido, los suelos superficiales (0-25 cm) presentaron altas concentraciones de As (129 mg $kg^{-1}$), Bi (1,64 mg $kg^{-1}$), Cd (1,44 mg $kg^{-1}$), Cu (115 mg $kg^{-1}$), Pb (210 mg $kg^{-1}$), Sb (13,8 mg $kg^{-1}$), Tl (1,17 mg $kg^{-1}$) y Zn (457 mg $kg^{-1}$). Las concentraciones de elementos traza en las plantas estudiadas estuvieron, en términos generales, dentro de los rangos normales para las plantas superiores. La excepción fue el álamo blanco (*Populus alba*), para el cual las concentraciones foliares de Cd y Zn alcanzaron los 3 y 410 mg $kg^{-1}$, respectivamente. Discutimos los resultados en relación al fitomanejo de zonas contaminadas por elementos traza.

# Trace element accumulation in woody plants of the Guadiamar Valley, SW Spain: a large-scale phytomanagement case study

María T. Domínguez[a], Teodoro Marañón[a], José M. Murillo[a], Rainer Schulin[b], Brett H. Robinson[c]

[a] Instituto de Recursos Naturales y Agrobiología, CSIC, P. O. Box 1052, E-41080, Seville, Spain
[b] Institute of Terrestrial Ecosystems, ETH Zürich,Universitätstrasse 16, CH-8092 Zurich, Switzerland
[c] Department of Agriculture and Life Sciences, Lincoln University, PO. Box 84, Lincoln 7647, Canterbury, New Zealand

## Abstract

Phytomanagement employs vegetation and soil amendments to reduce the environmental risk posed by contaminated sites. We investigated the distribution of trace elements in soils and woody plants from a large phytomanaged site, the Guadiamar Valley (SW Spain), seven years after a mine spill, which contaminated the area in 1998. At spill-affected sites, topsoils (0-25 cm) had elevated concentrations of As (129 mg $kg^{-1}$), Bi (1.64 mg $kg^{-1}$), Cd (1.44 mg $kg^{-1}$), Cu (115 mg $kg^{-1}$), Pb (210 mg $kg^{-1}$), Sb (13.8 mg $kg^{-1}$), Tl (1.17 mg $kg^{-1}$) and Zn (457 mg $kg^{-1}$). Trace element concentrations in the studied species were, on average, within the normal ranges for higher plants. An exception was white poplar (*Populus alba*), which accumulated Cd and Zn in leaves up to 3 and 410mg $kg^{-1}$ respectively. We discuss the results with regard to the phytomanagement of trace element contaminated sites.

Keywords: heavy metal; bioaccumulation; phytoremediation; *Populus alba*; *Quercus ilex*; *Olea europaea*

## 1.Introduction

Phytomanagement is the use of vegetation and soil amendments to reduce the environmental risk posed by contaminated sites (Bañuelos et al, 1999; Barceló and Poschenrieder, 2003). The primary aim of phytomanagement is to reduce contaminant mobility and the effects of contaminants on humans and ecosystems. A successfully phytomanaged site should have limited leaching and limited plant uptake of contaminants. The soil surface must be stabilised so that wind and water erosion are minimised and there is a reduced risk of direct soil consumption by humans and animals (Robinson et al., 2003). Here we investigate a large-scale phytomanagement programme, implemented after a toxic sludge spill in the Guadiamar River Valley (Southwestern Spain). This programme was one of the biggest cases of contamination remediation in Europe in the last years, which included the use of soil amendments and the revegetation of the affected area (about 55 $km^2$) with native woody plants.

As with the phytomanagement of other trace element-contaminated sites, the success of this programme depends on the combination of suitable soil amendments and well-chosen plant species able to tolerate the local conditions, including the elevated concentrations of trace elements. Plants should not accumulate high concentrations of trace elements into their aboveground parts, because this would facilitate their entry into the food chain (Salomons et al., 1995) and increase the trace element concentration on the soil surface upon litter fall (Johnson et al., 2003; Watmough et al., 2005). The revegetated plants should limit leaching by returning rainfall to the atmosphere via evapotranspiration (Tordoff et al., 2000).

Woody species constitute most of plant biomass in native Mediterranean forests and shrublands. They are important primary producers in local food webs. Woody species are long-lived organisms that can take up trace elements from the environment and store them for a long time. In the Guadiamar Valley, potential evapotranspiration exceeds rainfall (see description of study area below). Therefore, deep-rooting woody species would be most effective to reduce leaching, since they can access water from a greater depth in the soil profile allowing them to continue to transpire long after shallow-rooted plants have shut down due to drought stress. The dry buffer zone in the soil profile so created by deep-rooted plants can absorb water following a heavy rainfall event (Mills and Robinson, 2003).

In previous studies, trace element accumulation was analysed in surviving trees shortly after the mine-spill (Madejón et al., 2004, 2006a). High concentrations of Cd and Zn were found in leaves of *Populus alba*. The leaves of *Quercus ilex* had high concentrations of As and Pb and the fruits of *Olea europaea* had high concentrations of Cd and Pb. However, there is no previous published information on the response of afforested vegetation during the phytomanagement programme.

This paper aims to determine the environmental risk posed by trace element accumulation for the major tree and shrub species occurring in the Guadiamar Valley. These include existing trees that survived the spill as well as newly planted trees and shrubs. Specifically, we sought to determine the trace element burden under selected areas of the Guadiamar Valley, to investigate the vegetation uptake of trace elements in relation to plant species and soil characteristics, and to discuss their implications for the general phytomanagement of contaminated sites.

## 2. Material and Methods

### 2.1. Study area and studied species

The Guadiamar River Valley lies inside the Iberian Pyrite Belt, the largest massive sulphide province in Western Europe. It has a semi-arid Mediterranean climate with mild rainy winters and warm dry summers. Average annual temperature is 19 ºC (min. 9 ºC in January, max. 27 ºC in July). Average annual rainfall is 610 mm and potential evapotranspiration is 774 mm. Soils of the Guadiamar floodplain are mostly neutral or slightly alkaline, with the exception of some terraces (in the North right bank), which have low pH. Soil texture varies from loamy sand to silty clay (Madejón et al., 2004, 2006a). Currently, the vegetation in the Guadiamar Valley includes savannah-like oak woodlands in the upper reaches, cereal and olive crops (outside the spill-affected zone) in the middle, rice crops and halophytic shrubs in the saltmarshes southward to Doñana National Park, where the river drains. Fragmented riparian forests are dominated by white poplar (*Populus alba*) and narrow-leafed ash (*Fraxinus angustifolia*), while in terrace open woodlands the Holm oak (*Quercus ilex* subsp. *ballota*) and wild olive tree (*Olea europaea* var. *sylvestris*) are most abundant.

In 1998, the failure of a large mine tailing dam at Aznalcóllar (Seville) released about 4 M $m^3$ of trace element-contaminated sludge into the Guadiamar River (Garralón et al. 1999). The resulting flood inundated 55 $km^2$ of the basin southward towards the Doñana National Park (Grimalt et al., 1999). The affected soils, mostly under agricultural production, were burdened with high concentrations of As, Cd, Cu, Pb, Tl and Zn (Cabrera et al. 1999).

After the accident, an emergency cleanup removed sludge and contaminated topsoil, which were transported and deposited in the nearby opencast mine. Despite this remediation, the underlying soils still contained elevated amounts of trace elements (Moreno et al., 2001). Organic matter and Ca-rich amendments were added with the aim of immobilising trace elements and improving soil fertility (CMA, 2003).

The Regional Administration (RA) purchased affected lands, which were farms with some fragmented forests and savannah-like woodlands. The RA implemented the Guadiamar Green Corridor programme, with the goal of providing a continuous vegetation belt for wildlife to migrate along the Guadiamar River basin between the Doñana National Park in the south and the Sierra Morena mountains in the North (CMA, 2001). Revegetation on the alluvial terraces started in 1999. Depending on the local habitat conditions, the target tree and shrub species to afforest were those typical of Mediterranean riparian forests, such as *Populus alba*, *Tamarix africana*, *Fraxinus angustifoli*a and *Salix atrocinere*a or those typical of drier upland forests, such as *Quercus ile*x subsp. *ballota*, *Olea europaea* var. *sylvestris*, *Phillyrea angustifolia*, *Pistacia lentiscus*, *Rosmarinus officinalis* and *Retama sphaerocarpa*.

We focused our study on the most important trees and shrubs species, in terms of abundance, used in the restoration project of the Guadiamar Valley (since 1999). We also monitored adult trees of the same species, from some remnants forests affected by the spill. The selected tree species were: Holm oak (*Quercus ilex* subsp. *ballota* (Desf.) Samp.), wild olive tree (*Olea europaea* var. *sylvestris* Brot.) and white poplar (*Populus alba* L.). For shrubs, we selected narrow-leafed mock privet (*Phillyrea angustifolia* L.), mastic shrub (*Pistacia lentiscus* L.), rosemary (*Rosmarinus officinalis* L.), yellow retama brush *(Retama sphaerocarpa* (L.) Boiss.) and tamarisk (*Tamarix africana* Poir.). Nomenclature follows López-González, 2002.

2.2. Plant and soil sampling

Sampling occurred in the autumn of 2005. Nineteen sites along the Guadiamar floodplain were selected (Fig. 4.1), from the non-affected areas upstream of the Aznalcóllar tailings dam (37º 30’ N, 6º 13’ W) down to the southern limit of the Doñana saltmarshes (37º 13’ N, 6º 14’ W). Three of these sites were unaffected by the spill. Riparian species (*Populus alba* and *Tamarix africana*) were collected from ten sites and the rest of species (typical of drier upland forests) were collected from eleven sites. Two sites had a mixture of both suites of species.

For each species, we took samples from affected and unaffected sites, with exception of white poplar and tamarisk, which were collected only from affected sites.

The unaffected sites were located either outside the riparian areas (since the flood moved forward the riverbed) or upstream the mine tailing dam (Northern edge of the phytomanaged area, where acid soils are predominant). In neither of those habitats grow white poplar and tamarisk.

At each site, we selected between three and ten individual trees for each species, depending on their abundance. Around each tree, the leaf litter was removed and soil samples were taken from the rootzone at 0-25 cm and 25-40 cm, using a spiral auger of 2.5 cm diameter. Two cores were taken at opposite sides of the trunks to make a composite soil sample for each tree. Between 12 and 40 soil samples were taken at each site. The total number of soil samples was 234.

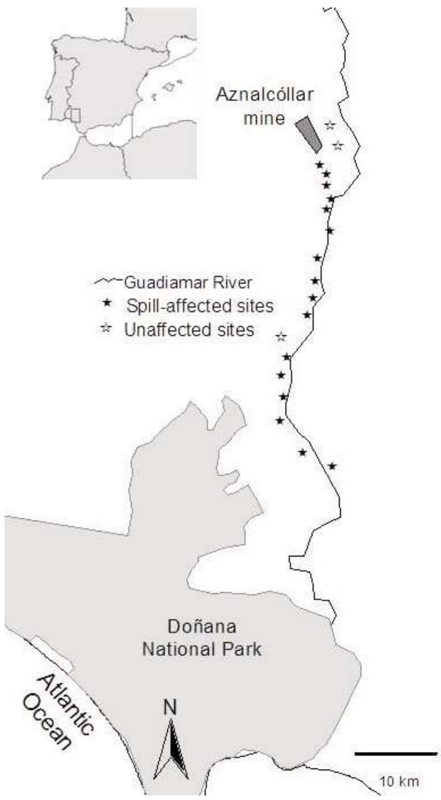


**Fig. 4.1**. Situation of the Guadiamar River Valley (SW Spain) inside the Iberian Peninsula and locations of the sampling sites.

For each selected tree, a composite leaf sample was taken from the outer canopy. Between 15 and 25 samples per tree species and life-stage (adult and sapling trees) were collected. For shrubs, four to six individuals of each species at each site were selected and leaf samples were obtained by combining the leaves of selected individuals of the same species. In the case of *Retama sphaerocarpa*, a shrub with short-lived leaves and photosynthetic stems, green stems were collected. The total number of plant samples was 160 (52 adult trees, 48 sapling trees and 43 shrubs) corresponding to eight species.

2.3. Sample preparation and chemical analyses

Soil samples were oven-dried at 40 ºC until a constant weight was obtained, then sieved to < 2 mm. A fraction of each sample was then ground in an agata morter to <1 mm for trace elements analysis. Soil samples were digested using concentrated $HNO_3$ and HCL (aqua regia). The values determined with this extraction are referred to as "total" concentration (Vidal et al., 1999).

The pH was determined potentiometrically in a 1:2.5 soil-water suspension. Equivalent calcium carbonate was determinated using a Bernard calcimeter (Hulseman, 1966) Organic matter content was analysed by dichromate oxidation and titration with ferrous ammonium sulphate (Walkley and Black, 1934).

Leaf samples were washed thoroughly with distilled water, dried at 70 ºC for at least 48 h and ground using a stainless-steel mill. Leaves were digested using concentrated $HNO_3$ (Jones and Case, 1990).

Trace element (As, Ba, Be, Bi, Cd, Co, Cu, Mn, Mo, Ni, Pb, Sb, Tl and Zn) concentrations of both soils and plants samples were determined by ICP-MS (inductively coupled plasma-mass spectroscopy). Quality assurance was obtained for soils by analysing reference material CRM 141R (calcareus loam soil, European Community Bureau of Reference). Recoveries from CRM 141R values ranged from 82 to 94%. The quality of the plant analyses was assessed by analysing two reference materials: NCS DC 73350 (white poplar leaves, China National Analysis Center for Iron and Steel) and BCR 62 (olive tree leaves, European Community Bureau of Reference; Colinet et al., 1982). For all elements except Sb our experimental values were 82 to 109% of the certified values. The lower recovery of Sb (60%) indicates that our results may be conservative estimations of the true values for this element.

2.4. Data analyses

Significant differences in soil and plant trace element concentrations between spill-affected and unaffected sites were analysed by the t-test. This test was also performed to compare contamination levels between soils beneath adult trees (from remnant woodlands) and beneath afforested saplings after the spill. Principal components analyses (PCA) were performed to investigate trace element variability trends both in soils and in plants. Data that were log-normally distributed were log-transformed for these statistical analyses.

Plant-soil relationships were assessed for the tree species, from individual plant/soil samples. Linear correlations were performed between plant and soil trace element concentrations. Bioaccumulation coefficients (BC), defined as the plant / soil concentration quotient (Adriano, 2001), were calculated. Average trace element concentrations through the sampled soil profile (0-40 cm) were considered for these coefficients. Relationships between BC and soil pH and organic matter were also evaluated by linear correlations.

Significance level was fixed at the 0.05. In order to avoid the increase of type I error derived from multiple testing, we controlled the 'false discovery rate', (FDR) at the 5% level, as suggested by García (2003). We used a powerful 'adaptive' FDR procedure (Hochberg and Benjamini, 2000) to calculate an overall threshold value (pt ≤ 0.05), to which individual p- values were compared. After applying the adaptive-FDR procedure to the overall p-value vector (including 420 p-values) we got a significance threshold value (pt) of 0.013. Therefore only p-values not exceeding this threshold value were considered as significant.

All statistical analyses were performed with STATISTICA v. 6.0. (StatSoft Inc., Tulsa, USA).

## 3. Results

### 3.1. Trace elements in soils

The concentrations of As, Bi, Cd, Cu, Pb, Sb, Tl and Zn were significantly higher in the spill-affected soils compared to the unaffected soils, at both 0-25 cm and 25-40 cm depths (Table 4.1). This result indicates that there was significant penetration of these eight contaminants (from the mine spill) into the soil profile. In contrast, the concentrations of other trace elements, namely Ba, Be, Co, Fe, Mn, Mo and Ni, were not significantly different between the contaminated and uncontaminated sites.

Most affected surface soils (86 % of the samples) had As concentrations above the range considered normal (0.1 – 40 mg $kg^{-1}$) for agricultural soils (Bowen, 1979). Similarly, other trace elements were above Bowen's range for normal concentrations in soils. These were (in decreasing order of the percentage of samples that exceeded these values) Tl (53%), Sb (52%), Pb (24%), Cd (17%), Zn (5%) and Cu (4%). Furthermore, 75% of affected soils exceeded the Dutch Intervention Values (DIV, see NIPHE, 2001) for As (55 mg $kg^{-1}$), and lower percentages of samples for other elements: Sb (40%), Zn (13%), Cu (8%) and Pb (4 %).

At a greater depth (25-40 cm) the concentrations of trace elements were generally lower than for the surface soils. Nevertheless, the DIV were still exceeded for As (60% of samples), Sb (27%), Cu and Zn (10% for both).

There were significant and positive correlations between all elements that occurred in elevated concentrations in the spill-affected soils (compared to the unaffected soils). This result supports the hypothesis that a single contamination event, the Aznalcóllar mine accident, deposited all these trace

**Table 4.1**. Mean (min.- max.) total concentration (mg $kg^{-1}$) of main trace element in soils in the Guadiamar Valley, from spill-affected and unaffected sites. Significance levels in the comparison (by t-test, controlling the overall FDR at the 5% level) between affected and unaffected sites are indicated (* p< 0.013, ** p< 0.001). Normal ranges in soils reported by Bowen (1979) and Dutch Intervention Values (DIV) are also indicated.

| | Surface soils (0-25 cm) | | Deep soils (25-40 cm) | | Normal ranges | DIV |
|---|---|---|---|---|---|---|
| | Affected (*N* = 100) | Unaffected (*N* = 17) | Affected (*N* = 100) | Unaffected (*N* = 17) | | |
| As | 129 (49-339)** | 17 (13-20) | 95 (18-438)** | 16 (14-17) | 0.1-40 | 55 |
| Bi | 1.64 (0.57-5.40) ** | 0.30 (0.15-0.40) | 1.35 (0.33-4.29) ** | 0.25 (0.18-0.35) | 0.1-13 | - |
| Cd | 1.44 (0.44-3.05)** | 0.23 (0.07-0.37) | 1.27 (0.22-3.28)** | 0.17 (0.10-0.25) | 0.01-2 | 12 |
| Cu | 115 (66-198)** | 32 (13-43) | 110 (30-238)** | 24 (14-31) | 2-250 | 190 |
| Pb | 210 (73-607)** | 47 (15-65) | 179 (38-519)** | 31 (18-38) | 2-300 | 530 |
| Sb | 13.8 (4.5-37.7)** | 3.0 (0.7-5.4) | 11.1 (2.1-30.0)** | 1.8 (0.9-2.8) | 0.2-10 | 15 |
| Tl | 1.17 (4.02-0.55)** | 0.29 (0.16-0.43) | 0.82 (0.20-3.15)** | 0.27 (0.23-0.33) | 0.01-0.8 | 15 |
| Zn | 457 (768-183)** | 109 (47-149) | 376 (103-954)** | 82 (53-99) | 1-900 | 720 |

elements. The first PCA component explained 47% of the total variance, and completely separated affected from unaffected soils (mean scores of -0.69 and 4.03 respectively). The factor loadings of the eight trace elements associated to the spill had the highest values for this first component (Table 4.2). Given that the contaminants are mutually correlated, the first component scores for each sample can be use as an index for the total contaminant burden. Detailed information about the level of trace element contamination at each sample site is given in the Appendix 1, along with other soil properties that affect the solubility of trace elements, namely pH, carbonate and organic matter content.

There was a large degree of spatial heterogeneity in the level of contamination between sites, as well as the factors affecting trace element solubility, namely carbonate, organic matter and pH (Appendix 1). The Northern and Central areas were the most contaminated sites; they were closest to the contamination source (tailing dam), and sludge was stored mostly in these sites during the clean-up operation. The degree of heterogeneity, as indicated by the coefficient of variation, increased in proportion to the described index for soil contamination (data not shown).

**Table 4.2**. Results of the Principal Component Analyses (factor loadings) of trace elements concentrations in soils (0-25 cm), and in leaves of three tree species (Holm oak, olive tree and white poplar), from the Guadiamar Valley. The percentage of variance explained by each component is also indicated.

| | Soils | | Plants | |
|---|---|---|---|---|
| | Comp. 1 (47 %) | Comp. 2 (29 %) | Comp.1 (26%) | Comp. 2 (20%) |
| As | -0.96 | -0.03 | -0.81 | -0.20 |
| Ba | 0.06 | -0.85 | -0.14 | 0.21 |
| Be | 0.34 | -0.79 | -0.42 | 0.28 |
| Bi | -0.95 | -0.10 | -0.39 | 0.19 |
| Cd | -0.76 | -0.10 | -0.35 | -0.86 |
| Co | -0.04 | -0.85 | -0.21 | -0.59 |
| Cu | -0.92 | -0.15 | -0.62 | 0.12 |
| Mn | 0.20 | -0.83 | -0.55 | 0.44 |
| Mo | -0.62 | -0.04 | -0.01 | -0.05 |
| Ni | 0.20 | -0.91 | -0.26 | 0.29 |
| Pb | -0.95 | -0.01 | -0.82 | 0.39 |
| Sb | -0.94 | -0.14 | -0.66 | 0.42 |
| Tl | -0.93 | -0.01 | -0.58 | -0.32 |
| Zn | -0.78 | -0.12 | -0.53 | -0.77 |

Soil acidity was also heterogeneous in the Guadiamar floodplain. The soil pH in Northern and Central regions was lower than in Southern regions, due to the different nature of bedrocks in Guadiamar Valley (slate and schist in the upper reaches and limestone and calcarenite in the lower reaches). In Northern and Central regions strongly acid soils were observed, having pH values below 4.5 and carbonate contents lower than 1% (21 % of the samples).

3.2. Trace element concentrations in plants

Table 4.3 shows the trace element concentrations in the leaves of studied species from the spill-affected sites. When compared with values from unaffected sites (for the six common species) there were significant differences for some elements and species (5 out of the 48 comparisons). Holm oak leaves accumulated significantly more As ($t = 2.95$, $p = 0.005$), Bi ($t = 3.26$, $p = 0.002$), Cu ($t = 2.62$, $p = 0.012$) and Zn ($t = 2.72$, $p = 0.010$) than in the unaffected sites (mean values of 0.10, 0.007, 6.14 and 29.9 mg $kg^{-1}$, respectively, in unaffected sites). Wild olive tree leaves had higher concentrations of Tl ($t = 2.71$, $p = 0.009$) than in unaffected sites (mean of 0.003 mg $kg^{-1}$). For the rest of elements and species, there was no significantly higher accumulation in the spill-affected sites.

Despite the higher accumulation of some trace elements in the leaves of some woody plant species, in comparison to uncontaminated sites, the concentrations were within the normal ranges for higher plants (as reported by Chaney, 1989, see Table 4.3). A notable exception was white poplar, which had foliar Cd up to 3 mg $kg^{-1}$,and Zn up to 410 mg $kg^{-1}$, well above normal ranges of 1 and 150 mg $kg^{-1}$, respectively.

The accumulation of trace elements was also influenced by the life-stage of the tree. In general, adult trees that survived the spill had higher foliar concentration of many trace elements than young saplings that were afforested after the spill. For example, adult wild olives had higher concentration of Zn (2 x) than saplings, adult poplars had higher Cd (5 x) and Zn (3.4 x) concentration than saplings, and adult oaks had higher Bi (2.3 x) and Cu (1.8 x) levels than saplings (Table 4.4).

In the leaves of tree species, sludge-associated trace elements were significantly and positively correlated. A principal components analysis (PCA) of the trace element composition of the leaf tissue revealed that the main trend (the first component accounted for 26% of the total variation) was related to elements contained in the sludge. Arsenic, Pb, and in a lower degree Cu, Sb, Tl (contained within the sludge) had high weightings, as well as Mn, which the PCA of the soil samples did not discriminate. The second component (20% of variance) was defined sharply by Cd and Zn, and in a lower degree by Co (Table 4.2). The first PCA axis may reflect a soil contamination gradient inducing a parallel gradient of leaf concentrations of trace elements, mostly As and Pb. The three tree species overlap in that accumulation gradient, although olive trees tend to have the lowest values (Figure 4.2). The second trend of variation seems to have a species-specific physiological nature, clearly associated to the higher accumulation of Cd, Zn, and Co in poplar leaves.

**Table 4.3**. Trace element concentration (mg kg$^{-1}$) in leaves (stems in the case of *Retama sphaerocarpa*) of woody plants from spill-affected sites in the study area. In the case of tree species, both adults and saplings are combined. Normal ranges for trace element in plants and maximum levels tolerated by livestock are indicated (see footnotes for references). Mean ± standard error.

| Species | As | Bi | Cd | Cu | Pb | Sb | Tl | Zn |
|---|---|---|---|---|---|---|---|---|
| *O. europaea* *N* = 31 | 0.32 ± 0.04 | 0.025 ± 0.005 | 0.07 ± 0.01 | 6.94 ± 0.49 | 0.89 ± 0.06 | 0.031 ± 0.023 | 0.013 ± 0.002 | 42.2 ± 3.8 |
| *P. angustifolia* *N* = 6 | 0.25 ± 0.07 | 0.019 ± 0.007 | 0.13 ± 0.03 | 5.67 ± 0.11 | 1.11 ± 0.18 | 0.046 ± 0.005 | 0.009 ± 0.004 | 79.9 ± 10.6 |
| *P. lenstiscus* *N* = 4 | 0.27 ± 0.12 | 0.026 ± 0.008 | 0.06 ± 0.01 | 4.48 ± 0.59 | 1.18 ± 0.39 | 0.028 ± 0.009 | 0.018 ± 0.006 | 14.9 ± 0.4 |
| *P. alba* *N* = 40 | 0.50 ± 0.04 | 0.014 ± 0.003 | 3.13 ± 0.45 | 8.11 ± 0.35 | 1.21 ± 0.05 | 0.037 ± 0.005 | 0.032 ± 0.008 | 412 ± 43 |
| *Q. ilex* *N* = 29 | 0.56 ± 0.08 | 0.026 ± 0.003 | 0.21 ± 0.05 | 10.2 ± 0.8 | 2.48 ± 0.26 | 0.070 ± 0.005 | 0.021 ± 0.005 | 80.0 ± 8.9 |
| *R. sphaerocarpa* *N* = 6 | 0.30 ± 0.10 | 0.014 ± 0.002 | 0.31 ± 0.20 | 15.5 ± 3.0 | 1.40 ± 0.48 | 0.067 ± 0.016 | 0.006 ± 0.002 | 114 ± 36 |
| *R. officinalis* *N* = 7 | 0.79 ± 0.18 | 0.023 ± 0.005 | 0.04 ± 0.01 | 13.2 ± 1.1 | 2.01 ± 0.52 | 0.046 ± 0.005 | 0.021 ± 0.007 | 51.2 ± 9.5 |
| *T. africana* *N* = 6 | 0.83 ± 0.19 | 0.022 ± 0.009 | 0.46 ± 0.21 | 11.3 ± 1.8 | 1.58 ± 0.27 | 0.070 ± 0.051 | 0.213 ± 0.059 | 54.7 ± 19.4 |
| Normal levels | 0.01-1[a] | 0.06[b] | 0.1-1[a] | 3-20[a] | 2-5[a] | 0.005-0.1[c] | 0.05[c] | 15-150[a] |
| Maximun levels for livestock[a] | 50 | | 0.5 | 300 | 30 | | | 1000 |

[a] Chaney, 1989; [b] Bowen, 1979; [c] Adriano, 2001;

**Table 4.4**. Trace element concentrations (mg kg$^{-1}$) in leaves of adult and sapling trees from affected sites. Significance levels (analysed by t-test) are indicated (* p< 0.013, ** p< 0.001). Values in italics are marginally significant (0.05 > p < 0.013), after controlling the overall FDR at the 5% level. Mean ± standard error.

| | *O. europaea* | | *P. alba* | | *Q. ilex* | |
|---|---|---|---|---|---|---|
| | Adult (*N* = 15) | Sapling (N = 16) | Adult (N = 22) | Sapling (N = 18) | Adult (N = 15) | Sapling (N = 14) |
| As | *0.42 ± 0.07* | 0.22 ± 0.03 | 0.63 ± 0.06** | 0.34 ± 0.05 | 0.59 ± 0.07 | 0.54 ± 0.14 |
| Bi | 0.016 ± 0.03 | 0.034 ± 0.010 | 0.018 ± 0.005 | 0.010 ± 0.03 | 0.036 ± 0.004** | 0.016 ± 0.001 |
| Cd | 0.06 ± 0.01 | 0.08 ± 0.02 | 4.90± 0.58** | 0.96 ± 0.13 | 0.14 ± 0.02 | 0.29 ± 0.09 |
| Cu | 6.55 ± 0.55 | 7.32 ± 0.80 | 8.16 ± 0.47 | 8.07 ± 0.54 | 13.1 ± 0.9** | 7.18± 0.31 |
| Pb | *1.02 ± 0.09* | 0.77 ± 0.07 | 1.28 ± 0.06 | 1.12 ± 0.08 | 3.18 ± 0.40** | 1.72 ± 0.21 |
| Sb | 0.037 ± 0.004** | 0.025 ± 0.002 | 0.050 ± 0.008** | 0.021 ± 0.004 | 0.079 ± 0.006** | 0.059 ± 0.008 |
| Tl | 0.014 ± 0.003 | 0.013 ± 0.003 | 0.053 ± 0.012** | 0.007 ± 0.002 | 0.029 ± 0.009 | 0.013 ± 0.003 |
| Zn | 57.0 ± 5.2** | 28.2 ± 2.5 | 605± 45** | 176 ± 22 | 83.5 ± 8.4 | 76.2 ± 16.4 |

### 3.3. Plant-soil correlations

The correlation coefficients between the concentrations of trace elements in surface soils and in leaves of trees (grouping adults and saplings) showed a low number of significant relationships (Table 4.5). Only 5 out of 48 possible correlations, corresponding to Holm oak saplings, were significant ($p < 0.013$). In other cases, marginally significant correlations ($0.05 > p < 0.013$) were found. In general, there were higher correlations between the trace element concentrations in plants and in the surface soils, than in deeper soils (data not shown). Tree saplings had higher plant – soil trace element correlations than the adult trees.

**Table 4.5.** Correlation coefficients (r) between trace elements in soils (total concentrations, 0-25 cm depth) and plants, for tree species, both for affected and unaffected sites. Non significant correlations (ns) are not shown. For the rest of correlations, significance levels are indicated (* $p < 0.013$, ** $p < 0.001$). Values in italics are marginally significant ($0.05 > p < 0.013$), after controlling the overall FDR at the 5% level.

| Species | | As | Bi | Cd | Cu | Pb | Sb | Tl | Zn |
|---|---|---|---|---|---|---|---|---|---|
| *O. europaea* | Adult ($N$ = 15) | ns | ns | ns | ns | ns | ns | ns | ns |
| | Sapling ($N$ = 25) | *0.46* | ns | ns | ns | ns | ns | ns | ns |
| *P. alba* | Adult ($N$ = 22) | ns | ns | *0.45* | ns | ns | ns | ns | ns |
| | Sapling ($N$ = 18) | ns | ns | ns | ns | ns | *0.54* | ns | ns |
| *Q. ilex* | Adult ($N$ = 15) | ns | ns | ns | ns | ns | ns | ns | ns |
| | Sapling ($N$ = 21) | 0.79** | 0.68** | 0.78** | ns | *0.53* | ns | 0.77** | 0.78** |

### 3.4. Bioaccumulation coefficients

For As, Bi, Pb, Sb and Tl, bioaccumulation coefficients (BC) were very low (< 0.03) and there were no significant differences between the species (Fig. 4.3). In contrast, Cd, Zn and Cu had the highest BCs, and there were greater differences between species. The BC of Cu was similar for all species, around 0.2. There were highly significant inter-specific differences in the BCs of Zn and Cd, in particular due to *Populus alba*, having values close to 2.0 for Cd, and about 0.9 for Zn (Fig. 4.3).

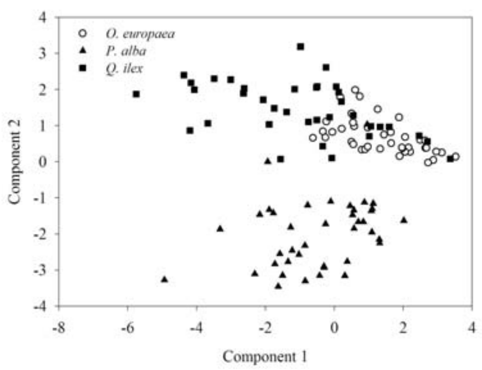


**Fig. 4.2**. A Principal Component Analysis (PCA) of trace element concentrations in the leaves of the studied tree species. Component loadings for each trace element are shown in Table 4. 2.

### 3.5. Effect of pH and soil organic matter on the bioaccumulation coefficients

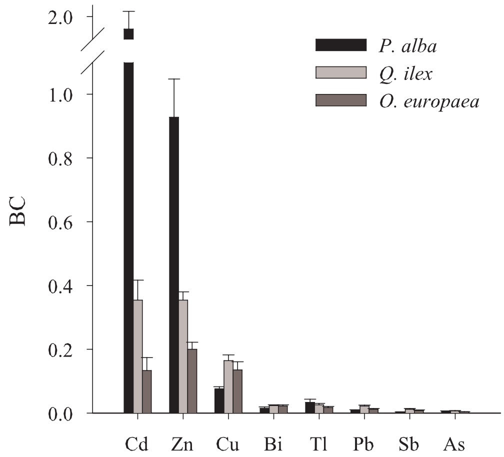


**Fig. 4.3**. Bioaccumulation coefficients (BC), defined as the plant / soil concentration quotients, of trace elements in leaves of trees from the Guadiamar Valley.

There were few significant correlations between pH and BCs. For white poplar, soil pH was negatively correlated with BC for Zn ($r = -0.60$, $p < 0.001$). A marginally significant positive correlation was found for As ($r = 0.35$, $p = 0.032$). A positive correlation between soil pH and the BC for Sb was found for olive trees ($r = 0.43$, $p = 0.012$). For the rest of elements and plant species, correlations pH - BC were not significant. Soil organic matter did not significantly affect the BC of any element in any species.

## 4. Discussion

The soils affected by a mine spill in the Guadiamar Valley were contaminated by several trace elements, namely As, Bi, Cd, Cu, Pb, Sb, Tl and Zn. These results are consistent with those reported by Cabrera et al. (1999) just after the mine spill. The contamination was spatially heterogeneous. Several factors may explain this non-uniform contamination. Firstly, it was a function of the irregular sludge deposition (Alastuey et al., 1999; López-Pamo et al., 1999). Secondly, the heterogeneous nature of the alluvial sediments along the Guadiamar River (Gallart et al., 1999) may also affect the distribution of residual soil contamination and may influence the different degree of leaching between different sites. Cabrera et al., (1999) showed that the clay content affected the degree of sludge penetration into the Guadiamar surface soils. Thirdly, the residual contamination is also a function of the irregular cleanup of affected soils. The contamination levels in soils around adult trees surviving the spill were significantly higher than those of soils with newly afforested saplings. This is probably due to the difficulty of removing sludge and topsoil from forested areas (Ayora et al., 2001).

Strong soil acidification was observed in the most contaminated sites. Soil acidification may have occurred due to the leaching of acids generated by the oxidation of sulphides in the remnant of sludges in the soils. Carbonates would have reduced the effects of such acidification. However, the natural pH of these sites was acidic and the soil's carbonate reserve was low, so there may have been less pH buffering in these sites.

Despite the relatively high soil trace element concentrations, sometimes well in excess of Dutch Intervention Values, their transfer into the woody plants from the Guadiamar Valley was limited. The concentrations in the aboveground parts of studied species were, on average, within the normal ranges for higher plants. The notable exception was the high Cd and Zn accumulation by poplar leaves in the spill-affected area. As a comparison, Madejón et al. (2004) reported foliar concentrations of Cd and Zn in the leaves of poplars from uncontaminated soils of the Guadiamar Valley (outside the phytomanaged area) of just 0.21 mg Cd $kg^{-1}$ and < 82 mg Zn $kg^{-1}$ on a dry matter basis, manifold lower than the values found in this study (3 and 410 mg $kg^{-1}$, respectively). The average Cd concentration in poplar leaves from the affected areas of the Guadiamar floodplain was greater than the concentration (0.5 mg $kg^{-1}$) that has been shown to adversely affect livestock (Chaney, 1985).

It is well known that poplar species accumulate Cd and Zn in their leaves (Di Baccio et al., 2003; Madejón et al., 2004; Robinson et al., 2005). Their high biomass production combined with high Cd and Zn accumulation may make poplar suitable for the phytoextraction of these elements from contaminated soils (Robinson et al., 2000¸ Giachetti and Sebastian, 2006;). The use of poplar for phytomanagement in this site may increase the risk of Cd and Zn entering the food chain. However, poplar is an integral part of the native riparian ecosystem and has a high landscape value. Moreover, poplar plays an important role in maintaining the stability of riparian zones. Before any recommendation to change the management programme, a risk assessment is required to determine the extent of Cd and Zn transfer to other organisms, as well as they accumulation in the topsoil due to litter fall.

Madejón et al. (2006a) reported that oak leaves had significantly higher concentrations of some trace elements than olive tree leaves, in spill-affected sites. We found similar results in this study. However, for both species the average trace element concentrations found in this study were lower than in the trees analysed in the first three years following the Aznalcóllar accident. The higher foliar concentrations reported in surveys taken immediately following the soil cleanup may have resulted from increased surface deposition of elements following the generation of dust during earth moving (Madejón et al., 2005).

With the exception of Holm oak saplings, very few significant soil-plant correlations were found in this study. Plant-soil relationships are always complex, since a suite of edaphic and climatic variables, in addition to the soil's trace element concentration, determines the trace element concentration in plants. Our soil analyses revealed a high variability in pH and organic matter content of the soil. Both these factors may affect the phytoavailability of trace elements (Greger, 1999). The nutrient status of each site is likely to have been different, further degrading leaf-soil correlations (Bargagli, 1998). In addition, plant physiology influences the uptake, transport, and accumulation rates, determining the foliar concentration of a trace element. Despite the correlations between leaf concentrations and soil total concentrations were low, the main trend of leaf chemical composition of the studied tree species reflected the gradient of soil trace element contamination. This indicates that leaf analyses may indicate soil quality with regard to trace element phytoavailability in contaminated sites. Since plants integrate several environmental variables over an extended time, they can provide information that is unobtainable from direct soil analyses (see Madejón et al. 2006b for a full discussion of this topic).

There were higher plant-soil correlations for afforested saplings than for adult trees. A possible explanation is that the roots of smaller plants occupy a smaller volume than large trees. Therefore, the soil that was sampled is more likely to reflect closely the local concentration that the roots of smaller plants were exposed to. Afforested shrubs and trees have been transplanted into the contaminated and remediated soil, therefore roots are growing and exposed to trace elements since the beginning. The roots of larger trees explore a larger volume of soil, which a single soil analysis may not accurately reflect.

Another factor may be that smaller saplings and shrubs are more affected by dust deposition than larger trees. Smaller plants, and those with pubescent leaves, are more likely to incorporate soil particles into the foliar tissues that even vigorous washing will not remove (Jones and Case, 1990). The foliar trace element concentrations could increase due to the deposition of soil, which is highly contaminated by trace elements, thus producing a positive correlation between plant and soil concentrations. The highest number of soil-plant correlations was observed for Holm oak saplings, low-growing trees with spiny and tomentose leaves. In this case, we found significant correlations for elements, such as Bi and Pb, that are relatively immobile in plant-soil systems, (Adriano, 2001; Jung et al., 2002). This may indicate that, in these saplings, a higher proportion of trace elements may arise from surface deposition. Foliar trace elements that occur via dust deposition still pose an ecological risk, since herbivores will ingest the trace elements, irrespective of their provenance. In taller trees with glabrous-leaves, the influence of surface deposition may be smaller. Olive tree leaves are glabrous, so they capture less aerial dust and are easier to wash before chemical analysis. White poplar is a fast growing tree, and the saplings can reach several meters in height after just three years, thus avoiding high surface deposition, despite their pubescent leaves. The contribution of surface deposition to the total metal burden of poplars in this study is likely to be low. In the case of Cd accumulation, the foliar concentration exceeded that of the soil. Here the effect of surface deposition would be to decrease the observed foliar Cd concentration, since the high concentration of Cd in the leaf is diluted by the addition of soil that has a lower Cd concentration.

For Bi, Tl, Pb, Sb, and As, bioaccumulation coefficients were below 0.03. This is consistent with previous works that report that these elements, with the exception of Tl, are immobile in plant – soil systems (Kabata-Pendias and Pendias, 1992; Ross, 1994). Thallium has limited mobility in the spill-affected soils in the study area (Martín et al., 2004; Vidal et al., 1999), especially in dry conditions, while during wet periods plants can uptake higher content of this element (Madejón et al., 2007). The BC of Cu was constant in the considered species, which probably is related to the plants' regulation of the uptake of this essential micronutrient. Several studies have reported a restricted transport of Cu from contaminated soils to aboveground parts in different species (Ait-Ali et al., 2002; Arduini et al., 1996; Kozlov et al., 2000). A single species, white poplar, showed BCs higher than 1 for some elements: Cd (all sites) and Zn (some sites). This indicates a high transfer of these elements from soil to leaves. Due to these high rates of metal transfer, concentrations in poplar leaves can be higher than those in soils. In the contaminated Guadiamar Valley, Cd and Zn accumulation by poplar represents one of the greatest environmental risks regarding the entry of trace elements into the food chain.

The solubility of cationic trace elements increases at low pH (Greger, 1999; Ross, 1994). Soil pH is the main determinant of cationic trace element solubility in the Guadiamar floodplain (Burgos et al., 2006; Clemente et al., 2003). Positive correlations between pH and BCs can occur for the anionic trace elements arsenate and antimonite, which are more mobile at higher pHs (Adriano, 2001). In this work, despite local acidic conditions, the BCs for cationic elements were not significantly correlated with soil pH, with the exception of Zn BC in white poplar. The positive correlations observed for As and Sb were weak. As discussed above, the physiology of these woody species may limit the uptake and transport of trace elements, despite increases in soil bioavailability due to pH conditions. Although low pH may be not greatly affect trace element uptake by woody plants in the Guadiamar Valley, the pH of the sites requires close monitoring, since acidification will result in increased trace element mobility and it may increase the rates of leaching into receiving waters. In the Guadiamar system, soil contamination is associated with sulphides, which will gradually oxidise and lower the soil pH. There is, therefore, a risk of a chemical time bomb where significant amounts of sludge remain in the soil. Maintaining neutral to basic soil pH should be an integral part of any phytomanagement programme that involves cationic trace-element contaminated soils. While there is little risk of plant uptake, the higher downward mobility of these elements may endanger groundwater.

## 5. Conclusions

Despite the high concentrations of several trace elements in the soils from the Guadiamar Valley, there was a limited transfer of these elements to the aboveground parts of woody plants. With the exception of white poplar, the ecological risk of the foliar trace accumulation in these plants is low. Future work could focus on the effect of the trees on the downward mobility of trace elements in the Guadiamar basin. This should include not just the effect of transpiration, but also the generation of preferential flow pathways along root-macropores, and the solubilisation of trace elements by organic acids generated from decaying leaf litter. Horizontal migration of diluted elements and solid matter as surface run-off during heavy rainfall events should also be taken into account.

Also warranted is a better understanding of the effect of the contaminating trace elements on the vegetation dynamics in the Guadiamar Basin. While the accumulation of trace elements is unlikely to pose an ecological threat, the effects of the contaminants may nonetheless have ecological consequences in terms of plant growth or nutrient status of plant tissue. Such information would be helpful in the selection of most suitable species for the phytomanagement of trace element-contaminated areas under semi-arid climate.

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## Appendix

Soil Contamination levels (indicated by a contamination index) and general properties (geometric mean and range) in studied sites from the Guadiamar Valley (SW Spain). Sites 1, 2 and 13 were not affected by the mine spill. For each site, the contamination index is the average value of PCA first component scores, calculated from the total concentrations of 14 trace elements in soil. Further explanation is given in Results.

| Site | Contamination index (0-25 cm) | Contamination index (25-40 cm) | pH (0-25 cm) | pH (25-40 cm) | CaCO3 (%) | OM (%) |
|---|---|---|---|---|---|---|
| **1** | **3.33** | **3.38** | 8.4 (8.3-8.5) | 8.1 (7.9-8.2) | 17 (17-18) | 2.32 (1.73 - 2.90) |
| **2** | **2.96** | **2.92** | 5.4 (5.1-5.6) | 5.1 (4.3-5.6) | < 0.5 | 1.72 (0.85 - 2.57) |
| 3 | -0.66 | -0.51 | 5 (3.5-7.5) | 5.0 (4.0-6.2) | 1 (< 0.5 - 20) | 3.16 (2.85-3.56) |
| 4 | 0.40 | -0.71 | 7.4 (6.5-9.2) | 7.0 (6.4-7.6) | 2 (< 0.5 - 6) | 2.21 (1.39 - 2.95) |
| 5 | 1.21 | 0.57 | 7.7 (6.2-8.4) | 6.5 (3.0-8.4) | 2 (< 0.5 - 6) | 1.8 (1.50 - 2.84) |
| 6 | -2.46 | -1.14 | 6.3 (3.7-7.9) | 5.2 (3.5-7.7) | 2 (< 0.5 - 6) | 1.6 (0.53 - 3.27) |
| 7 | -1.78 | 0.24 | 7.6 (7.2-8.1) | 6.6 (5.2-7.5) | 2 (< 0.5 - 3) | 1.13 (0.81 - 1.51) |
| 8 | -1.06 | -0.45 | 5.5 (3.1-7.9) | 4.6 (2.6-7.4) | 2 (< 0.5- 12) | 2.35 (1.40 - 4.54) |
| 9 | -1.78 | -2.48 | 7.3 (6.1-7.9) | 6.9 (4.6-8.1) | 3 (< 0.5- 12) | 2.73 (2.16 - 3.56) |
| 10 | -3.17 | -1.60 | 3.5 (2.4-4.5) | 4.4 (2.9-5.8) | < 0.5 | 2.26 (1.82 - 3.88) |
| 11 | -1.87 | -4.48 | 7.6 (7.3-7.8) | 7.1 (6.6-7.8) | 13 (10-15) | 2.56 (2.40 - 2.67) |
| 12 | 1.41 | 1.38 | 7.9 (7.5-8.1) | 8.0 (7.9-8.2) | 12 (6-16) | 1.63 (1.45 - 1.92) |
| **13** | **6.34** | **5.06** | 7.8 (7.5-8.2) | 7.7 (7.1-8.3) | < 0.5 (< 0.5 - 1) | 1.57 (1.16 - 2.26) |
| 14 | 0.72 | 0.56 | 7.7 (7.6-8.2) | 8.1 (7.9-8.2) | 11 (8-13) | 2.43 (1.78 - 2.63) |
| 15 | -0.70 | -2.30 | 7.8 (7.0-8.1) | 7.3 (6.3-7.8) | 8 (3-10) | 1.6 (0.89 - 3.18) |
| 16 | 0.42 | -0.68 | 7.7 (7.4-8.1) | 8.0 (8.0-8.1) | 13 (11-14) | 2.55 (2.39 - 2.84) |
| 17 | -0.18 | 1.11 | 8.1 (7.5-8.5) | 7.9 (7.7-8.2) | 9 (6-12) | 2.14 (1.33 - 3.05) |
| 18 | 0.54 | 3.52 | 8.3 (8.2-8.4) | 8.2 (8.1-8.2) | 6 (6-7) | 2.48 (1.45 - 1.47) |
| 19 | 0.32 | -1.85 | 8.0 (8-8.1) | 7.7 (7.5-7.9) | 9 (8-11) | 2.02 (1.68 - 2.80) |

**Foto 4.1**. Detalle de una muestra de suelo, tomada con barrena de 2.5 cm de diámetro. Foto: Teodoro Marañón.

**Foto 4.2.** Vista de zona reforestada en las terrazas aluviales.

**Foto 4.3.** Reforestaciones en la ribera de río Guadiamar.

**Foto 4.4.** Detalle de hojas de álamo blanco (*Populus alba*), especie acumuladora de Cd y Zn.

# Capítulo 5

# Capítulo 5. Evaluación del riesgo por pastoreo en zonas contaminadas por elementos traza

Este capítulo reproduce el siguiente manuscrito:

**Madejón, P., Domínguez, M.T., Murillo, J.M. 2009. Evaluation of pastures for horses grazing on soils polluted by trace elements. Ecotoxicology 18, 417–428.**

## Resumen

Los pastizales que se desarrollan sobre suelos contaminados pueden suponer un riesgo para los herbívoros y el ganado, limitando los posibles usos y aprovechamientos de la zona. En ausencia de ganado, los herbazales pueden alcanzar gran desarrollo y competir con las plantas leñosas jóvenes por los recursos, requiriéndose un control mecánico que es, por lo general, bastante costoso. En este trabajo, se evalúa el riesgo asociado al consumo de pastizales por parte de ganado equino (no destinado a consumo humano) en la cuenca del río Guadiamar (SO España), donde existen suelos contaminados por elementos traza. La contaminación del suelo no afectó especialmente a la producción de biomasa ni a la composición florística de los pastos, aunque ambas variables influyeron en la acumulación de elementos traza en los pastizales. Las concentraciones de estos elementos en los pastizales fueron inferiores a los máximos niveles tolerables por el ganado. El análisis de las heces de los animales mostró diferentes patrones de absorción de los elementos traza esenciales y no esenciales, siendo los elementos no esenciales preferentemente excretados. La composición de las crines fue similar en animales pastando tanto en las zonas contaminadas como en pastizales no contaminados. De contemplarse el pastoreo como medida de control de los herbazales en la cuenca del Guadiamar, es recomendable realizar un seguimiento de la exposición crónica del ganado y otros herbívoros a estos elementos traza.

# Evaluation of pastures for horses grazing on soils polluted by trace elements

Paula Madejón, M.T. Domínguez y J.M. Murillo

Instituto de Recursos Naturales y Agrobiología, CSIC, P. O. Box 1052, E-41080, Seville, Spain

## Abstract

Pasture established on polluted soil may pose a risk to grazing livestock creating a requirement for mechanical management which may affect biodiversity and expend energy. The risk associated with managing pasture by grazing horses (non-edible livestock) is being assessed in the Guadiamar Valley (SW Spain), where soils are polluted with trace elements following a major pollution incident. Soil pollution does not affect biomass production or floristic composition of pasture, although both variables influence trace element accumulation in herbage. Element concentrations in herbage are below maximum tolerable limits for horses. Faecal analysis showed regulated absorption of essential elements, while non-essential elements seemed preferentially excreted. Elemental content of horse hair did not differ in animals from polluted and control pastures. If pastures are managed by grazing, periodic monitoring is recommended in view of the long-term chronic trace element exposure in these systems.

Keywords: Pasture composition; trace element ingestion; horses' hair; horses' faeces.

# 1. Introduction

Mineral content of plants eaten by herbivores can be strongly influenced by the soils and geology on which they are located (Sutton et al., 2002). Accumulation of potentially toxic trace elements in grazing animals may occur on soils that are naturally rich in metals or polluted by human activities as occurred following the release of metal-rich sludge when a tailings dam burst at Aznalcóllar in SW Spain (Grimalt and Macpherson, 1999).

This accident (April 1998) affected an area of 4286 ha along the Agrio and Guadiamar River valleys. To mitigate the environmental impact of this accident, a large-scale restoration project was launched, including the compulsory purchase of the land (formerly devoted to crops and pastures) and the designation of a public nature reserve: 'Green Corridor' (CMA , 2003). This project was one of the largest soil remediation operations in Europe (Domínguez et al., 2008). However, despite the clean-up and partial restoration of soils, the affected zone is still polluted with trace metals, but with an irregular distribution pattern (Cabrera et al., 2008).

Partial restoration of soil fertility helped in the establishment of herbaceous vegetation cover, based on autochthonous ruderal species well adapted to the local conditions and apparently unaffected by comparatively high trace element content in soil. The area was also planted with native trees and shrubs and these, together with the herbaceous cover, contribute to the sustainable phytostabilisation of the polluted soils, as their root systems physically bind the soil and prevent excessive re-entrainment (Pulford and Watson, 2003).

Grazing of livestock was forbidden when the Green Corridor was created. As a result, the vigorous and healthy herbaceous cover competes with planted woody species for water and nutrients. In addition, the desiccated remains of the herbaceous vegetation present a fire hazard during summer droughts. Mechanical control of these herbaceous species is expensive, may affect biodiversity and generates greenhouse gas emissions. For these reasons the possibility of grazing with horses (non human-edible livestock) is being currently considered by the regional Government as a benign and sustainable management tool for control of the herbaceous cover.

In the context of changing agricultural and conservation policies in Europe, free-ranging herbivores are being more widely used to achieve conservation objectives. Cattle, sheep and horses are used to maintain open grasslands and marshes and their associated fauna (Menard et al., 2002). There is limited data available concerning the use of grazing horses for herb control in remediation programmes (Menard et al., 2002). Currently, horses are the only livestock found along the study area (Guadiamar basin). Despite the ban on grazing in the Green Corridor, there are numerous horses grazing illegally at present. In consequence, there is a clear need to assess risks associated with grazing these pastures. The objective of this paper is to evaluate the potential ingestion of As, Cd, Cu, Fe, Mn, Pb, Tl and Zn from pasture by horses and to assess the possibility of using grazing as a management tool for herbaceous vegetation in the Green Corridor. We analyzed the floristic composition of pastures, trace element concentrations in the dominant herbs, and trace element concentrations in horse faeces and hair. Soil-to-plant uptake relationships will be reported in a subsequent paper.

## 2. Material and Methods

### 2.1 Study area and Sampling sites

The Guadiamar River Valley, in SW Spain (37º 30-13' N, 6º 13' W), lies inside the Iberian Pyrite Belt, the largest massive sulphide province in Western Europe. The area has a semi-arid Mediterranean climate with mild rainy winters and warm dry summers. Average annual temperature is 19 ºC (min. 9 ºC in January, max. 27 ºC in July) and annual average rainfall is 484 mm. Soils of the Guadiamar floodplain are mostly neutral or slightly alkaline, with the exception of some terraces (on the northern right bank), which have low pH. Soil texture varies from loamy sand to silty clay (Cabrera et al., 1999). In 1998, the failure of a large mine tailing dam at Aznalcóllar (Seville) released about 6 M $m^3$ of trace element-polluted sludge into the Guadiamar River (see general description of the accident in Grimalt et al., 1999). The resulting flood inundated 55 $km^2$ of the basin southward towards the Doñana National Park. The affected soils, mostly under agricultural production, were contaminated with high concentrations of As, Cd, Cu, Pb, Tl and Zn (Cabrera et al., 1999). After the accident, an emergency cleanup removed the sludge and the first 0-10 cm of soil and organic matter and calcium-rich amendments were added with the aim of immobilizing trace elements and improving soil fertility (CMA, 2003). Revegetation started in 1999, after the purchase of affected lands by the Regional Administration. At present, the plant cover is composed by native shrubs and trees planted at different densities, and a dense matrix of ruderal herbs, methods for the control of which are currently under consideration.

Sampling was carried out in the spring and autumn 2007. Samples of pasture and horse faeces and hair were collected at 8 sites along the Guadiamar Green Corridor. Sites 1 to 7 were affected by the spill and still present different levels of residual pollution and site 8 was not affected by the spill and is used as the control site for this study. To evaluate soil pollution, we used the pollution load index (PLI, as defined by Tomlinson et al., 1980). This index is based on the value of the concentration factor (CF) of each metal in the soil. The CF is the ratio obtained by dividing the concentration of each metal in the soil by background values (As, Cd, Cu, Pb and Zn) of soils in the Guadiamar valley. For each sampling site, PLI was calculated as the nth root of the product of the obtained nCF. Values of PLI ~ 1 indicate metal loads near background level, while values > 1 indicate soil pollution (Cabrera et al., 1999). The pH values, As and Pb (main soil pollutants) total concentrations and PLI values of the studied soils are shown in Table 5.1.

## 2.2 Sampling of pasture, horse faeces and hair

Plots of 1 ha (sites 3 and 7) or 0.5 ha (sites 1, 2, 4, 5, 6 and 8) were delineated. Plot size depended on plant diversity. Pasture vegetation was sampled by collecting all the aboveground biomass found in a 25 x 25 cm quadrant. Pasture height and species present were noted before biomass sampling. The percentage of the plant cover in each quadrant was visually estimated, as well as the percentage of the cover that corresponded to each species ("relative plant cover"). Twenty (1 ha) or ten (1/2 ha) samples were taken at each site, each sampling quadrant being at least 3 m distant from the previously sampled replicate. A total of 100 samples of pasture were taken along the Corridor. All plant species were included in herbage analysis, despite the known preferential feeding of horses on grasses (Menard et al., 2002). Identification of the plant species was checked in the laboratory; most autumnal species had to be grown under greenhouse conditions before they were sufficiently mature for final identification. Nomenclature follows that of Castroviejo (1986, 2005).

Horse faeces (three samples per site) were collected at each sampling site, except site 6, where none were found. Horse hair was collected from the mane of horses that had been grazing freely, if illegally, along the Green Corridor, for two years (n= 7). Hair and faeces were also collected from horses grazing on similar pasture in the vicinity of the Green Corridor,

**Table 5.1**. pH, As and Pb concentrations and PLI (Pollution Load Index) values calculated for the 8 sampled soils. For each element, values followed by the same letter do not differ significantly ($p<0.05$).

| Site | Latitude/Longitude | pH | As (mg $kg^{-1}$) | Pb (mg $kg^{-1}$) | PLI |
|---|---|---|---|---|---|
| 1 | 37º28'09.8'', 6º12'42.0'' | 7.6 ± 0.18 ab | 56.2 ± 20.1 b | 128 ± 25.7 bc | 5.45 |
| 2 | 37º27'41.4'', 6º12'42.0'' | 8.3 ± 0.0 4b | 87.6 ± 42.0 bcd | 148 ± 69.3 bcde | 7.38 |
| 3 | 37º25'45.0'', 6º13'05.0'' | 8.2 ± 0.48 b | 145 ± 85.6 d | 290 ± 185 cde | 8.90 |
| 4 | 37º23'13.5'', 6º13'38.0'' | 7.0 ± 0.90 a | 147 ± 9.88 d | 263 ± 35.7 e | 11.18 |
| 5 | 37º22'39.5'', 6º13'45.2'' | 8.2 ± 0.11 b | 44.0 ± 14.1 c | 62.8 ± 34.8 bc | 2.31 |
| 6 | 37º17'25.7'', 6º15'46.2'' | 8.1 ± 0.13 b | 62.2 ± 9.95 c | 128 ± 16.1 cd | 7.49 |
| 7 | 37º14'27.0'', 6º15'22.0'' | 8.0 ± 0.07 b | 93.3 ± 32.2 cd | 188 ± 79.6 de | 9.90 |
| 8 | 37º19'21.5'', 6º15'17.8'' | 7.8 ± 0.33 ab | 21.5 ± 2.53 a | 14.3 ± 1.93 a | 0.73 |

outside of the spill-affected area (n= 5). It was not always possible to take hair at each sampling point, as for faeces, because horses move freely.

Plant material was oven-dried at 70º C to constant weight, weighed to obtain dry biomass and directly ground without prior washing (to study the actual pollution of trace elements in the grass and their possible toxic impact through consumption as forage); dry biomass was passed through a 500 µm stainless-steel sieve.

Faeces were washed (for approximately 10 s) with a 0.1 N HCl solution, then with distilled water, and finally oven-dried at 70 ºC to constant weight, ground and passed through a 500 µm stainless-steel sieve. The hair samples were washed in deionised water to remove dust and superficial contamination. This was followed by sequential washing with acetone, and distilled water. The washed hair samples were oven-dried for 24h at 70 ºC, then cut into lengths of less than 0.5 cm.

2.3 Analytical methods

Plant material was analysed for N by Kjeldahl digestion. Total protein in plants was calculated by multiplying Kjeldhal N content by 6.25. Plant material, faeces and horse hair were digested by wet oxidation with concentrated $HNO_3$ under pressure in a microwave digester. Three consecutive steps (5 min. each) of power (250 W, 450 W and 600 W) were applied. Analysis of mineral nutrients and Fe in the digests was performed by ICP-OES (inductively coupled plasma spectrophotometry; Thermo Jarrell Ash Corporation). Analysis of trace elements (As, Cd, Cu, Mn, Pb, Tl and Zn) was performed by ICP-MS (inductively coupled plasma-mass spectroscopy; Perkin Elmer, Sciex-Elan 5000), using an internal standard (Rh) and multielement standard solutions for calibration. The accuracy and precision of the analytical method was assessed by routine analyses of the following reference samples (NCS DC73350, China National Analysis Center for Iron & Steel, 2004, leaves of poplar, and BCR reference material no. 279, sea lettuce), and hair (BCR reference material no. 397, human hair). Recovery rates for reference plant samples were between 90% and 110% and for reference hair samples between 90% and 100%.

2.4 Data analysis

To assess the abundance of the different species compounding of pastures we calculated the relative frequency of each species (Table 5.2). This value indicates the number of observations of a given species in the whole sample set (N = 100 sample units), given as a fraction of unity. The relative plant cover (%) of different plant groups (families) was also calculated (Table 5.3).

Mean and standard deviation were determined for all variables. Normality of the data was tested prior to analysis and, when necessary, variables were transformed logarithmically. A Student t-test was used to assess significant differences between affected and unaffected sites. One way ANOVAs were used to analyse the differences in the biomass productivity and trace element concentration among sites. Significant statistical differences of all variables between sites were established by Tukey's test. If, after logarithmic transformation, the data did not fit a normal distribution, the non-parametric Kruskal–Wallis analysis of ranks was used. Significant differences in Tables and Figures are marked by letters or asterisks.

Principal Component Analyses (PCA) were performed to explore the patterns of variation of trace element accumulation in the different pastures. Variables introduced into the PCAs were trace element concentrations, pasture production (biomass per area unit) and relative percentage of cover of Poaceae, Fabaceae and Asteraceae (main species composing the pastures) in each sample. General Linear Models (GLM) were used to analyse the possible effects of sampling site, productivity and plant composition (predictor variables) on the concentration of trace elements in the pasture (dependent variables). The variables were log-transformed or arcsin-transformed (those variables expressed as percentage) prior to these analyses. All analyses were performed with Spss 15.0.

## 3. Results and Discussion

3.1. Biomass production and plant composition

The productivity of pastures in the study area showed significant differences between sites (only in spring) and between seasons (Fig. 5.1). In spring, mean values of biomass production for each sampling site ranged from 200 to 700 g $m^{-2}$. In some cases there was considerable within-site variability, as indicated by a high standard deviation, especially at sites 4, 6 and 8 (Fig. 5 1). These sites showed the highest biomass production, reaching up to 2217 g $m^{-2}$. In autumn, mean values for each site were below 350 g $m^{-2}$ and there were no significant differences in biomass production between spill-affected and unaffected sites. The lack of effect of residual pollution on pasture productivity was reflected by the fact that the highest biomass production (site, 4, Fig. 5.1) corresponded to the most polluted site (PLI 11.2, Table 5.1). This may be because bioavailability of trace elements in these soils is low, with little influence over plant establishment and subsequent growth. Previous studies have shown that, in field conditions found in the Guadiamar Green Corridor, pH is the most important factor controlling the trace element bioavailability and that, in general, the bioavailable concentrations are low where pH values exceed 5.0, as found in the soils considered here (Domínguez et al. unpublished).

The total number of species identified in the sampled pastures was 39 in spring and 23 in autumn (Table 5.2). The most frequent species belong to the Poaceae (especially *Bromus* spp. and A*grostis pouretii*), Fabaceae (*Medicago polymorph*a) and Asteraceae family (*Senecio* spp.). Members of the Poaceae family accounted for the highest proportion of herbaceous cover, especially during autumn when, on average, 77 % of the plant cover at each sampling site consisted of grass species (Table 5.3).

3.2. Trace element concentrations and nutritional value of pastures

The majority of trace element concentrations (As, Cd, Cu and Zn) were below the maximum tolerable levels (MTL) for horses reported by NRC (2005) (Fig. 5.2). These are based on indices of animal health (levels in brackets were derived from interspecies extrapolation). Excessive elemental content of herbage was only found at sites 1 and 2 (close to the mine) in autumnal sampling, where concentrations of Pb and especially Fe were excessive, possi-

**Table 5.2**. Species composition of the pastures (affected and control sites). The relative frequency of observations of each species (N = 100) during the spring and autumn sampling is indicated.

| Family | Sp | Spring | Autumn | Family | Sp | Spring | Autumn |
|---|---|---|---|---|---|---|---|
| Poaceae | *Agrostis pourretii* | 0.08 | 0.35 | Fabaceae | *Biserrula pelecinus* | 0.01 | |
| | *Avena barbata subsp. barbata* | 0.03 | | | *Medicago doliata* | 0.06 | 0.01 |
| | *Avena sterilis* | 0.1 | 0.05 | | *Medicago murex* | 0.07 | 0.01 |
| | *Bromus diandrus* | 0.17 | 0.11 | | *Medicago polymorpha* | 0.13 | 0.03 |
| | *Bromus lanceolatus* | 0.21 | | | *Scorpiurus muricatus* | 0.04 | 0.04 |
| | *Bromus madritensis* | 0.1 | | | *Vicia villosa subsp. varia* | 0.02 | |
| | *Cynodon dactylon* | 0.01 | 0.25 | Others | *Anagallis arvensis* | 0.07 | 0.01 |
| | *Hordeum marinum* | 0.06 | | | *Beta vulgaris* | 0.01 | |
| | *Lamarckia aurea* | 0.03 | | | *Convulvulus arvensis* | 0.01 | 0.06 |
| | *Lolium perenne* | 0.1 | | | *Echium plantagineum* | | 0.05 |
| | *Phalaris minor* | 0.05 | 0.03 | | *Equisetum arvense* | 0.01 | 0.03 |
| | *Piptatherum miliaceum* | 0.08 | 0.1 | | *Euphorbia helioscopia* | 0.01 | |
| Asteraceae | *Andryala integrifolia* | 0.1 | | | *Heliotropum europaeum* | | |
| | *Carduus pycnocephalus* | 0.02 | | | *Hirchsfeldia incana* | | |
| | *Chrysanthemum coronarium* | 0.09 | | | *Malva hispanica* | 0.04 | 0.05 |
| | *Coleostephus myconis* | 0.21 | 0.12 | | *Plantago coronopus* | 0.01 | o |
| | *Conyza bonariensis* | 0.05 | | | *Plantago lagopus* | 0.05 | o |
| | *Galactites tomentosa* | 0.02 | | | *Ranunculus trilobus* | 0.03 | o |
| | *Lactuca serriola* | 0.01 | | | *Scirpoides holoschoenus* | | 0.02 |
| | *Leontodon longirostris* | 0.09 | 0.02 | | *Torilis arvensis* | 0.04 | 0.07 |
| | *Senecio lividus* | 0.22 | 0.01 | | | | |
| | *Senecio vulgaris* | 0.14 | 0.16 | | | | |
| | *Silybum marianum* | 0.03 | 0.02 | | | | |
| | *Xanthium strumarium* | | 0.01 | | | | |

**Table 5.3**. Relative composition of plant groups in the sampling units (expressed by the relative percentage of cover, mean ± SE), per site and season.

| Site | Spring | | | | Autumn | | | |
|---|---|---|---|---|---|---|---|---|
| | Poaceae | Fabaceae | Asteraceae | Others | Poaceae | Fabaceae | Asteraceae | Others |
| 1 | 72 ± 6 | 19 ± 6 | 8 ± 4 | 1 ± 1 | 61 ± 11 | 11 ± 7 | 24 ± 10 | 4 ± 1 |
| 2 | 58 ± 10 | 16 ± 9 | 10 ± 5 | 16 ± 9 | 63 ± 14 | 10 ± 9 | 3 ± 1 | 24 ± 13 |
| 3 | 40 ± 9 | 27 ± 7 | 27 ± 8 | 5 ± 4 | 85 ± 7 | 0 | 7 ± 5 | 8 ± 5 |
| 4 | 71 ± 12 | 6 ± 6 | 14 ± 10 | 8 ± 7 | 67 ± 15 | 0 | 9 ± 8 | 24 ± 13 |
| 5 | 58 ± 15 | 9 ± 9 | 31 ± 15 | 2 ± 1 | 77 ± 13 | 0 | 0 | 22 ±12 |
| 6 | 64 ± 14 | 5 ± 5 | 24 ± 13 | 8 ± 3 | 79 ± 13 | 0 | 20 ± 13 | 1 ± 1 |
| 7 | 54 ± 10 | 21 ± 8 | 24 ± 8 | 1 ± 1 | 82 ± 7 | 14 ± 7 | 4 ± 2 | 0 |
| 8 | 62 ± 15 | 0 | 18 ±12 | 20 ± 12 | 99 ± 1 | 0 | 0 | 0 |

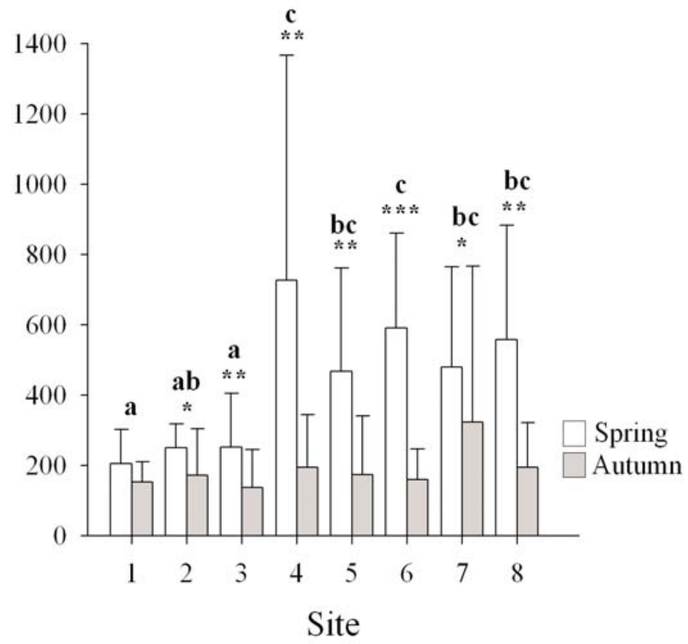


**Fig. 5.1**. Spring and Autumn biomass production (mean+ standard deviation) at each study site. Asterisks mark significant differences between seasons, within each sampling site (*p <0.05, **p <0.01, ***p <0.001). Letters indicate differences among sites, during the spring season. For the autumn season there were no significant differences among sites.

bly due to contamination of herbage with soil (Fig. 5.2). High levels of Fe in soil-contaminated pastures for other areas of SW Spain, not affected by a mine spill, were reported by Murillo et al. (1985). Soil contamination of fodder appears to be an important source of iron for horses, especially for foals (Brommer and Oldruitenborgh-Oosterbaan, 2001). In general, it has been demonstrated that young horses have a high susceptibility to trace elements, especially in case of lead (Schmitt et al., 1971; Casteel, 2001).

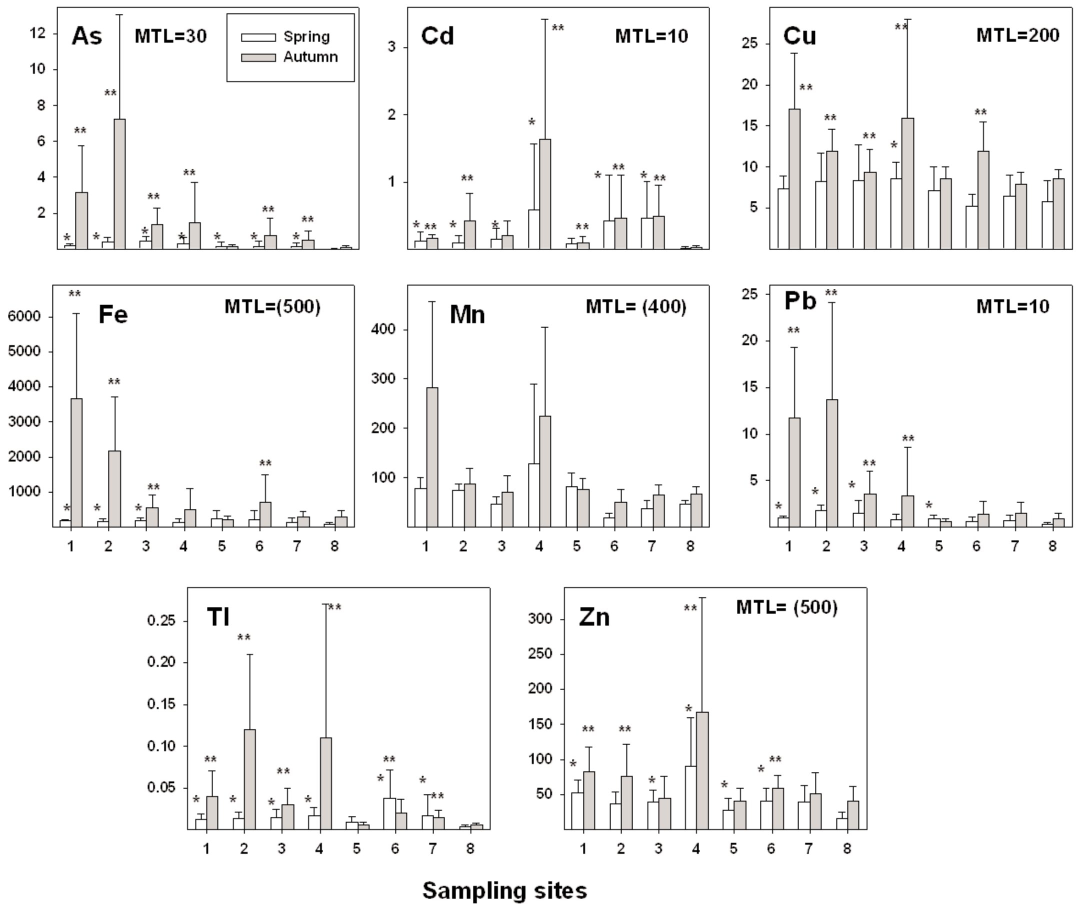


**Fig. 5.2.** Trace element concentration (mean + standard deviation) of pasture in the Guadiamar Green Corridor (1 – 7) and the control site (8). Significant differences between each site and site 8 (Control) for each season are marked with * (spring) or ** (autumn).

In the case of Pb, although the MTL has been established as 10 mg kg$^{-1}$, its tolerance could be greater under adequate Ca levels in the diet (NRC, 2005), as is found in these pastures (see below).

There is no published information on the MTL for Tl. According to Hapke (1984), 'safe' Tl concentrations in pasture are > 0.5 mg kg$^{-1}$ dry wt. In this study, Tl levels are, in general, far lower than this value and also lower than the safe value for the trophic web (2.5 mg kg$^{-1}$ dry matter, Makridis and Amberger, 1996).

Cadmium is mobile in plants and may become concentrated in leaves; therefore grazing animals may potentially receive the highest exposure to this element. Although chronic dietary levels of 10 mg kg$^{-1}$ dry matter can be tolerated by non-ruminants, these levels can result in unacceptable levels of Cd in kidneys, livers and, in some cases, in muscle (NRC 2005) taking to account animal and human health. Lower values in the diet are thus desirable (e.g., those in Fig. 5.2). An MTL of 0.5 mg kg$^{-1}$ dry wt has been recommended for livestock (Chaney, 1989), although Beyer (2000) argues that Cd toxicity levels for wildlife have been exaggerated. Marginal deficiencies of essential nutrients (Zn, Fe and Ca) can enhance Cd absorption by animals (Reeves and Chaney, 2008). Such deficiencies are not present in these pastures (Fig. 5.2 and Table 5.4).

In general, apart from other factors (very young, old, reproducing, sick, exposed to stressful environments), animals consuming nutritional imbalanced diets may be more sensitive to toxicoses (NRC, 2005). Apart from the Ca/P ratio, which is comparatively high, the pastures in the study area have a reasonable nutritive value (Table 5.4). Protein contents of the pasture would satisfy horse requirements, typically about 1 g crude protein $kg^{-1}$ weight $day^{-1}$. Concentrations of S, P, Ca and Mg do not exceed MTL levels for horses (0.5%, 1%, 2% and 0.8% respectively; NRC, 2005). Only K content is greater than the MTL of 1%, although this is a conservatively safe maximum tolerable level for non-ruminants; the NRC (2005) set the maximum tolerable amount of K at 3 % for both ruminant and non-ruminant species.

Ratios of Ca/P measured in autumn forage appear excessive for non-ruminants, although this ratio was lower in spring, when there is a longer grazing period (Table 5.4). Horses feed on forage that is usually low in phosphorus (Jordan et al., 1975). According to data reported by Schryver et al. (1983), the high Ca/P ratio in the pasture was corroborated by the comparatively high ratio Ca/P in faeces collected in the affected soils: 2.25 ± 1.47 (see section 3.5).

**Table 5.4.** Seasonal differences in protein and nutrient concentrations (mean ± standard deviation), and Ca/P ratios in pasture growing on affected (n = 90) and non-affected soils (n = 10).

| Element | Season | Affected soils | Control soils |
|---|---|---|---|
| Proteins (%) | Spring | 8.67 ± 4.00 | 9.54 ± 5.47 |
| | Autumn | 10.8 ± 3.87 | 6.41 ± 1.44 |
| S (%) | Spring | 0.34 ± 0.15 | 0.18 ± 0.09 |
| | Autumn | 0.37 ± 0.15 | 0.18 ± 0.04 |
| P (%) | Spring | 0.22 ± 0.05 | 0.20 ± 0.06 |
| | Autumn | 0.18 ± 0.07 | 0.12 ± 0.06 |
| K (%) | Spring | 2.00 ± 0.55 | 2.04 ± 0.76 |
| | Autumn | 2.09 ± 1.02 | 1.04 ± 0.54 |
| Ca (%) | Spring | 0.90 ± 0.61 | 0.64 ± 0.55 |
| | Autumn | 1.03 ± 0.70 | 0.57 ± 0.19 |
| Mg (%) | Spring | 0.15 ± 0.07 | 0.13 ± 0.07 |
| | Autumn | 0.18 ± 0.08 | 0.17 ± 0.07 |
| Ca/P | Spring | 4.02 ± 2.68 | 3.13 ± 2.46 |
| | Autumn | 6.11 ± 5.54 | 5.47 ± 1.96 |

3.3. Plant composition and trace element accumulation

Principal Component Analyses (PCA) of the studied plant variables revealed that the floristic composition of the pastures had some influence on patterns of trace element accumulation, especially in the spring growing season. Application of PCA to spring samples identified three factors with eigenvalues > 1, explaining 68 % of the total variability (Table 5.5). The first factor was the most important one; it was positively related to the potentially toxic element concentrations, and negatively related to the dry biomass and the grass composition of the samples. The second factor related Cd concentrations and the relative abundance of Asteraceae species. The third factor showed a slight relationship between Tl concentrations and the relative abundance of Fabaceae. These relationships can be observed in Fig. 5.3a. Pasture biomass was associated with the presence of a high proportion of grasses; both variables were situated in the negative side of the Factor 1-axis. Therefore, it is possible that samples with a high grass cover tend to show lower trace element concentrations, due to a dilution effect caused by high biomass production of these species. The ‘dilution effect’ of elements due to biomass increase has been profusely considered in literature (e.g. Jarrel and Beverly, 1981). In our study, this effect must be considered not only as a plant physiological phenomenon but also as a mechanical phenomenon, reducing the herbage exposure to trace element (soil contamination of pastures) (Healy, 1973). Sampling units with a high proportion of Asteraceae tended to have higher Cd concentration. These patterns were not so clear for autumn sampling, due to reduced biomass production and species diversity (Table 5.5). Nevertheless, biomass production and trace element concentration were still in opposite sides of the Factor 1- axis (Fig. 5.3b) for this sampling period. Apart from the dilution effect, grasses can accumulate comparatively less trace elements than other species (e.g. Asteraceae). Considering only pasture samples that had a grass cover ranging between 90-100 %, autumnal concentrations of As, Fe and Pb at site 1, and Cd at site 4, decrease to values of 1.50, 1980, 5.50 and 0.90 mg $kg^{-1}$, respectively, compared to those shown in Fig. 5.2. This could be important, given that horses feed preferentially on graminaceous patches.

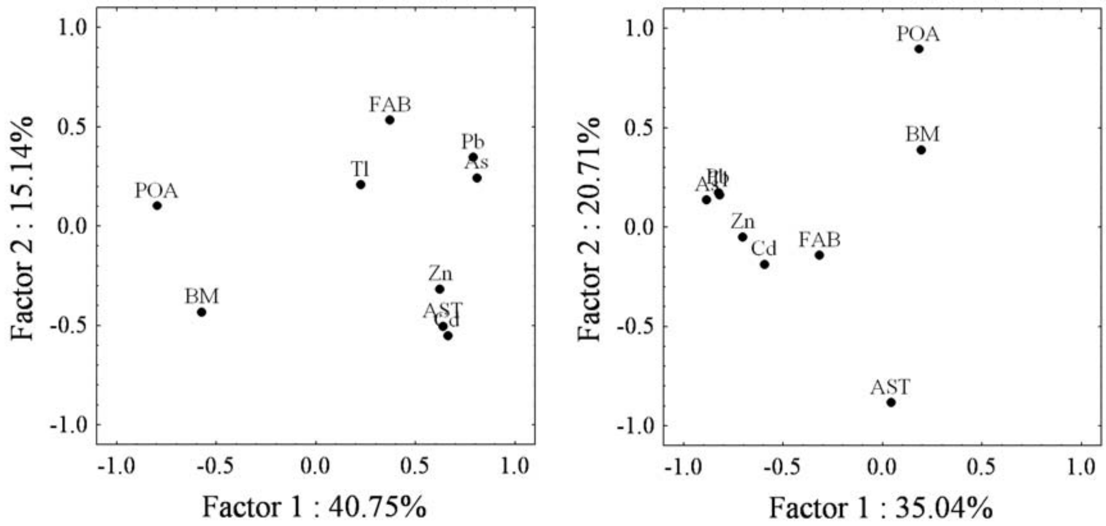


**Fig. 5.3.** Plot of the two first factors extracted by a Principal Component Analyses of the pasture characteristics and trace element concentrations, applied to the spring (a) and to the autumn (b) samples. The percentage of variance explained by each factor is indicated in their respective axes. Factor-variable correlations are shown in Table 5.5.

**Table 5.5.**. Results of the Principal Component Analyses (factor-variable correlations) applied to the trace element concentrations (mg $kg^{-1}$) and other characteristics of the studied pastures: biomass production (BM, g $m^{-2}$) and relative cover (%) of Poaceae (POA), Fabaceae (FAB) and Asteraceae (AST) species.

| Variable | Spring | | | Autumn | | |
|---|---|---|---|---|---|---|
| | Factor 1 | Factor 2 | Factor 3 | Factor 1 | Factor 2 | Factor 3 |
| As | 0.81 | 0.24 | -0.19 | -0.88 | 0.14 | -0.31 |
| Cd | 0.66 | -0.55 | 0.25 | -0.59 | -0.19 | 0.63 |
| Pb | 0.79 | 0.35 | -0.24 | -0.83 | 0.17 | -0.37 |
| Tl | 0.22 | 0.21 | 0.51 | -0.82 | 0.16 | 0.15 |
| Zn | 0.62 | -0.32 | 0.32 | -0.70 | -0.05 | 0.50 |
| BM | -0.58 | -0.43 | 0.42 | 0.20 | 0.39 | 0.03 |
| POA | -0.80 | 0.10 | -0.11 | 0.18 | 0.89 | 0.28 |
| FAB | 0.37 | 0.53 | 0.58 | -0.32 | -0.14 | -0.65 |
| AST | 0.64 | -0.51 | -0.30 | 0.04 | -0.89 | 0.15 |

The General Linear Models applied to the trace element concentrations confirmed that increased biomass production negatively affected the accumulation of most of these elements, and that this dilution effect was more significant in spring (Table 5.6). In these samples, all trace element concentrations, except Tl, were significantly influenced by either biomass production or the proportion of grass cover. The sampling site also significantly influenced trace element accumulation. In contrast, for the autumn pastures, sampling site was the only significant factor, with the exception of Pb concentrations, for which biomass production was also a significant predictor (Table 5.6). Therefore grazing on poor grown or partial covered pastures may increase exposure to metals derived from comparatively greater concentration of trace elements in forage which may be due to the "dilution effect" as exposed above. Horses usually feed closer to the ground, using shorter grass than other animals (Menard et al., 2002; Fleurance et al., 2007).

3.4. Ingestion of trace elements by grazing horses

Animals' estimated daily intake of trace elements via food intake, both essential and non-essential, are shown in Table 5.7. These are based on an average pasture ingestion of 21 g dw per kg body weight (Aronson, 1978; Liu, 2003). Although other authors (Fleurance et al., 2001) consider that horses could ingest more than this amount, most available data in the literature are close to this ingestion value. For example, for 500 kg BW horses, Dulphy et al. (1995) reported an ingestion within a range of 20.3 (grasses) – 23.9 (alfalfa) g dm $kg^{-1}$ BW $day^{-1}$.

Trace element intake in autumn was higher than in spring, due to the spring dilution of trace element content, attributable to better pasture growth (see section 3.3). In general, the ratios between daily element ingestions in affected sites and control sites (A/C, Table 5.7) were higher in autumn than in spring, apart from Zn.

High rates of Fe intake were predicted from our data, arising as a result of herbage contamination by the Fe-rich soil. However, iron toxicity is not a common problem in most domestic animals, probably because of the limited absorption and uptake of Fe when intakes are high (NRC, 2005). To determine the effect of iron excess on liver function, adult ponies were given 50 mg Fe $kg^{-1}$ BW $day^{-1}$ (Pearson and Andreasen, 2001). Treated individuals showed no adverse clinical signs or development of hepatic lesions. Maximum predicted ingestion from the present study was 20.9 mg Fe $kg^{-1}$ BW $day^{-1}$; even if this was doubled, it would not exceed 50 mg Fe $kg^{-1}$ BW $day^{-1}$.

Manganese is considered to be one of the least toxic of the essential elements (NRC, 1980) and ingestion by horses at the Green Corridor is far below toxic thresholds. The main effect of chronic excess Zn intake is a reduced efficiency of Cu absorption (NRC, 2005). However, the predicted ingestion level in the Green Corridor was always below that which could be considered excessive. In feeding experiments using horses, inclusion of 500 mg Zn $kg^{-1}$ in the diet had no obvious effects on Cu metabolism (Hoyt et al., 1995).

In general, higher A/C ratios (ingestion values at affected sites/ingestion values at control site), were found for non essential elements (Table 5.7). Although As was one of the elements that caused social alarm due to its toxicity, the predicted ingestion levels do not seem high for horses. According to NRC (2005), a daily food intake of 2.66-4.00 mg As $kg^{-1}$ BW in horses did not produce any discernible injury. This ingestion was 70 to 100-fold greater than the maximum predicted values found in our study (autumn, Table 5.7).

In the case of Cd, ingestion levels in the Green Corridor could be tolerable. The maximum predicted Cd intake from food found in this study (Table 5.7) was much lower (110-fold) than lethal ingestion levels of 1.1 mg Cd $kg^{-1}$ BW $day^{-1}$ reported by Liu

**Table 5.6**. Results of the general linear models applied to the concentrations of trace elements in the pastures as dependent variables, with the site sampling, biomass production and the relative composition of Poaceae, Fabaceae and Asteraceae as predictor variables. Only predictors with a significant effect on each concentration are shown (n.s.= non-significant)

| Element | Spring | | | | | Autumn | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Factor | $F$ | $p$ | $R^2$ model | $p$ model | Factor | $F$ | $p$ | $R^2$ model | $p$ Model |
| As | Biomass | 8.97 | 0.003 | 0.49 | < 0.001 | Site | 19.16 | < 0.001 | 0.61 | < 0.001 |
| | Site | 5.81 | < 0.001 | | | | | | | |
| Cd | Asteraceae | 9.68 | 0.002 | 0.47 | < 0.001 | Site | 8.55 | < 0.001 | 0.35 | < 0.001 |
| | Site | 5.38 | < 0.001 | | | | | | | |
| Pb | Biomass | 7.35 | 0.08 | 0.47 | < 0.001 | Biomass | 4.36 | 0.039 | 0.63 | < 0.001 |
| | Site | 3.45 | 0.003 | | | Site | 20.13 | < 0.001 | | |
| Tl | n.s. | n.s. | n.s. | n.s. | ns | Site | 7.20 | < 0.001 | 0.32 | < 0.001 |
| Zn | Poaceae | 4.00 | 0.048 | 0.45 | < 0.001 | Site | 8.19 | < 0.001 | 0.33 | < 0.001 |
| | Site | 9.18 | < 0.001 | | | | | | | |

**Table 5.7.** Daily predicted intake of trace elements (Mean values ± standard deviation) by consumption of pasture growing on affected (n=90) and control (n=10) soils. Estimated food intake for horses in mg element $kg^{-1}$ body weight $day^{-1}$ (data based on a daily food intake of 21 g of plant dry weight per kg of body weight). A/C is the ratio between values at affected and control sites. Significant differences between soils are marked with an asterisk.

| Elements | Season | Soil | Cu | Fe | Mn | Zn |
|---|---|---|---|---|---|---|
| Essentials | Spring | Affected (A) | 0.15 ± 0.06* | 3.60 ± 2.96* | 1.27 ± 1.31 | 0.94 ± 0.70 |
| | | Control (C) | 0.12 ± 0.05 | 1.75 ± 0.92 | 0.97 ± 0.15 | 0.34 ± 0.18 |
| | | **A/C** | **1.25** | **2.06** | **1.31** | **2.76** |
| | Autumn | Affected (A) | 0.23 ± 0.12* | 20.9 ± 31.3* | 2.32 ± 2.41* | 1.44 ± 1.48 |
| | | Control (C) | 0.18 ± 0.02 | 5.90 ± 3.53 | 1.40 ± 0.31 | 0.86 ± 0.45 |
| | | **A/C** | **1.28** | **3.54** | **1.66** | **1.67** |
| | | | As | Cd | Pb | Tl |
| Non essentials | Spring | Affected (A) | 0.006 ± 0.006* | 0.006 ± 0.01 | 0.02 ± 0.02* | 0.001 ± 0.004* |
| | | Control (C) | 0.0004 ± 0.0005 | 0.0005 ± 0.0005 | 0.007 ± 0.002 | 0.0001 ± 0.00005 |
| | | **A/C** | **15.0** | **12.0** | **2.86** | **10.0** |
| | Autumn | Affected (A) | 0.04 ± 0.06* | 0.010 ± 0.02* | 0.10 ± 0.14 | 0.001 ± 0.001* |
| | | Control (C) | 0.002 ± 0.002 | 0.0006 ± 0.0006 | 0.02 ± 0.01 | 0.0001 ± 0.00004 |
| | | **A/C** | **20.0** | **16.6** | **5.0** | **10.0** |

(2005) for horses. Lead has been incriminated as a cause of accidental poisoning in domestic animals more than any other element, particularly in cattle, sheep and foals and horses (Liu, 2003; Schmitt et al., 1971). This element is often present with Cd and their effects could be additive. A minimum cumulative fatal dosage of 1.7 mg Pb $kg^{-1}$ BW $day^{-1}$ has been reported for horses (Palacios et al., 2002). This value is 17-fold greater than maximum predicted values found in the present study (Table 5.7). Palacios et al. (2002) found negative effects in horses that received a diet which resulted in a dose of between 2.4-99.5 mg Pb $kg^{-1}$ BW $day^{-1}$, and Liu (2005) found lethal effects in horses at 6 mg Pb $kg^{-1}$ BW $day^{-1}$ (about 60-fold greater than maximum predicted values found in this study).

Thallium is more toxic to mammals than Cd, Pb, or even Hg (Nriagu, 1998), but there is very little published information concerning lethal ingestion rates for grazing animals. Frerking et al. (1990) reported non-fatal Tl poisoning in cattle at an ingestion rate of 0.75 mg Tl $kg^{-1}$ BW $day^{-1}$ over a 6-week ingestion period. This value is 750-fold higher than maximum ingestion values predicted from the data presented above (Table 5.7). Konermann et al. (1982) found no injury in pigs that received a daily intake of either 0.05 or 0.1 mg Tl $kg^{-1}$ BW $day^{-1}$. Therefore, a daily ingestion of Tl predicted to be approximately 0.001 mg $kg^{-1}$ BW in the affected areas seems to be tolerable for horses and other animals (Table 5.7).

3.5. Trace elements in horse hair and faeces

The elemental composition of animal excrement can reflect changes in diet and the level of metal contamination in the diet. The concentrations of some minerals in excreta can be greater than those found in feed (NRC, 2005). Our results show this for non-essential elements in horse faeces (Table 5.8). Concentrations of As were 2.7-fold greater in faeces than in autumnal pasture, 1.6-fold greater for Cd, 3.0-fold greater for Pb and 2.6-fold greater for Zn. The A/C ratios were also greater for non essential elements than those of essential elements (Table 5.8).

In mammals, the absorption mechanisms for essential metals are controlled by homeostatic or homeorhetic mechanisms (Wilkinson et al., 2003), that could also be utilised for other non-essential metals, although, in general, non-essential elements are often characterised by low absorption rates. For example, the transport of Cd from the mucosa to the bloodstream is much less (about 1%) than that of essential metals such as Zn (up to 50%). Cadmium

**Table 5.8**.Trace element concentrations (mg $kg^{-1}$, mean ± standard deviation) in horses' faeces from affected and control sites. A/C is the ratio between values at affected and control sites. Significant differences between sites are marked with an asterisk.

| Elements | Soil | Cu | Fe | Mn | Zn |
|---|---|---|---|---|---|
| Essential | Affected (A) | 34.9 ± 23.9 | 1914 ± 774* | 187 ± 64.5* | 142 ± 60.3 |
| | Control (C) | 19.4 ± 8.81 | 914 ± 78.8 | 90.6 ± 6.75 | 68.7 ± 19.0 |
| | **A/C** | **1.8** | **2.1** | **2.1** | **2.1** |
| | | As | Cd | Pb | Tl |
| Non essential | Affected (A) | 4.93 ± 3.69* | 0.78 ± 0.57 | 13.8 ± 12.6 | 0.13 ± 0.11 |
| | Control (C) | 0.58 ± 0.08 | 0.15 ± 0.13 | 2.02 ± 0.37 | 0.02 ± 0.001 |
| | **A/C** | **8.5** | **5.2** | **6.8** | **6.5** |

**Table 5.9**. Trace element concentrations (mg $kg^{-1}$, mean ± standard deviation) in mane hair of horses grazing affected and control sites. A/C is the ratio between affected and control sites. Significant differences between sites are marked with an asterisk. [a] Reference range of trace elements in mane hair of healthy race horses (Asano et al., 2002).

| Elements | Soil | Cu | Fe | Mn | Zn |
|---|---|---|---|---|---|
| Essentials | Affected (A) | 14.7 ± 5.05* | 334 ± 151* | 14.1 ± 7.53* | 185 ± 23.8 |
| | Control (C) | 9.84 ± 0.71 | 21.7 ± 3.57 | 4.29 ± 1.04 | 208 ± 6.82 |
| | **A/C** | **1.5** | **15.4** | **3.3** | **0.9** |
| *Reference range[a]* | | *5.9 ± 1.0* | *22.4 ± 17.5* | *4.99 ± 4.25* | *101.7 ± 14.9* |
| | | As | Pb | Tl | |
| Non essentials | Affected (A) | 0.85 ± 0.49* | 1.34 ± 0.66* | 0.015 ± 0.01* | |
| | Control (C) | 0.19 ± 0.12 | 0.37 ± 0.02 | 0.001 ± 0.001 | |
| | **A/C** | **4.47** | **3.62** | **15.0** | |
| *Reference range* | | *1.11 ± 0.17* | *0.77 ± 0.29* | - | |

absorbed into mucosal cells is bound to cell membranes and returns to the gastrointestinal tract following the desquamation of these cells. In contrast, Zn is retained and may be released as required, depending on the body burden (Wilkinson et al., 2003). Mammals convert inorganic As into methylated metabolites which are rapidly excreted; hence the carry-over of As compounds from feeds to edible tissues of mammalian species is very low (Kan and Meijer, 2007). Analysis of horse faeces seems to show a preferential excretion of non-essential trace elements compared to essential metals (Table 5.8). The lowest A/C ratio in faeces was found for Cu; excessive accumulation of this essential element could be controlled by metallothioneins in animal tissues (Yin at al., 2008).

Hair has often been proposed for biomonitoring environmental contaminants but suffers from problems with external contamination. This fibre is metabolically very active during its growth and its composition is highly influenced by the health and nutritional status of the individual before it leaves the epidermis (Hasan et al., 2004). Thus, long-term exposure to heavy metals can be readily identified by hair. Trace element content of mane hair of horses has been used to assess diseases, metabolic disorders and nutritional status, because sampling and storage of hair is straightforward when compared to other biological materials (Asano et al., 2002).

Higher As levels have been reported in the wool of sheep exposed to dietary arsenic (Raab et al., 2002). Ward and Savage (1994) investigated exposure of horses to several toxic heavy metals, including Cd and Pb, from vehicle emissions using hair analysis. They detected increases in Pb and Cd concentrations in equine hair and blood, with a significant correlation between blood and hair levels of Pb. According to Combs et al. (1983) little Cd is incorporated into hair, making this tissue a poor indicator of exposure. In the present study, despite greater concentrations of Cd in hair of horses from the affected areas than in control horses, accumulation seems rather low in both cases (<0.1 mg $kg^{-1}$).

Element ratios in the hair of horses grazing in the Green Corridor and horses from outside this area are shown in Table 5.9. Copper and Zn had the lowest ratios, similar to the ingestion values and faecal ratios for these elements. Iron is abundant in soils, so despite the rigorous hair washing procedure, the high A/C ratio for Fe could indicate some degree of soil contamination; the highest ratio was found for Tl.

In general, trace elements in hair of ‘control horses’ were in the same range as those reported for healthy horses (Table 5.9). In comparison with horses from the Green Corridor, the greatest difference was in Fe content. This could be explained by greater exposure to Fe-rich soil in their diet, compared to the race horses studied by Asano et al. (2002). In the case of non-essential elements, values were always in the same range as reported for healthy horses (no data was found for Tl), and even lower in the case of As. Although there was no attempt to correlate concentrations in horse hair to a measure of toxicity in horses, results from hair seem to indicate an acceptable situation for grazing in the Corridor.

## 4. Conclusions

Biomass production of the pasture appears to be unaffected by soil pollution in the Guadiamar Green Corridor. Trace element concentrations in pasture plants were below the maximum tolerable level for horses, although in autumn concentrations were higher than in spring, due to a dilution effect of greater biomass production in the latter season. The floristic composition of pastures had some influence on patterns of elemental accumulation. Pastures composed mostly of grasses had lower trace element concentrations, since these species produced the greatest biomass. Asteraceae species tended to show higher concentrations of other elements, such as Cd. Therefore, grazing should preferably occur in areas and during periods where high biomass grasses are dominant. Grazing on regenerating pastures in autumn may increase exposure to metals derived from comparatively greater concentrations of trace elements in, and soil adhering to, herbage, especially in the case of Pb and Fe. Predicted values of daily elemental intake for horses, in spring and autumn, seem to be much lower than critical values reported to induce toxicity. In general, faecal analyses showed that absorption of essential elements is regulated by homeostatic mechanisms which control their accumulation. However, non-essential elements tend to be preferentially excreted by horses. Finally, trace elements in horse hair from the Green Corridor were in the same range as reported for healthy horses, therefore there appears to be no evidence that supports a toxic risk to horses grazing this region. The use of horses as management tools for the long-term restoration of this ecosystem should be further investigated, but any long-term strategy should incorporate systematic monitoring of both forage and animals.

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**Foto 5.1.** Caballo pastando en las inmediaciones de la mina de Aznalcóllar. Al fondo, el vertedero de la mina.

**Foto 5.2.** Pastizales de gran altura que se desarrollan en algunas zonas contaminadas del Corredor Verde del Guadiamar, durante la primavera de 2007.

**Foto 5.3**. Detalle de una de las unidades de muestreo de pastos.

**Foto 5.4**. Recogida de excrementos de caballo, para el análisis de su composición química.

# Capítulo 6

# Capítulo 6. Estado nutricional de especies arbóreas mediterráneas en zonas contaminadas

Este capítulo reproduce el siguiente manuscrito:

**Domínguez, M.T., Marañón, T., Murillo, J.M., Schulin, R. and Robinson, B.H. 2009. Nutritional status of Mediterranean trees growing in a contaminated and remediated area. Water, Air and Soil Pollution (en prensa).**


## Resumen

La contaminación del suelo puede contribuir al decaimiento forestal, mediante alteraciones en los ciclos de nutrientes y en la adquisición de estos nutrientes por las plantas. Esto podría dificultar el establecimiento de la cubierta vegetal en zonas contaminadas, y por lo tanto, reducir el éxito de los programas de restauración. En este trabajo, estudiamos el estado nutricional de plantones de encina (*Quercus ilex* subsp. *ballota* (Desf.) Samp.), álamo blanco (*Populus alba* L.) y acebuche (*Olea europae*a var. *sylvestris* Brot.) en el Corredor Verde del Guadiamar (SO de España), así como de árboles adultos de las mismas especies establecidos en la zona. Los suelos de esta zona fueron alterados por un accidente minero en 1998, que ha provocado la acidificación del suelo en ciertas zonas, debido a la oxidación de los restos de pirita, así como el enriquecimiento de los suelos con algunos elementos traza. En algunas zonas la proporción N:P en las hojas de *Q. ilex* y *O. europae*a fue mayor que 16, indicando cierta deficiencia de fósforo. Para el acebuche, la contaminación del suelo explicó un 40 % de la variabilidad de fósforo en las hojas, y estuvo negativamente relacionada con el contenido de clorofila foliar. El pH del suelo fue el factor edáfico que más influyó en las concentraciones de ciertos nutrientes, como Mg, P y S. Las concentraciones de Mg y S en *P. alba* fueron superiores en los suelos acidificados. El seguimiento de los niveles de pH en la zona es muy recomendable, ya que los posibles afectos a largo plazo de la acidificación del suelo podrían en el futuro agravar las deficiencias nutricionales de los árboles.

# Nutritional status of Mediterranean trees growing in a contaminated and remediated area

María T. Domínguez[a], Teodoro Marañón[a], José M. Murillo[a], Rainer Schulin[b] and Brett H. Robinson[c]

[a]Instituto de Recursos Naturales y Agrobiología, CSIC, Seville, Spain
[b] Institute of Terrestrial Ecosystems, ETH Zürich, Universitätstrasse 16, Zurich, Switzerland
[c] Department of Agriculture and Life Sciences, Lincoln University, , Canterbury, New Zealand

## Abstract

Soil contamination may contribute to forest decline, by altering nutrient cycling and acquisition by plants. This may hamper the establishment of a woody plant cover in contaminated areas, thus limiting the success of a restoration programme. We studied the nutritional status of planted saplings of Holm oak (*Quercus ilex* subsp. *ballota* (Desf.) Samp.), white poplar (*Populus alba* L.) and wild olive tree (*Olea europae*a var. *sylvestris* Brot.) in the Guadiamar Green Corridor (SW Spain), and compared it with established adult trees. Soils in this area were affected by a mine spill in 1998 and a subsequent restoration programme. The spill resulted in soil acidification, due to pyrite oxidation, and deposited high concentrations of some trace elements. In some sites, we detected a phosphorus deficiency in the leaves of *Q. ilex* and *O. europaea* saplings, as indicated by a high N:P ratio (>16). For *O. europaea*, soil contamination explained 40 % of the variability in leaf P, and was negatively related to chlorophyll content. Soil pH was a significant factor predicting the variability of several nutrients, including Mg, P and S. The uptake of Mg and S by *P. alba* was greater in acidic soils. The monitoring of soil pH is recommended, since long term effects of soil acidification may negatively affect the nutritional status of the trees.

Keywords: heavy metal; tree nutrition; phosphorus; *Quercus ilex*; *Olea europaea*; *Populus alba*; soil remediation

## 1. Introduction

Soil contamination can affect nutrient cycling and acquisition by trees, leading to nutrient disorders in forests sites. Competitive interactions between pollutants, such as trace elements, and nutrients in the soils and in the roots may alter the availability or uptake of essential elements. For example, these interactions have been described for As and P (Lambkin and Alloway, 2003), Tl and K (Kwan and Smith, 1991), or Cd and Ca (Perfus-Barbeoch et al., 2002). Root growth and morphology can also be altered by trace elements (Arduini et al., 1994; Reichman et al. 2001; Domínguez et al., 2009), reducing root ability to absorb essential nutrients. Elevated trace element concentrations can alter microbial activity and composition in soils (Pennanen et al., 1998; Tuomela et al., 2005), affecting plant-microorganism interactions that are essential for plant nutrition such as mycorrhizae (Kieliszewska-Rokicka et al., 1997; Hartley-Whitaker et al., 2000).

Mediterranean environments are frequently deficient in N and P (Henkin et al., 1998; Rodà et al., 1999). Nutrient limitations may affect the growth of afforested woody plants in Mediterranean areas (Romanyà et al., 2004; Villar-Salvador et al., 2004). Therefore, fertilization often improves the performance of planted seedlings (Querejeta et al., 1998; Vallejo et at., 2000; Fuentes et al., 2007a). The management of contaminated sites should address possible tree nutrient deficiencies, associated with the contamination event or its subsequent remediation. However, little is known about the effects of soil trace elements on the nutrition of Mediterranean trees, despite the increasing use of metal-rich amendments in the afforestation of Mediterranean sites. On one hand, soil contamination may exacerbate nutrient deficiencies in plants. On the other hand, many semi-arid areas in the Mediterranean region are characterized by a relatively high pH and carbonate content, which, may result in a low availability of some micronutrients such as Cu or Zn for plants (Adriano, 2001). The application of metal-enriched material such as sewage-sludge to semi-arid calcareous soils may improve the seedling performance, by releasing micronutrient limitations (Fuentes et al., 2007a). Thus, the influence of soil contamination on plant nutrition depends on the type of contaminant (essential vs. non-essential trace element), the dose, the type of soil and the soil organic matter content. The latter two factors determine the background availability of essential trace elements, the capacity for the retention of the added contaminants, and, therefore, the possible phytotoxic effects (Illera et al., 2000; Oudeh et al., 2002; Toribio and Romayà, 2006).

The Guadiamar Green Corridor (SW Spain) is one of the largest cases of soil remediation in Europe in the last decade. This area was contaminated by a mine-spill in 1998 and a large-scale restoration programme was implemented, which included the addition of soil amendments and the afforestation with native Mediterranean woody plants. Soil types in the area range from sandy loam to calcareous clay loam. Therefore, this wide variability allows exploring the influence on soil type on the dynamics and effects of contamination. Several recent studies have shown the dynamics of trace elements in soils and plants from the Guadiamar Green Corridor following remediation (Madejón et al., 2004; Cabrera et al., 2008; Domínguez et al., 2008). However, there is a lacuna of information on the ecophysiological responses of the trees growing in this area. Given the high trace element concentrations in soils, the pollutants may detrimentally affect plant processes at the root level, and hence affect plant nutrition.

In this work, we studied the chlorophyll content and nutritional status, as plant-health indicators of trees in the Guadiamar Green Corridor. We assessed the influence of soil conditions on these plant variables. We selected three tree species (*Olea europaea* var. *sylvestris*, *Populus alba* and *Quercus ilex* subsp. *ballota*), which are the most abundant in the Guadiamar Green Corridor. We aimed to determine: 1) whether there was any disorder in nutritional status or chlorophyll content of trees growing under these altered soil conditions; 2) the relative influence of soil contamination on plant nutrient status over a range of soil properties, including different pH, texture, organic matter content and nutrient availability. As a secondary objective, we sought to elucidate relationships (synergistic, antagonistic or neutral) that exist between essential and non-essential trace element concentrations at the leaf level, since these relationships could help to explain any nutritional changes induced by soil contamination.

## 2. Material and Methods

2.1. Study area and studied species

The Guadiamar River Green Corridor (SW Spain) has a semi-arid Mediterranean climate with mild rainy winters and warm dry summers. The average annual temperature is 19 ºC (monthly mean min. 9 ºC in January, and max. 27 ºC in July). The average annual rainfall is 484 mm and potential evapotranspiration is 1139 mm (period 1971 - 2004). Soils of the area are mostly neutral or slightly alkaline, with the exception of some terraces on the Northern bank, which are acidic.

In 1998 a mine-spill affected some 4286 ha of the river basin. The sludge that covered the soils was composed by polymetalic sulfphides (mainly pyrite), with average concentrations of 380 g $kg^{-1}$ of Fe, 9.3 g $kg^{-1}$ of Zn; 8.6 g $kg^{-1}$ of Pb, 5.7 g $kg^{-1}$ of As, 1.5 g $kg^{-1}$ of Cu, 40 mg $kg^{-1}$ Cd and 43 mg $kg^{-1}$ of Tl, as main contaminants (ITGE, 1999).

After the accident, an emergency cleanup removed sludge and contaminated topsoil. Organic matter and Calcium-rich amendments were added with the aim of immobilising trace elements and improving soil fertility. Sugar beet lime was the most used amendment, which had 70-80 % of $CaCO_3$ (pH 9) and N, P and K concentrations up to 9.8, 5.1 and 5.3 g $kg^{-1}$, respectively (Madejón et al. 2006). Application rates ranged from 3 to 50 t $ha^{-1}$, depending of the degree of contamination of the site (Arenas et al., 2008).

The revegetation of around 2700 ha with native Mediterranean tree and shrub species started in 1999. One of the goals of this programme was the long-term establishment of a continuous vegetation belt for wildlife to migrate along the Guadiamar River between the Doñana National Park in the South (were the river flows into) and the Sierra Morena Mountains in the North. Depending on the local conditions, the target tree and shrub species used for afforestation were those typical of riparian forests, such as *Populus alba*, *Fraxinus angustifolia* and *Salix atrocinerea* or those typical of drier upland forests, such as *Quercus ilex* subsp. *ballota*, *Olea europaea* var. *sylvestris*, *Ceratonia siliqua, Phillyrea angustifolia, Pistacia lentiscus, Rosmarinus officinalis* and *Retama sphaerocarpa*. Seedlings were grown in a local nursery, and then planted out after one year. The planting density ranged from 480 to 980 plants per hectare.

For this study we selected the most abundant tree species used in this restoration programme: Holm oak (*Quercus ilex* subsp. *ballota* (Desf.) Samp.), wild olive tree (*Olea europae*a var. *sylvestris* Brot.) and white poplar (*Populus alba* L.).

2.2. Plant and soil sampling

Sampling was carried out during autumn of 2005, seven years after the remediation works. Nineteen sites along the Green Corridor were selected (Fig. 6. 1), from the unaffected areas upstream of the tailings dam (37º 30’, 6º 13’ W), 38 km down to the Southern limit of the Doñana saltmarshes (37º 13’ N, 6º 14’ W). Three of these sites were unaffected by the spill, but also were afforested and included within the Corridor; they will serve as "blank" references for this study. Several soil types are present along the sampled area. Bedrocks in the North of the basin (including the unaffected sites 1 and 2) are mostly slate and schist, and soils are mostly acidic sandy loams. In the Central part of the basin, predominant bedrocks are lime and calcarenite, and soils are neutral and basic loams (including the unaffected site 13). Soils in the South of the basin are mostly clay loams, due to the vicinity of the saltmarshes in the South of the basin.

Samples of *P. alba* and *Q. ilex* were collected from ten sites, and *O. europae*a from eleven sites. Where possible, at each site we also sampled adult trees that survived the spill, for comparison with planted saplings. There were no individuals of *P. alba*, nor adult trees of the other two species at the non-affected sites.

At each site, we selected three to ten individual trees of each species, depending on their abundance. Around each tree, the leaf litter was removed and soil samples were taken from the root-zone at 0-25 cm, using a spiral auger with a diameter of 2.5 cm. Two cores were taken at opposite sides of the trunks to make a composite soil sample for each tree. A composite leaf sample was taken for each selected tree, by randomly collecting 4-8 leaf subsamples around the crown. We collected fully expanded leaves from the outer canopy, avoiding shaded leaves from the inner parts of the crown. For adult trees, we used a pole to reach the leaves of the medium and upper parts of the crown. Leaves were placed in a chilled, dark storage container for transportation to the laboratory. Between 15 and 25 plant samples per species and life-stage (adults and saplings) were collected. The total number of plant (and correspondent soil) samples was 116 (52 adults and 64 saplings).

2.3. Sample preparation and chemical analyses

Immediately upon returning from the field, we selected ten leaves from each plant sample and carefully washed them with deionised water. In these leaves we used a SPAD-502 chlorophyll meter (Minolta Camera Co. Ltd. Osaka, Japan) to determine the Chlorophyll Content Index (CCI) of each leaf, by taking three measurements per leaf. The CCI is a non-destructive, dimensionless variable, which is linearly related to the total chlorophyll concentration per unit of area. This relationship differs for leaves of different species, so we did not compare across species. The rest of each plant sample was washed thoroughly with deionised water, dried at 70 ºC for at least 48 h and ground using a stainless-steel mill.

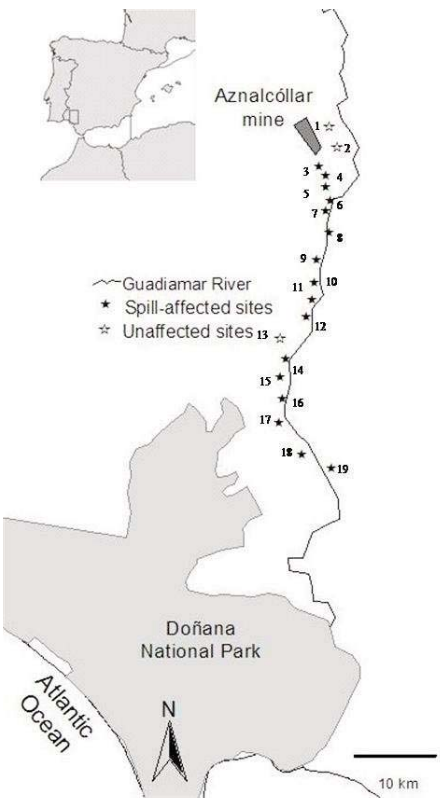


**Fig. 6.1.** Map of the Guadiamar River Valley (SW Spain) in the Iberian Peninsula (insert) and location of the sampling sites.

Plant material was analyzed for N using a Kjeldahl digestion. The rest of macronutrients (Ca, K, Mg, P and S) and trace elements (As, Bi, Cd, Cu, Pb, Tl and Zn) were extracted by wet oxidation with concentrated $HNO_3$ under pressure in a microwave digester. Macronutrients (except N) were analyzed by ICP-OES (Inductively Coupled Plasma Optical Emission Spectrophotometry; Thermo Jarrel Ash Corporation). Trace elements were analyzed by ICP-MS (Inductively Coupled Plasma Mass Spectroscopy; Perkin Elemer, Sciex-Elan 5000).

To ensure that the CCI was a reliable measurement of the total leaf chlorophyll of the studied species, a calibration was conducted in the autumn 2007. Ten trees per species and life-stage were selected in four of the sampling sites; in each of them 2-3 leaf were collected and treated as described above. The chemical analysis of the chlorophyll concentration was performed in two disk of 5 mm diameter of each leaf; for *P. alba* and *Q. ilex* chlorophyll was extracted with N,N-dimethylformamide and determined by spectrophotometry according to Moran (1982). For *O. europaea*, the extraction with N,N-dimethylformamide was not complete, and methanol was used as extractant in a subsample of leaves; chlorophyll was then determined by spectrophotometry according to Wellburn (1994). The best fits between CCI and chlorophyll concentrations were obtained by exponential regressions (Fig. 6.2).

All soil samples were oven-dried at 40 ºC until a constant weight was obtained, then sieved to < 2 mm, for the analysis of general properties. A fraction of each sample was then ground in an agate mortar to < 1 mm for trace element analysis.

Soil texture was determined by the hydrometer method (Gee and Bauder, 1979). The pH was determined potentiometrically in a 1:2.5 soil-water suspension. Organic matter content was analyzed by

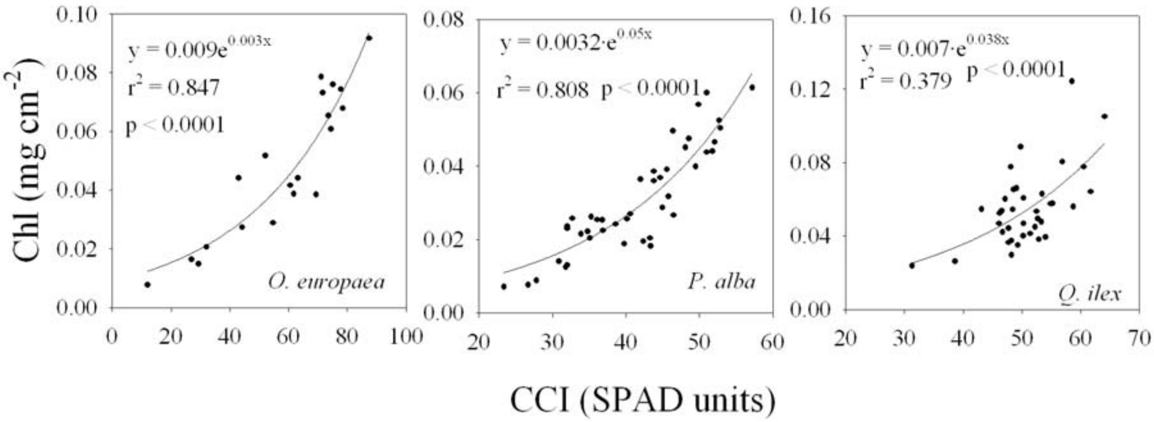


**Fig. 6.2.** Relationship between the Chlorophyll Content Index (CCI) estimated by a SPAD meter and total chlorophyll concentration (Chl) for the three studied species. Exponential function and parameters are indicated.

dichromate oxidation and titration with ferrous ammonium sulphate (Walkley and Black, 1934). Available P was determined by sodium bicarbonate extraction (Olsen et al., 1954), and available K was analyzed by extraction with ammonium acetate (Bower et al., 1952). Total organic N was analyzed by the Kjeldahl method (Kammerer et al., 1967). For total trace element concentrations (As, Bi, Cd, Cu, Pb, Tl and Zn), the < 1 mm soil fraction was digested using concentrated $HNO_3$ and HCL (1:3 v/v, aqua regia) and analyzed by ICP-MS.

The quality of the analyses was assessed by analyzing different reference materials: NCS DC 73350 (white poplar leaves, China National Analysis Center for Iron and Steel) for macronutrients and trace elements; BCR-62 (olive tree leaves, European Community Bureau of Reference) only for plant trace elements, and CRM 141R (calcareous loam soil, European Community Bureau of Reference) for soil trace elements). Our experimental values showed recoveries from the certified values of 86 to 96 % (plant macronutrients), 81 to 105% (plant trace elements) and 83 to 91 % (soil trace elements).

2.4. Data analyses

Principal Component Analyses (PCAs) were performed to explore the relationships between soil factors. Factor-variable correlations were considered as relevant when factor loadings were ≥ 0.60. Significant differences in the concentrations of plant nutrients and the chlorophyll content between saplings and adult trees of each species were analyzed using the t-test. Linear correlation analyses were performed within the concentrations of elements and the chlorophyll content in the leaves, to investigate possible interferences of trace element accumulation on the leaf nutrient content. With the mean values of nutrient and chlorophyll concentrations in each sampling site, we performed univariate linear regressions with some of the soil properties of the corresponding sites (pH and an index of soil contamination, see below). Weighted regressions were used because the variances were highly different among sites. The inverse of the variance of the studied concentrations was used as the weight variable.

On an individual tree basis, multiple regression models were used to investigate the variation of nutrient concentrations and the chlorophyll content in leaves in relation to various soil predictors. We used the Mallow's Cp coefficient for the selection of the best subset of the models. Mallow's Cp is a special case of the Akaike Information Criterion and is less dependent than R-square on the number of predictors in the model. We selected models with minimum Cp coefficients values (Mallow, 1973). The soil variables used as predictors were pH, clay content, N, P, K concentrations and a Contamination Index (CI). The CI incorporates all the major soil contaminants, since all contaminants were mutually correlated and were therefore unsuitable individually as predictors. CI is the first component of a PCA of the total elemental concentrations in the soil, with the soil contaminants (As, Bi, Cd, Cu, Pb, Tl, and Zn) having factor loadings > 0.75 (Domínguez et al., 2008). The more contaminated the soil is, the more positive the

**Table 6.1**. Soil properties (mean and min., max. values in parentheses) in the Guadiamar Green Corridor on affected and unaffected sites. OM: Organic Matter; CI: Contamination Index (see Material and Methods for further explanations)

| Variable | Unaffected Sites | | | Affected Sites | | |
|---|---|---|---|---|---|---|
| | Site 1 (*N*= 5) | Site 2 (*N*=6) | Site 13 (*N*=6) | North (sites 3-7, *N*=25) | Central (sites 8-12, *N*=41) | South (sites 14-19, *N*= 33) |
| pH | 8.4 (5.5, 8.3) | 5.5 (5.1, 6.3) | 7.9 (7.4, 8.1) | 7.1 (3.5, 8.4) | 6.0 (2.4, 8.1) | 8.1 (7.0, 8.5) |
| Silt (%) | 32.1 (31.7, 32.5) | 31.2 (29.7, 32.2) | 24.4 (17.6, 31.3) | 25.1 (12.7, 32.7) | 33.3 (19.3, 45.4) | 29.9 (18.7, 41.9) |
| Clay (%) | 54.2 (53.1, 55.5) | 21.1 (17.3, 28.4) | 23.3 (11.5, 34.0) | 25.1 (14.2, 39.2) | 23.8 (14.8, 33.6) | 24.5 (17.0, 39.2) |
| Sand (%) | 13.7 (12.7, 14.9) | 47.7 (39.8, 52.9) | 52.3 (34.7, 70.9) | 49.8 (32.2, 73.1) | 42.9 (21.3, 62.9) | 45.6 (27.0, 62.5) |
| OM (g kg$^{-1}$) | 66.3 (63.0, 100) | 18.1 (7.8, 25.7) | 16.3 (11.6, 22.6) | 22.4 (3.0, 71.2) | 24.1 (12.7, 45.4) | 22.6 (8.99, 71.2) |
| N (g kg$^{-1}$) | 1.24 (1.12, 1.31) | 0.99 (0.6, 1.4) | 0.89 (0.48, 1.17) | 1.16 (0.39, 2.17) | 1.33 (0.59, 3.1) | 0.65 (0.08, 1.21) |
| P (mg kg$^{-1}$) | 8.4 (6.3, 10.0) | 10.8 (5.7, 20.6) | 12.4 (4.6, 17.6) | 35.6 (14.3, 100) | 15.9 (1.7, 101) | 18.7 (3.7, 41.5) |
| K (mg kg$^{-1}$) | 463 (438, 482) | 201 (125, 309) | 267 (230, 295) | 144 (22, 357) | 172 (18, 378) | 223 (95, 404) |
| CI | -3.37 (-3.4, 3.30) | -2.87 (-3.33, -2.27) | -4.99 (-5.23, -4.88) | 0.44 (-3.59, -4.62) | 1.31 (-2.8, 9.21) | -0.17 (-2.68, 2.91) |

CI is. In each model, we checked that the tolerance of the selected soil predictors was ≥ 0.40, to avoid error inflation due to possible collinearity of the predictors.

The significance level was fixed at the 0.05. To avoid the increase of type I error derived from multiple testing, we controlled the 'false discovery rate', (FDR) at the 5% level, as suggested by García (2004). We used an adapted FDR procedure (Hochberg and Benjamini, 2000) to calculate a threshold value (pt ≤ 0.05) for each test, to which individual p-values were compared. Therefore, only p-values not exceeding a threshold (pt) value were considered as significant. The pt values for each test are reported in the Results section.

Data that were log-normally distributed were log-transformed for all these statistical analyses. All analyses were performed with STATISTICA v. 6.0. (StatSoft Inc., Tulsa, USA).

## 3. Results and Discussion

### 3.1. Soil conditions

Soil properties were heterogeneous in the afforested Guadiamar Green Corridor (Table 6.1). Soils at the Northern areas were mostly sandy-loam and loam; while most frequent soils at Central and Southern areas were loam and clay-loam. The pH values were highly variable within the affected sites, and included extremely acid soils (pH < 4). Many soils (45 %) had N-concentrations < 1 g kg$^{-1}$, a value considered low for Southern Spanish loamy soils (CAC, 1992). Similarly, 50 % of the sampled soils had low organic matter content, of < 20 g kg$^{-1}$. The availability of P was highly variable, especially within the affected area, ranging from 1.7 to 101 mg kg$^{-1}$. Nearly 40 % of the soils had a comparatively low P availability (< 13 mg kg$^{-1}$). However, 40 % of soils had a high Olsen-P concentration (> 20 mg kg$^{-1}$). Most of the soils had normal to low levels of available K (< 200 mg kg$^{-1}$). Trace element concentrations (As, Bi, Cd, Cu, Pb and Zn) were relatively high in the spill-affected sites (Appendix 1, Table 1). Arsenic was the most important contaminant, with an average concentration of 129 mg kg$^{-1}$ in affected sites; it is remarkable that this average value is much higher than the upper limit of the range of normal values for agricultural soils (40 mg kg$^{-1}$, Bowen, 1979). The most contaminated sites (as indicated by the Contamination Index) were located at the Northern and Central areas of the Guadiamar Green Corridor.

The PCA of the soil data revealed three factors with eigenvalues > 1, explaining 73 % of the variance (Table 6.2). Factor 1 had high weightings for soil texture, organic matter and available K, the latter two were negatively correlated with sand content. Factor 2 was defined by soil pH, negatively correlated with total N and contamination (CI). Factor 3 was associated with Olsen-P. The negative correlation between CI and pH was especially high in the Central areas ($r = -0.50$, $p = 0.001$). The distribution of the soil samples in the PCA diagram (Fig. 6.3) illustrates the high variability of the soil properties. Even the unaffected soils had distinct textures and organic matter contents, as indicated by their positions along the Factor 1 axis. The range of coordinates in the Factor 2 axis indicates the variability in pH values, and the trend of lower pH in the contaminated soils.

**Table 6.2**. Results of the Principal Component Analysis (factor loadings) applied to the soil properties (N = 116). The percentage of variance explained by each factor is also indicated. OM: Organic Matter; CI: Contamination Index.

| Variable | Factor 1 (38.7%) | Factor 2 (22 %) | Factor 3 (12.3%) |
|---|---|---|---|
| pH | -0.40 | **0.79** | 0.11 |
| Silt | **-0.68** | -0.31 | -0.21 |
| Clay | **-0.87** | -0.16 | 0.03 |
| Sand | **0.92** | 0.20 | 0.09 |
| OM | **-0.66** | -0.34 | 0.25 |
| N | -0.23 | **-0.65** | 0.47 |
| P | 0.14 | 0.29 | **0.86** |
| K | **-0.73** | 0.48 | 0.09 |
| CI | 0.47 | **-0.59** | 0.12 |

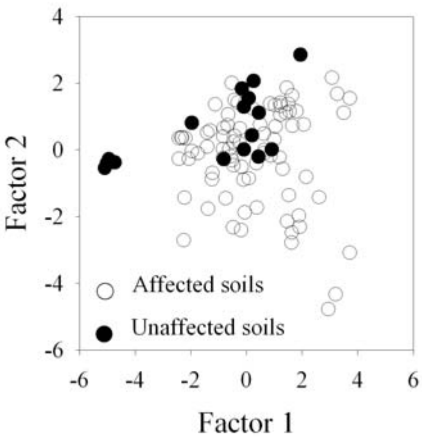


**Fig. 6.3.** Principal Component Analysis of the studied soil properties (N = 116). Soil-factor correlations are shown in Table 6.2

3.2. Leaf chlorophyll and nutrients in trees

The average Chlorophyll Content Index values (CCI, hereafter referred as "chlorophyll" for simplicity) were 67, 37 and 50 for the leaves of O. europaea, *P. alba* and *Q. ilex*, respectively. The afforested *Quercus ilex* saplings had lower leaf chlorophyll than adult trees (Table 6.3). For the other two species, there were no differences between life-stages. Considering the average chlorophyll content in the leaves of each site individually, and for each species, there was no significant relationship with soil contamination (Fig. 6.4).

Leaf nitrogen levels were similar for the three species, ranging between 12 and 16 g $kg^{-1}$. Phosphorus concentrations varied from 0.70 g $kg^{-1}$ (*Q. ilex* saplings) up to 1.25 g $kg^{-1}$ (*P. alba* adults). *Populus alba* had the highest concentrations for Ca, Mg and S (Fig. 6.5) For each species, there were some differences between adult trees and the afforested saplings (Table 6.3). In most cases, these differences indicated a higher leaf concentration of nutrients in adult trees, with the exception of P concentrations for *O. europaea,* which were lower in the adult trees. The ratios between N and S, P, K and Mg (respectively) also varied between life-stages (Table 6.4). In particular, there was a significant difference in the ratio N:P between *Q. ilex* saplings (mean value of 19) and adults (mean value of 15) which indicates a relative deficiency in P content of the afforested oak saplings.

**Table 6.3**. Results of the t-test for the comparison of Chlorophyll Content Index (CCI) and nutrient concentrations in adult trees and saplings, for each species. Significance levels after controlling the FDR at the 5% level was pt = 0.0315. Significant values are indicated in italics. A: Adult trees; S: saplings.

| | *O. europaea* N = 15 (A), 25 (S) | | *P. alba* N = 22(A), 18 (S) | | *Q. ilex* N = 15 (A), 21 (S) | |
|---|---|---|---|---|---|---|
| | *t*-value | *p* | *t*-value | *p* | *t*-value | *p* |
| CCI | 0.65 | 0.5201 | -0.06 | 0.9493 | *-3.86* | *0.0006* |
| Ca | -1.82 | 0.0761 | *-3.00* | *0.0048* | *-3.05* | *0.0044* |
| K | 1.42 | 0.1630 | *-2.52* | *0.0159* | -1.78 | 0.0846 |
| Mg | *-3.68* | *0.0007* | -1.34 | 0.1890 | -0.21 | 0.8350 |
| N | 1.25 | 0.2176 | -0.04 | 0.9706 | *-2.24* | *0.0316* |
| P | *2.95* | *0.0054* | -0.92 | 0.3610 | *-4.30* | *0.0001* |
| S | *-2.97* | *0.0051* | *-3.20* | *0.0028* | -0.56 | 0.5796 |
| N:K | -0.53 | 0.5994 | *2.62* | *0.0126* | 1.36 | 0.1826 |
| N:Mg | *3.53* | *0.0011* | 0.81 | 0.4248 | 1.73 | 0.0935 |
| N:P | -1.66 | 0.1049 | 0.91 | 0.3681 | *3.95* | *0.0004* |
| N:S | *2.92* | *0.0058* | *2.61* | *0.0130* | 0.83 | 0.4151 |

**Table 6.4**. Ratios between macronutrients (mean ± standard error) in the leaves of the studied plant species. Significance levels in the comparison between adult trees and saplings are indicated in Table 6.3.

| Specie | N:K | N:Mg | N:P | N:S |
|---|---|---|---|---|
| *O. europaea* Adult (N = 15) | 2.70 ± 0.35 | 7.85 ± 0.86 | 16.9 ± 0.6 | 7.15 ± 0.50 |
| *O. europaea* Sapling (N = 25) | 2.52 ± 0.23 | 14.8 ± 1.4 | 15.0 ± 1.1 | 9.34 ± 0.43 |
| *P. alba* Adult (N = 22) | 3.45 ± 0.30 | 4.03 ± 0.38 | 14.1 ± 1.0 | 5.78 ± 0.32 |
| *P. alba* Sapling (N=18) | 4.36 ± 0.24 | 4.5 ± 0 .49 | 14.9 ± 0.7 | 7.78 ± 0.68 |
| *Q. ilex* Adult (N = 15) | 3.02 ± 0.20 | 8.93 ± 0.79 | 15.0 ± 0.6 | 9.08 ± 0.56 |
| *Q. ilex* Sapling (N = 21) | 3.59 ± 0.29 | 10.9 ± 0.9 | 19.2 ± 0.8 | 10.2 ± 0.7 |

An analysis at the site level revealed a negative relationship between soil contamination and the P concentrations in the leaves of *O. europaea* (Fig. 6.6). For this species, soil pH was positively correlated with P concentrations and negatively correlated with the N:P ratio (Fig. 6.6). Consequently, in the most contaminated sites, the P concentrations were much lower than in the unaffected sites. For example, the wild olive saplings growing on sites 4 and 10 (affected) had some 33% less P than those on unaffected sites.

For *P. alba*, there was an increase in leaf S with higher soil contamination levels (Fig. 6.7). Soil pH was highly correlated with the average leaf S at each site (Fig. 6.7), and showed marginally significant correlations ($0.05 > p > 0.004$) with leaf Mg (negative) and N:S (positive). For *Q. ilex*, we did not find any similar relationship between the soil contamination level and the leaf concentration of any nutrient.

3.3. Soil factors influencing leaf chlorophyll and nutrient concentrations

The models obtained by multiple regressions, considering each tree and its surrounding soil individually, reflect the influences of soil conditions on leaf chlorophyll and nutrients. In the case of P concentrations in the leaves of *O. europaea* adults, soil contamination was the only significant predictor in the model, and explained a 40% of the variance of leaf P (Table 6.5). In these trees, contamination was also negatively related to the N:S ratio, although soil texture had a higher relative importance in the variance of this variable (indicated by the β coefficient of the model). In the *O. europaea* saplings, soil contamination negatively influenced the plants' chlorophyll content and the N:Mg ratio. For chlorophyll, the model was highly significant and explained a high percentage of the variance ($r^2 = 0.72$, $p < 0.001$). For N:Mg ratio, soil pH was also a significant predictor, and its influence was higher than that corresponding to contamination. In these saplings, soil pH positively influenced leaf Mg; thus, under acidic conditions the N:Mg increased, due to a probable decrease in Mg uptake. Likewise, there was a negative correlation between soil pH and the N:P ratio (Table 5).

In contrast to *O. europaea*, the uptake of some nutrients by *P. alba* responded positively to soil contamination or acidification. For example, soil contamination was a positive predictor for leaf K in adult trees; decreasing pH increased the leaf S and, marginally

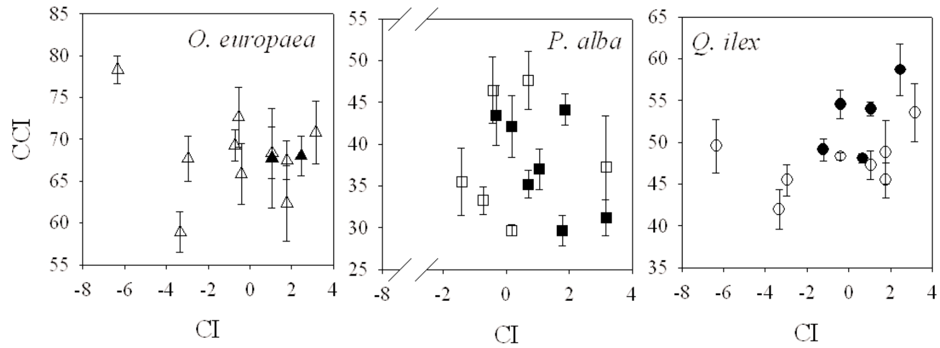


**Fig. 6.4**. Relation between Chlorophyll Content Index (CCI, mean ± standard error) in the leaves of the studied species and the soil degradation level (indicated by a Contamination Index, CI). Filled and open symbols correspond to adult trees and saplings, respectively.

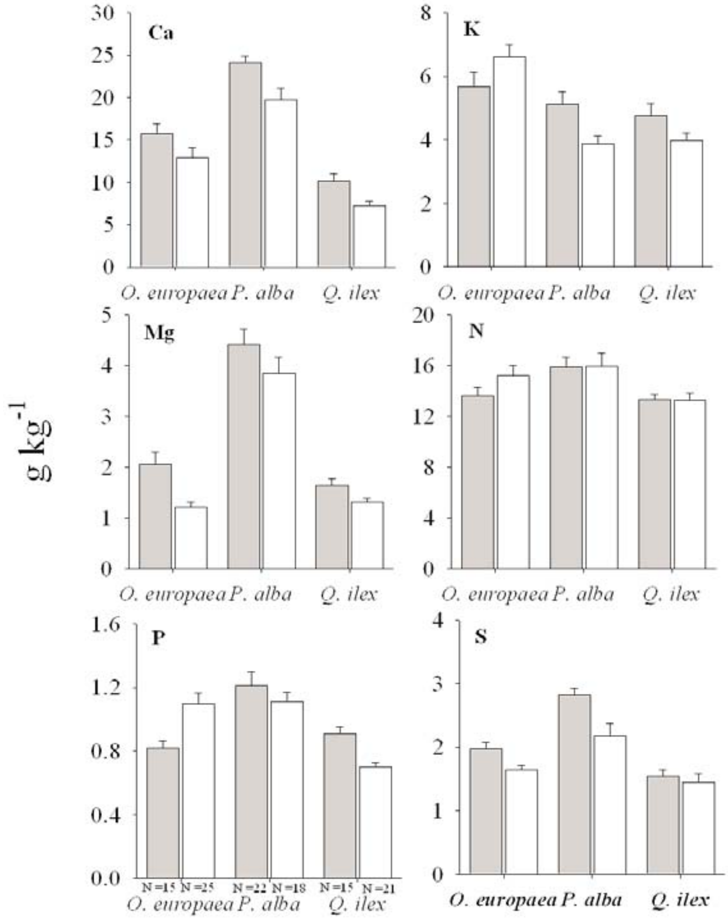


**Fig. 6.5**. Nutrient concentrations (mean and standard error) in the leaves of the studied species. Grey and white bars correspond to adult trees and saplings, respectively.

($p > 0.025$), the leaf Mg in the saplings. The exception was the N to P ratio: for this variable soil pH was a negative predictor, explaining a 30% of the variance; this indicates that, under acidic conditions, the trees showed a relative P deficiency.

Similarly to *P. alba* trends, contamination was positively correlated with leaf S in *Q. ilex* saplings, and negatively with the N:S ratio.

3.4. Interactions between leaf trace elements and chlorophyll and nutrients

Some of the above-mentioned patterns were related with the interactions between non-essential elements (such as As, Pb, and Cd) and nutrients in the leaves of the trees. For example, in *O. europaea* saplings chlorophyll and Pb showed a negative relationship ($r = -0.73$, $p < 0.001$). Likewise, P was negatively correlated with As in the adult olive trees ($r = -0.68$, $p = 0.005$), and, marginally, in the saplings ($r = -0.436$, $p = 0.037$). A marginal influence of Bi on P levels was also observed for the saplings ($r = -0.439$, $p = 0.036$). The K concentration showed a highly significant relationship with As in the leaves of the adult olive trees ($r = -0.81$, $p < 0.001$).

In contrast, for the other two species there were some positive interactions between trace elements and some nutrients, especially for *P. alba*. For example, between K and Cu ($r = 0.62$, $p = 0.002$) and Tl and Mg ($r = 0.711$, $p = 0.001$) in adult trees. Again, the exception was related to leaf P, having a marginal and negative interaction between As and P in P. alba saplings ($r = -0.57$, $p = 0.016$).

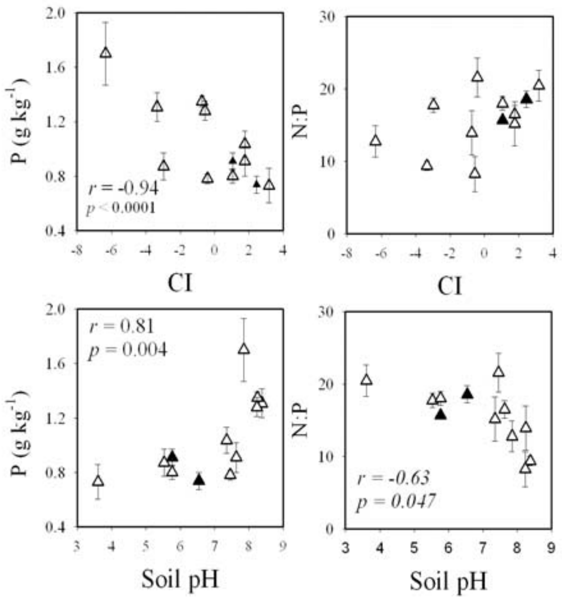


**Fig. 6.6**. Relationship between Contamination index (CI, plots in top row) or soil pH (plots in bottom row) and leaf P and N:P in the leaves of *O. europaea* (mean ± standard error). Filled and open symbols correspond to adult trees and saplings, respectively. If significant (pt = 0.004), correlation coefficients and p-values are indicated. Values in italics are marginally significant (0.05 > p > 0.004) respectively.

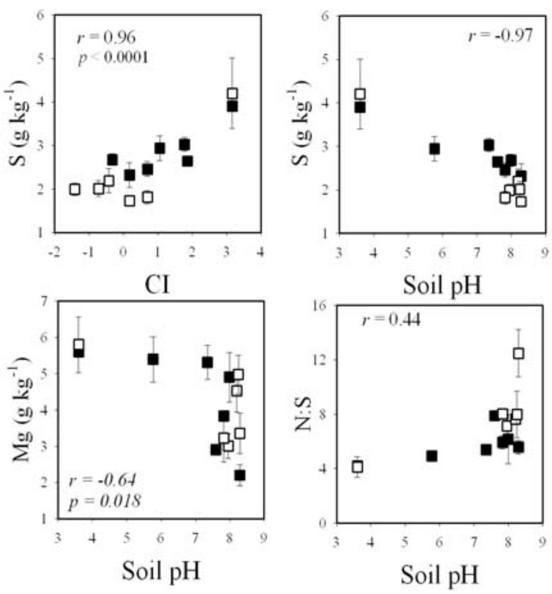


**Fig. 6.7**. Relationship between Contamination index (CI) or soil pH on each sampling site and leaf S and Mg and N:S ratio in the leaves of *P. alba* (mean ± standard error). Filled and open symbols are adult trees and saplings, respectively. Values in italics are marginally significant (0.05 > p > 0.004).

## 4. Discussion

### 4.1. Nutritional status of the trees

Soil contamination frequently leads to nutrient disorders in plants. Thus, the monitoring of the nutritional status of trees should be addressed in polluted areas, since the enhanced nutrient deficiencies could limit the success of a restoration programme. Tree nutritional and health status depends on many soil factors, which determine the availability of nutrients, as well as the bioavailability of potentially toxic elements. In this study, we assessed the nutritional status of three different tree species, growing in afforested sites with different degree of soil degradation (elevated trace element concentrations and acidification).

We found some evidences of nutrient deficiencies in the studied species. In general, the afforested saplings had lower nutrient concentrations than the adult trees. For adult *O. europaea*, N and K leaf concentrations were lower than the values reported for trees growing on uncontaminated sites (Madejón, 2004), and were below the optimal thresholds for cultivated olive (according to Fernández-Escobar, 1997). At some affected sites, the N:P ratio in both adult and sapling trees of *O. europaea* was above 16, indicating a P limitation (Koerselman and Meuleman, 1996).

The concentrations of all macronutrients in *P. alba* (in both adult trees and saplings) were suboptimal (according to Kopinga and van den Burg, 1995). However the nutrient levels were similar to those reported for adult trees growing in a nearby uncontaminated riparian forest (Madejón et al., 2004).

The leaf concentrations of N and P in *Q. ilex* saplings were below the levels reported for seedlings of the same species, but growing under optimal conditions (Cornelissen et al., 1997). Mean N:P ratios in saplings over the whole study area were much higher than 16 (Table 6.3), indicating a strong P limitation (Koerselman and Meuleman, 1996).

**Table 6.5**. Results of the General Multiple Regression Models, where either the Contamination Index (CI) or soil pH significantly affected the leaf Chlorophyll Content Index (CCI) or nutrient concentrations. Significance level (after controlling the FDR at the 5% level) was pt = 0.025. Values in italics are marginally significant (0.05 > p < 0.025).

| Species | Leaf Variable | Soil Predictor | β | *p* | Model adjusted $r^2$ | Model *p* |
|---|---|---|---|---|---|---|
| *O. europaea* adults | P | CI | -0.53 | 0.032 | 0.39 | 0.025 |
| *N* = 15 | *N* | *CI* | *-0.49* | *0.049* | *0.43* | *0.033* |
| | | *Clay* | *0.70* | *0.010* | | |
| | *S* | *CI* | *0.53* | *0.048* | *0.23* | *0.048* |
| | N:S | CI | -0.67 | 0.002 | 0.65 | 0.003 |
| | | Clay | 0.70 | 0.002 | | |
| *O. europaea* saplings | CCI | CI | -0.47 | 0.007 | 0.72 | < 0.001 |
| *N* = 25 | | Clay | -0.61 | 0.003 | | |
| | | OM | -0.46 | 0.002 | | |
| | K | Clay | -0.52 | 0.008 | 0.66 | < 0.001 |
| | | pH | -0.89 | < 0.001 | | |
| | | P | 0.34 | 0.021 | | |
| | | K | 1.21 | < 0.001 | | |
| | Mg | pH | 0.73 | 0.006 | 0.32 | 0.022 |
| | | P | -0.55 | 0.014 | | |
| | | K | -0.64 | 0.012 | | |
| | N:P | pH | -0.61 | < 0.001 | 0.62 | < 0.001 |
| | | P | 0.68 | < 0.001 | | |
| | N:Mg | CI | -0.39 | 0.047 | 0.50 | 0.003 |
| | | pH | -0.74 | 0.002 | | |
| | | P | 0.71 | < 0.001 | | |
| | | K | 0.86 | 0.003 | | |
| *P. alba* adults | K | CI | 0.97 | 0.013 | 0.37 | 0.009 |
| *N* = 22 | | pH | 0.53 | 0.047 | | |
| | S | pH | -0.46 | 0.014 | 0.70 | < 0.001 |
| | | N | 0.28 | 0.046 | | |
| | *N:K* | *CI* | *-0.79* | *0.012* | *0.24* | *0.047* |
| | N:P | pH | -0.57 | 0.010 | 0.25 | 0.024 |
| *P. alba* saplings | *Mg* | *pH* | *-0.56* | *0.030* | *0.26* | *0.030* |
| *N* = 18 | S | pH | -0.81 | < 0.001 | 0.87 | < 0.001 |
| | | OM | 0.31 | 0.030 | | |
| | N:P | pH | -0.61 | 0.020 | 0.32 | 0.020 |
| *Q. ilex* saplings | S | CI | 0.73 | < 0.001 | 0.65 | < 0.001 |
| *N* = 21 | N:S | CI | -0.68 | < 0.001 | 0.43 | < 0.001 |

4.2. Influence of soil conditions on leaf nutrients and chlorophyll

In the Guadiamar Green Corridor we found a high level of heterogeneity in soil properties, seven years after the soil remediation works, which partly explained the differences in the concentrations of some nutrients in the trees growing there.

The soils in the affected area still presented high concentrations of trace elements, with a high variability even within each site, due to the irregular sludge deposition and the irregular cleanup of the soils. For example, in a same site soils beneath adult individuals were more contaminated and had lower pH than those beneath afforested saplings, due to the difficulty of removing the sludge and topsoil from forested areas (data not shown). The bioavailability of the cationic trace elements in the Guadiamar area is primarily determined by soil pH, with soil organic matter content or soil texture having little influence (Domínguez et al., 2009). Thus, in the acidic sites the interactions between soil contaminants and plant nutrition may be greater. Soil pH was one of the most important soil factors explaining nutrient variability in the leaves of trees.

A decrease in soil pH was associated with contamination, due to the leaching of acids generated by the oxidation of sulphides in the remnant of sludge in the soils (Kraus and Wiengand, 2006). Sugar beet lime, which had 70-80 % $CaCO_3$, was the most used amendment. The application rates during the remediation work were higher in the most contaminated sites (Arenas et al., 2008). Nevertheless, the most contaminated sites were highly acidified, and carbonate content was lower than 1 % (Domínguez et al,. 2008). Therefore, the carbonates supplied in the amendments may have already been attenuated by acid generation from the contaminating sludge. In contrast, the nutrient concentrations, particularly P, in the soils still reflected the application of the amendments. On average, the spill-affected sites had significantly higher Olsen-P than non-affected sites (data not shown). Some 40% of the soils had P concentracions higher than 20 mg $kg^{-1}$, which is significantly higher than the background values for this region. Contaminated soils in central areas had P concentrations up to 100 mg $kg^{-1}$ (Table 1). This reflects the high P content of the sugar beet lime (up to 5.1 g $kg^{-1}$, Madejón et al., 2006). Under experimental conditions, the addition of sugar beet lime to soils from the Guadiamar Green Corridor resulted in the enrichment in P to levels up to three times higher than P levels in the unamended soils, 30 months after application. The relative increase of nutrient concentrations after application was higher for P than for other nutrients (Pérez de Mora et al., 2007).

Some of the detected nutrient deficiencies, particularly P, in the afforested trees were significantly related to either the level of soil contamination or to soil acidification. For example, there was a strong negative interaction between the index of soil contamination and the P uptake for *O. europaea*.

There are several possible explanations for these negative interactions among trace elements and nutrients. Arsenic may interfere with plant P uptake, since arsenate and phosphate are analogue ions; they compete in the soil matrix and As is transported across the plasma membrane through phosphate transport systems (Meharg and Hartley-Whitaker, 2002). Arsenic was one of the most important soil contaminants in the Guadiamar Green Corridor; the mean As concentration of 130 mg $kg^{-1}$ and maximum of 340 mg $kg^{-1}$ (Appendix 1) were high in comparison with P concentrations (mean of 23 mg $kg^{-1}$ and maximum of 100 mg $kg^{-1}$, Table 1). In fact, for *O. europaea* we found negative correlations at the leaf level between As and P; therefore, some competitive interaction may be taking place at the root level. However, this effect would be greater on basic soils, since anionic trace elements such as arsenate are more mobile at higher pHs (Adriano, 2001). In contrast, we found that trees growing in acidic sites showed the highest P deficiencies.

This could be explained by a decrease in P availability in acid soils, due to increases in P sorption capacity and decreases in the rate of organic phosphorus mineralization (Paré and Bernier, 1989; Carreira et al., 1997). Oxides of Al, Fe and Mn dominate P sorption in acid soils. Under acidic conditions, P sorption by Fe oxides could thus reduce the P availability. However, acidity and contamination were not related to P availability in the studied soils. Therefore, the decrease in the P concentrations in the leaves of some plants (mainly *O. europaea*) in the most degraded sites cannot be atributable to a limited availability of soil P, but to some interaction between soil degradation and P uptake by roots or P translocation to the leaves.

The toxicity at the root level may be hampering the nutrient acquisition in the most contaminated and acidic soils, since there the solubility of toxic trace elements is higher at low pH (Greger 1999). Several studies with woody species have reported root damage by trace elements, but low contaminant transport from roots to shoots (Arduini et al. 1996; Wisniewski and Dickinson 2003; Fuentes et al. 2007b). However, non-specific morphological damage at the root level may hamper the uptake of several nutrients, and we have found that P uptake was reduced, while the uptake of other nutrients was enhanced in the most acidic and contaminated sites.

That P is the most affected nutrient may be due to the precipitation of phosphates with soluble cationic trace elements in the rhizosphere (Rolfe, 1973; Ruby et al,. 1994), which might be enhanced in the most contaminated and acidic sites. The precipitation of cationic elements with phosphate can reduce the bioavailability of these elements in soils, ameliorating their toxicity and reducing the accumulation of trace elements in the leaves (Brown et al., 2004; Bosso et al., 2008). This mechanism of trace element-phosphate interaction could contribute to keep the low trace element accumulation in the leaves of *O. europaea* at low levels, even in highly contaminated and acidic sites.

For the other two species, *Q. ilex* and *P. alba*, the interactions between soil degradation and P uptake were less clear. Nevertheless, there were also some effects of soil contamination on the N:P ratios of the leaves. The reason why *O. europaea* is the most sensitive species, in terms of P nutrition, to soil contamination is unclear and deserves further research. Different plant species can have different mechanisms for P uptake, and *O. europaea* may be less efficient in accessing P from metal-phosphate complexes than the other two studied species. In general, phosphorous is a frequent limiting nutrient in Mediterranean ecosystems, and can limit the growth of Mediterranean woody plant seedlings (Sardans et al., 2004; Villar-Salvador et al., 2004). Thus, the inhibition of P uptake by soil degradation could hamper the development of a woody plant cover on this remediated and afforested area.

In contrast to the negative effects on phosphorus nutrition, for *P. alba* (and to a lesser extent also for *Q. ilex*) the altered soil conditions (with high level of trace elements and low pH) enhanced the uptake of several nutrients. This unexpected result may be a short-term result of thesoil acidification. In forest soils with a pH < 4, $Ca^{2+}$, $Mg^{2+}$ and $K^{+}$ are released to the soil solution (Rehfuess, 1989; Foster et al., 1989). In the short-term, this may enhance the nutrient uptake and tree growth (Likens et al., 1996; Tomlinson, 2003). *Populus alba* (a fast-growing deciduous tree) may respond faster to this release of cationic nutrients than *O. europaea* and *Q. ilex* (slow-growing evergreen trees). However, if cation inputs to the soil are low, a possible long-term loss of cationic bases due to thesoil acidification may negatively influence the uptake of cationic nutrients by trees in the future.

In general, the chlorophyll content was not sensitive to the nutritional changes induced by soil degradation. In particular, chlorophyll content did not reflect the P deficiencies, which were the main detrimental effect of soil degradation on tree nutrition. The only significant interaction between chlorophyll and soil contamination occurred for *O. europaea*, coinciding with a decrease in leaf Mg, and therefore with an alteration in the N:Mg ratio. Leaf N and Mg are mostly contained in chlorophyll, so changes in chlorophyll content may be more related to N or Mg limitations. The use of the chlorophyll estimates (CCI) as a rapid and non-destructive measurement of tree health may become more important if some Mg deficiencies appear in the future, as a possible long-term effect of soil degradation.

## 5. Conclusions

There are nutrient deficiencies, particularly of phosphorus, in the trees growing in many sites of the Guadiamar Green Corridor. Soil contamination and subsequent soil acidification contribute to these P deficiencies, especially for *O. europaea*. High levels of available P were found on affected soils. Since there were no important soil nutrient limitations in these afforested lands, the benefits of soil fertilisation are likely to be small. Soil pH was found to be the most important factor affecting the nutritional status of the trees. In contrast to P, the uptake of some cationic nutrients was enhanced by soil acidification, especially in *P. alba*. Soil pH monitoring in the area is advisable, since acidification may led to nutrient disorders in the trees, influencing the long-term growth and ecosystem functioning in the Guadiamar Green Corridor.

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**Foto 6.1**. Restos de lodos en el suelo, sometidos a procesos de óxido-reduccción, en algunas de las zonas más contaminadas del Corredor Verde. Junio 2005.

**Foto 6.2.** Restos de espuma de azucarera en alguno de los suelos del Corredor Verde, aplicada durante las tareas de remediación posteriores al accidente. Junio 2005.

**Foto 6.3.** Individuo juvenil de acebuche, en una de las zonas reforestadas del Corredor Verde. Diciembre de 2005.

# Capítulo 7

# Capítulo 7. Disponibilidad de cadmio en suelos y retención en las raíces de encina: potencial para la fitoestabilización

Este capítulo reproduce el siguiente manuscrito:

**Domínguez, M.T., Madrid, F., Marañón, T., Murillo, J.M. 2009. Cadmium availability in soil and retention in oak roots: potential for phytostabilization. Chemosphere 76, 480–486.**


**Resumen**

La reforestación de zonas contaminadas se perfila como una técnica viable para la estabilización dela contaminación en zonas extensas. En este trabajo, estudiamos los patrones de disponibilidad de algunos metales (Cd, Cu, Pb y Zn) en una zona contaminada y reforestada. En particular, estudiamos la respuesta de las hojas de la encina (*Quercus ilex* subsp. *ballota*) a cambios en la disponibilidad de los metales en el suelo, con especial atención al caso del Cd. En condiciones controladas, estudiamos la respuesta de una serie de plántulas de encina expuestas a distintas concentraciones de Cd, con el objetivo de analizar los patrones de translocación de Cd y la tolerancia de las plántulas a este elemento. El cadmio fue el elemento más disponible, en términos relativos; un 15 % de la cantidad total de Cd fue extraíble con $NH_4NO_3$. La disponibilidad de Cd, Cu y Zn se relacionó exponencialmente con el pH del suelo (rango de pH de 2.4 a 8.4). La acumulación de Cd en las hojas de encina no estuvo relacionada con los cambios en la disponibilidad de Cd. Los ensayos en invernadero mostraron que las plántulas tienen una alta capacidad de retención de Cd en las raíces finas (concentraciones de hasta 7 g $kg^{-1}$ en este tipo de raíces) y unas bajas tasas de translocación de Cd a las hojas (coeficientes de transferencia menores de 0.03). La biomasa total de las raíces y el grosor de las raíces principales en los tratamientos con Cd disminuyó, en comparación con el tratamiento control A pesar de ello, las medidas de fluorescencia de clorofilas (como indicador del grado de estrés de las plantas) sólo fueron ligeramente distintas, en comparación con el tratamiento control, a exposiciones de Cd de 200 mg $L^{-1}$. Debido a la relativa buena tolerancia al Cd y a la capacidad de retención radical de este metal, la encina podría ser empleada en la fitoestabilización de suelos contaminados con Cd.

# Cadmium availability in soil and retention in oak roots: potential for phytostabilization

María T. Domínguez, Fernando Madrid, Teodoro Marañón and José M. Murillo

Instituto de Recursos Naturales y Agrobiología, CSIC, P. O. Box 1052, E-41080, Seville, Spain

## Abstract

Afforestation of contaminated land by trees is considered a feasible strategy for the extensive stabilization of contaminants. In this work, we studied the patterns of metal availability (Cd, Cu, Pb and Zn) in a contaminated and afforested area. Specifically, we observed the response of Holm oak (*Quercus ilex* subsp. *ballota*) leaves to changes in the availability of metals under field conditions, focusing on Cd. Under controlled conditions we studied the performance of oak seedlings exposed to high levels of Cd, with the aim of analyzing the patterns of translocation and tolerance of the seedlings. Cadmium was the most available metal, in relative terms; 15 % of the total Cd in the soil was extracted with $NH_4NO_3$. The availabilities of Cd, Cu and Zn showed exponential relationships with soil pH (pH values ranged from 2.4 to 8.4). Cadmium accumulation in the leaves was not related to the changes in Cd availability. Greenhouse studies showed that seedlings had a high Cd retention capacity in fine roots (up to 7 g $kg^{-1}$) and low rates of Cd translocation to the leaves (transfer coefficients below 0.03). Root biomass and thickness was altered by exposure to Cd. In spite of this, the chlorophyll fluorescence measurements (an indicator of plant stress) only differed slightly from the control treatment at a Cd dose of 200 mg $L^{-1}$. Due to the relatively high tolerance to Cd and the capacity of roots to retain this metal, Holm oak may be useful for the phytostabilization of soils contaminated by Cd.

Keywords: *Quercus*; fine roots; phytomanagement; metal; chlorophyll fluorescence

## 1. Introduction

During the last decade, the afforestation of contaminated areas has been accepted as a feasible strategy for the long-term, extensive management of these sites. The use of trees for reclamation of contaminated land can be a low-cost and ecologically sustainable alternative to other techniques, such as washing or containment, especially for large areas (Dickinson, 2000). The main benefit of the use of trees in soil remediation is their high stabilization potential. Phytostabilization potential comprises different processes in the soil that lead to a decrease in the spread of contaminants through the ecosystem (Vangronsveld et al., 1995; Mendez and Maier, 2008). Due to their vast root systems, trees bind the soil, reducing wind and water erosion. In addition, the high transpiration rates of the trees may help to reduce the migration of contaminants to surface and ground waters (Garten, 1999). The pool of available heavy metals may decrease by absorption and accumulation of these metals by tree roots, by adsorption onto roots, or by precipitation within the rhizosphere (Pulford and Watson, 2003; Wong, 2003). Phytostabilization may be a more feasible approach for the management of contaminated sites than other strategies using trees, such as phytoextraction, which is the removal of metals from the soil by tree uptake and translocation into the aboveground biomass of the vegetation (Kumar et al. 1995), because the long-term accumulation of metals in the aboveground biomass of the trees may pose a risk of transfer to the food chain (Mertens et al., 2004), especially in extensive areas where metal fluxes cannot be fully controlled.

Afforestation with native Mediterranean woody plants, such as the Holm oak (*Quercus ilex* subsp. *ballota*), has been encouraged in the management of degraded areas in southern Spain, such as contaminated sites or abandoned croplands. Often, the establishment of trees in these sites is limited by high irradiance, low water availability and weed competition (Rey-Benayas et al., 2003; 2005). The soils in these areas are commonly characterized by low levels of organic matter and poor substrate structure; in addition the soils of mining areas are generally acidified (Conesa et al., 2007). All these soil conditions may enhance metal bioavailability (Greger, 1999).

Mediterranean tree species may have a high potential for phytostabilization of contaminated soil. They have massive root systems that allow them to survive in low-water and low-nutrient conditions, which are typical of Mediterranean environments (Canadell et al., 1996). It has been found that Mediterranean sclerophyllous species growing on substrates with a high availability of metals normally show a low transfer of metals to the leaves (Arduini et al., 1996; Fuentes et al., 2007), so these species may have important mechanisms of metal tolerance in the roots.

For a tree species to be suitable for phytostabilization, the root system should be able to both retain and tolerate high concentrations of metals. Inhibition of root growth is one of the first symptoms of toxicity observed in tree seedlings (Kahle, 1993; Wisniewski and Dickinson 2003). This reduction in root growth could reduce the resistance of plants to summer drought, which is one of the main causes of mortality in young plants in the Mediterranean climate (Pausas et al., 2004), and could thus reduce the success of afforestation programs.

In this work, we studied the patterns of metal availability (Cd, Cu, Pb and Zn) in the Guadiamar Valley in southwest Spain, an area that was contaminated by a mine spill in 1998 and later afforested with woody plants. In a previous paper, we analyzed the accumulation of trace elements in the leaves of the tree and shrub species used in the remediation program (Domínguez et al. 2008). In this paper, we focus on the response of the foliar chemistry of the Holm oak, one of the most planted species, to changes in the availability of metals under field conditions. As Cd was the element with the highest potential soil-plant transfer, we studied the response of Holm oak seedlings to exposure to different amounts of Cd under controlled conditions. Specifically, we monitored biomass production, root growth and chlorophyll fluorescence, as well as the Cd accumulation patterns. We addressed four specific questions: 1) How is the availability of Cd and other metals affected by soil conditions seven years after soil remediation? 2) What is the relationship between available Cd in the soil and Cd in the leaves under field conditions? 3) What is the process of Cd translocation from the substrate to oak roots and leaves? 4) Is the Holm oak tolerant to high Cd levels in the soil, thus suggesting that it could be used for phytostabilization of contaminated soils?

## 2. Material and Methods

2.1. Study area and study species

The field study was conducted in the Guadiamar River Valley in southwest Spain, which has a semi-arid Mediterranean climate with mild, rainy winters and warm, dry summers. This area was affected by a toxic flood that inundated 4286 ha of the river basin with trace element-polluted sludge and acidic water after the failure of a mine-tailing dam (details of the accident in Grimalt et al. 1999). After the accident, a large remediation program was implemented, which included removal of the polluted topsoil, the addition of soil amendments and the revegetation of around 2700 ha with native Mediterranean tree and shrub species. In spite of these efforts, the underlying soil still contained elevated amounts of trace elements, specifically As, Cd, Cu, Pb, Tl and Zn (Cabrera et al., 2008).

The Holm oak (*Quercus ilex* subsp. *ballota* (Desf.) Samp.) is one of the most abundant trees in the study area. Currently, this species is present in scattered and fragmented savannah-like woodlands in the terraces of the river, since most of the land was used for agriculture before the accident. Holm oak saplings were widely used during the afforestation of the polluted sites, although survival of the saplings during the first several years after planting was low (Domínguez et al., 2009).

2. 2. Soil and plant sampling

Sampling was conducted in autumn 2005. The Guadiamar River Valley was divided into four zones, 40 km along the river in the north-south direction, with different soil characteristics and contamination levels. Zone 1 was located in the surroundings of the mine tailing, and soils were mostly acidic sandy loam. Zone 2, which had similar soil properties to Zone 1, was one the most contaminated areas along the Guadiamar Basin because the sludge was temporally stored in this area during the soil clean-up operations. Zone 3 also contained highly contaminated areas, but the soils were mostly neutral and basic loam. Finally, the soils in Zone 4 were mostly clay loam, due to the vicinity of the salt marshes in the south of the basin.

Along the first three zones, we selected 18 Holm oak trees (10 adult trees and 8 saplings). Around each tree, the leaf litter was removed and two soil cores were taken from the root zone at two depths (0-25 cm and 25-40 cm) to make a composite soil sample for each tree. A composite leaf sample was taken from the outer canopy of each tree. To increase the data set for the study of trace element availability in the soil (objective 1), additional soil samples were taken underneath 18 white poplar trees (*Populus alba*) in Zones 3 and 4, where this species is more abundant than the Holm oak. Thus, we got a broad range of soil types, especially in relation to pH and clay content. The total number of soil samples was 72 (36 for each depth). We also took leaf samples from the 18 white poplars, but the corresponding leaf data have not been included in this work.

2. 3. Soil and plant analyses

The soil samples were oven-dried at 40ºC, then sieved to < 2 mm for the analysis of their general properties. A fraction of each sample was then ground to < 1 mm for metal analysis.

The soil texture was determined by the hydrometer method (Gee and Bauder, 1979). The pH was determined potentiometrically in a 1:2.5 soil-water suspension. The organic matter content was analyzed according the method of Walkley and Black (1934). The cation exchange capacity was determined by the ammonium acetate method (Peech et al., 1947). To determine the total metal concentrations (Cd, Cu, Pb and Zn), one replicate of each soil was digested using concentrated $HNO_3$ and HCl (1:3 v/v, aqua regia) and analyzed by ICP-MS (Inductively Coupled Plasma Mass Spectroscopy; Perkin Elemer, Sciex-Elan 5000). The available concentrations of these elements were analyzed by extraction with $NH_4NO_3$, according to the Deutsches Institut für Normung protocol (1993), and determination by atomic absorption spectrophotometry (Perkin Elmer 1100B). In this method, the pH of the extracting solution is not adjusted; therefore, the extraction takes place under pH conditions similar to those for the native soil solution (Gryschko et al., 2005).

Three replicates per sample were analyzed.

Leaf samples were washed thoroughly with distilled water, dried at 60ºC for at least 48 h and ground. One replicate of each sample was digested using concentrated $HNO_3$ in a microwave digester (ETHOS D, Milestone, Italy), and Cd, Cu, Pb and Zn concentrations were determined by ICP-MS.

The quality of the trace element analyses was assessed by analyzing different reference materials: NCS DC 73350 (white poplar leaves, China National Analysis Center for Iron and Steel), BCR-62 (olive tree leaves, European Community Bureau of Reference) and CRM 141R (calcareous loam soil, European Community Bureau of Reference). Our experimental values showed recoveries of 81 to 105% for the plant samples and 83 to 91% for the soils samples when compared to the certified values.

2. 4. Greenhouse experimental design

Under controlled conditions in a greenhouse (University of Seville, Spain), Holm oak seedlings were exposed to different concentrations of Cd. Acorns were germinated in forestry trays of twenty 8 x 20 cm cells, each containing 550 g of pure silica sand. One hundred twenty seedlings with similar heights were selected. Then, the seedlings were exposed to four different treatments (two trays of 15 plants for each treatment, N = 30 per treatment). The control treatment trays received a nutritive solution of 1 M $KNO_3$, 1 M $Ca(NO_3)_2$, 1 M $KH_2PO_4$, 1 M $MgSO_4$ and 0.01 M Fe-EDTA (pH = 7), which was renewed weekly. The other three treatments consisted of different levels of Cd, which was provided as $CdCl_2$ added to the nutritive solution (20, 80 and 200 mg $L^{-1}$, respectively). The exposure to Cd for the 20 mg $L^{-1}$ and the 80 mg $L^{-1}$ treatments was increased gradually. During the first week, both treatments received the lowest dose (20 mg $L^{-1}$), and then the dose was increased to 80 mg $L^{-1}$ during the second week. The dose was increased again during the third week to 200 mg $L^{-1}$ for the last treatment. Plants grew in the greenhouse over a period of six months, with a photoperiod of 16 h and a maximum radiation of 1000 $\mu$mol $m^{-2}$ $s^{-1}$.

2. 5. Chlorophyll fluorescence and plant analysis

After six months, six plants per treatment were selected. The maximum photochemical efficiency of photosystem II (Fv/Fm) was calculated from the minimum (Fo) and the maximum (Fm) fluorescence values measured in three leaves per plant These measurements were taken at midday with an FM2 fluorimeter (Hansatech, Norwolk, UK) after adaptation of the leaves to darkness for at least 20 minutes. After the chlorophyll measurements, ten plants per treatment were harvested and separated into leaves, stems and roots. Morphometrical measurements of the taproot were taken (total length and diameter of four sections of the root). Each part was washed, dried and weighed separately. In a subsample of the harvested plants (five per treatment), the roots were separated into taproots and lateral fine roots (≤ 2 mm diameter), and each type of root was weighed separately. This subsample was used for chemical analysis. Fine roots, taproots and leaves were ground and digested as described above. The Cd concentration in each sample was determined by ICP-MS, and the concentrations of other elements (Ca, K, Mg, Mn, S, P) were determined by ICP-OES (Inductively Coupled Plasma Optical Emission Spectroscopy; Thermo Jarrell Iris Advantage).

2.6. Field and greenhouse data analyses

Linear regression models were used to analyze the influence of pH, organic matter content and cation exchange capacity on the availability of the trace metals. The corresponding total concentrations of metals in soils were also considered as predictors in the models. The best subset of models for each studied metal was selected as those showing the minimum value of Cp coefficients (Mallow, 1973). Log-transformations of the variables were applied previously.

Bioaccumulation coefficients (BC) were calculated using the field data. The bioaccumulation coefficient is defined as the ratio of the Cd concentration in the plant to the total Cd concentration in the soil (Adriano, 2001).

Using the greenhouse data, one way ANOVAs were used to compare the different variables among treatments and the chemical compositions among tissues (fine roots, taproots and leaves). Previously, normality and homoskedasticity of the data were checked.

The translocation coefficient (TC), which is the ratio of concentrations between different plant organs, was used to quantify the transfer of Cd within the plant. All statistical analyses were performed with STATISTICA v. 6.0. (StatSoft Inc., Tulsa, USA).

**3. Results and Discussion**

3.1. Influence of soil conditions on metal availability

The total and available metal concentrations were highly variable within and among zones, as indicated by the high standard deviations (Table 7.1). Under field conditions, Cd was the most available element, in relative terms. On average, 15% of the total soil Cd was extracted by $NH_4NO_3$. The so-extracted Cd comprises the water-soluble and easily exchangeable fraction in the soil. Mean available fractions of Zn, Cu and Pb were 5.6, 2.6 and 0.035%, respectively. In all of the zones, the $NH_4NO_3$-extracted concentrations of Cd were higher than 0.04 mg $kg^{-1}$, which is the intervention value for $NH_4NO_3$-extracted Cd in Germany (BBodSchV, 1999). The maximum concentration of available Cd was 1.86 mg $kg^{-1}$, which was found in some subsoil samples (25-40 cm depth) from Zone 3 (Table 7.1). Likewise, the available concentrations of Pb and Zn exceeded the German intervention values (0.1 and 2 mg $kg^{-1}$, respectively) in all of the zones. However, the available Cd concentrations found in this work were not much higher than the $NH_4NO_3$-extracted Cd concentrations in some unpolluted reference soils (average of 0.049 mg $kg^{-1}$, Mèers et al., 2007).

The highest total concentrations of Cd and Zn were found in the southern zone (Zone 4), while Cu and Pb showed the highest concentrations in the central areas (Zone 2 and 3). This pattern can be related to the different solubility of each metal. The more labile elements (Cd and Zn) would have been more easily transported through the river basin to Zone 4, where the soils are predominantly calcareous clay loams and had a higher capacity for metal retention than soils in Zones 2 and 3. Cu and Pb, on the other hand, would have been previously deposited upstream nearer the spill source due to their higher affinities for the soil solid phase. These results are consistent with other investigations in the study area (Cabrera et al., 2008).

The high variations in the metal availability may be due to differences in the soil properties. The lowest soil pH values were observed in Zones 2 and 3, which also had the highest sand content. In these sites, extremely acid soils (pH < 4) were observed. In general, organic matter content was moderately low in the whole area and was similar among zones (Table 7.2). Soil pH was the most important factor explaining the patterns of solubility of the studied metals. In the regression models, the β coefficient (which indicates the relative importance of each predictor in the variance of the dependent variable) for soil pH was higher than the β coefficients for the rest of the predictors (Table 7.3). Therefore, the availability of Cd, Cu and Zn could be well predicted from the soil pH values (see example for surface soils, Fig. 7.1). For Cd, the organic matter content

**Table 7.1**. Trace element concentrations (total and $NH_4NO_3$-extracted, mg $kg^{-1}$) in the studied soils. Mean ± standard deviation. N = 72 (36 for each depth).

| | | Upper soils (0-25 cm) | | | | Subsoils (25-40 cm) | | | |
|---|---|---|---|---|---|---|---|---|---|
| Zones | | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 |
| Total | Cd | 0.59 ± 0.42 | 1.3 ± 0.8 | 1.3 ± 0.9 | 2.4 ± 1.4 | 0.46 ± 0.27 | 1.1 ± 1.2 | 2.0 ± 1.8 | 2.5 ± 0.6 |
| | Cu | 74 ± 36 | 150 ± 68 | 173 ± 52 | 128 ± 51 | 70 ± 45 | 140 ± 97 | 189 ± 96 | 138 ± 23 |
| | Pb | 173 ± 131 | 268 ± 157 | 511 ± 625 | 97 ± 28 | 185 ± 130 | 244 ± 189 | 293 ± 320 | 252 ± 165 |
| | Zn | 239 ± 141 | 425 ± 236 | 440 ± 267 | 768 ± 395 | 170 ± 85 | 361 ± 325 | 555 ± 550 | 718 ± 206 |
| Available | Cd | 0.06 ± 0.03 | 0.17 ± 0.23 | 0.18 ± 0.12 | 0.08 ± 0.05 | 0.07 ± 0.03 | 0.17 ± 0.14 | 0.34 ± 0.55 | 0.12 ± 0.05 |
| | Cu | 0.29 ± 0.21 | 5.5 ± 11.3 | 10 ± 15 | 0.45 ± 0.17 | 0.91 ± 2.48 | 8.2 ± 11.7 | 4.5 ± 8.8 | 0.37 ± 0.14 |
| | Pb | 0.39 ± 0.25 | 0.37 ± 0.18 | 5.0 ± 13.7 | 0.47 ± 0.12 | 0.47 ± 0.35 | 0.90 ± 0.78 | 1.4 ± 2.2 | 0.75 ± 0.17 |
| | Zn | 4.3 ± 4.8 | 29 ± 60 | 30 ± 36 | 0.92 ± 0.98 | 2.3 ± 3.0 | 29.9 ± 42.5 | 53± 103 | 1.5 ± 2.7 |

**Table 7.2**. Soil properties in the studied zones. OM = organic matter content; CEC = cation exchange capacity. Mean ± standard deviation. N = 72 (36 for each depth)

| Depth (cm) | Variable | Zone | | | |
|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 |
| 0-25 | pH | 6.6 ± 1.1 | 6.4 ± 1.8 | 5.5 ± 2.4 | 7.7 ± 0.5 |
| | OM (g $kg^{-1}$) | 22 ± 10 | 21 ± 9 | 24 ± 8 | 19 ± 8 |
| | CEC (cmol $kg^{-1}$) | 21 ± 19 | 21 ± 15 | 17 ± 10 | 16 ± 11 |
| | Sand (%) | 43 ± 16 | 52 ± 18 | 46 ± 17 | 44 ± 9 |
| | Silt (%) | 28 ± 4 | 25 ± 11 | 32 ± 11 | 31 ± 11 |
| | Clay (%) | 29 ± 13 | 22 ± 8 | 23 ± 7 | 25 ± 8 |
| 25-40 | pH | 6.3 ± 0.8 | 5.4 ± 1.9 | 5.9 ± 2.0 | 7.3 ± 0.6 |
| | OM (g $kg^{-1}$) | 16 ± 7 | 19 ± 7 | 26 ± 19 | 21 ± 15 |
| | CEC (cmol $kg^{-1}$) | 25 ± 21 | 22 ± 15 | 20 ± 17 | 15 ± 6 |
| | Sand (%) | 45 ± 18 | 55 ± 20 | 52 ± 14 | 39 ± 10 |
| | Silt (%) | 26 ± 6 | 29 ± 16 | 28 ± 3 | 30 ± 17 |
| | Clay (%) | 29 ± 13 | 19 ± 8 | 23 ± 16 | 31 ± 14 |

and the cation exchange capacity had also significant influences on the metal availability in the surface soils (Table 7.3). Finally, Pb availability showed a non-linear relationship to soil pH, with large increases in availability in extremely acid soils (pH < 3).

The different fractions of organic carbon in the soil play an important role in metal mobility. For example, Cu and Pb form complexes with dissolved organic carbon, while Cd and Zn usually interact with the electrostatic binding sites of organic matter (McBride, 1989). In our study area, the structure of the soil in many sites was severely affected by the clean-up procedure after the accident, which removed the contaminated topsoil (10 to 20 cm depth). This could have enhanced the penetration of the remnants of sludge into deeper depths of the soils, as reflected by similar metal concentrations at 0-25 cm and at 25-40 cm depth. In general, the organic matter content of the soil was drastically reduced, so the effect of organic matter content is expected to be low. However, the afforested woody species act as sources of organic matter via litterfall. Therefore, the organic matter content in the soil may increase due to litter deposition, and this edaphic factor may have a greater impact on metal availability in the future.

The long-term effect of tree growth on metal mobility is an issue under discussion. On one hand, metal retention in the rhizosphere can reduce metal mobility, but, on the other hand, the production of organic acids by root exudation and by litter decomposition may enhance metal solubility depending on the types of acid produced (Jones and Darrah, 1994; Marschner, 1995). The afforestation of grasslands or former agricultural lands normally results in the acidification of the soil (Jobbágy and Jackson, 2003; Mertens et al., 2007), which could influence the metal solubility (Strobel et al., 2001; Andersen et al., 2004). The effect of trees on the soil properties can be very different depending on the species planted (Mertens et al., 2007). In the Guadiamar Valley, different tree species, with different foliar chemistries, were planted. In the future, it would be interesting to analyze the different footprint of each species on the soil properties. The possible soil acidification after afforestation should be monitored, since there are already some extremely acid soils in the area where metals have a higher mobility.

3.2. Accumulation of Cd and other metals in Holm oak leaves

Under field conditions, the mean leaf concentrations of Cd, Cu, Pb and Zn were 0.20, 10.8, 2.66 and 75.7 mg $kg^{-1}$, respectively. These values are within the normal ranges for higher plants (Chaney et al., 1989). The highest BC were found for Cd and Zn, with mean values around 0.30, followed by Cu

(0.14) and Pb (0.022). In the case of Cd, the maximum BC was 1.1, but for most of the trees (78%), the BC was below 0.6. Neither the leaf concentrations nor the bioaccumulation coefficients were related to the available concentration of metals in the soils (see example for Cd, Fig. 2). No correlation was neither found with soil pH, organic matter content, nor CEC. Thus, oaks growing on acidic soils did not significantly accumulate more metals than those growing on neutral or basic soils (data not shown).

**Table 7.3**. Results of the regression models, explaining the influence of the studied soil properties on metal availability. N = 72 (36 for each depth).

| Detph (cm) | Variable | Significant predictors | β coefficient, *p*-value | model $r^2$ |
|---|---|---|---|---|
| 0-25 | Cd | pH | -0.89, < 0.0001 | 0.69 |
| | | OM | -0.25, 0.028 | |
| | | CEC | 0.25, 0.023 | |
| | | $Cd_{total}$ | 0.50, < 0.0001 | |
| | Cu | pH | -0.67, < 0.0001 | 0.57 |
| | | CEC | 0.28, 0.034 | |
| | | $Cu_{total}$ | 0.38, 0.009 | |
| | Pb | pH | -0.68, < 0.0001 | 0.73 |
| | | $Pb_{total}$ | 0.27, 0.014 | |
| | Zn | pH | -0.9, 0.004 | 0.33 |
| 25-40 | Cd | pH | -0.73, < 0.0001 | 0.63 |
| | | $Cd_{total}$ | 0.58, < 0.0001 | |
| | Cu | pH | -0.58, < 0.0001 | 0.79 |
| | | $Cu_{total}$ | 0.57, < 0.0001 | |
| | Pb | $Pb_{total}$ | 0.74, < 0.0001 | 0.54 |
| | Zn | pH | -0.85, < 0.0001 | 0.67 |
| | | $Zn_{total}$ | 0.34, 0.002 | |

Some studies have shown that the concentrations of metals extracted from soil with neutral salts, in particular with ammonium nitrate, are well correlated with the metal concentrations in plant shoots or plant yield, and the use of these salts has been proposed to measure metal phytoavailability (Bhogal et al., 2003; Menzies et al., 2007). In contrast, other studies have shown that ammonium nitrate is not the best extractant for the prediction of metal uptake (Hall et al., 1998; Grønflaten and Steinnes, 2005). Plant species differ in their mechanisms of uptake and translocation of metals, and these differences may result in variable tissue concentrations, which could be much different from the potentially available metal concentrations in the soil. For example, leaves of *Populus alba* have been shown to be good indicators of the available Cd and Zn (but not Pb) levels in soil under the conditions of the Guadiamar River Valley (Madejón et al. 2004). In contrast, oak leaves do not reflect such levels, probably due to important mechanisms of avoidance of metal uptake by the roots, or to low traslocation rates to the leaves (Dickinson et al., 1991). In soils with a heterogeneous distribution of contaminants, root proliferation is promoted in the less contaminated spots (Turner and Dickinson, 1993; Menon et al., 2007). The roots of trees can also exudate organic acids, which have been suggested to prevent metal uptake (Heim et al., 1999; Ahonen-Jonnarth et al., 2000). Immobilization of the metals in the cell walls of the roots is one of the most common mechanisms of metal detoxification in trees (Kahle, 1993; Brunner et al., 2008).

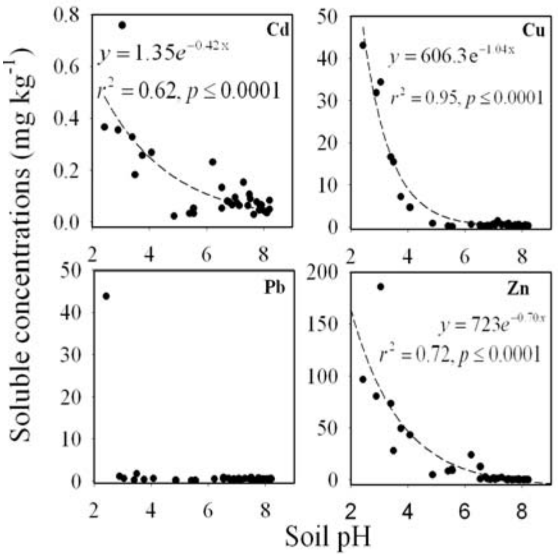


**Fig. 7.1.** Prediction of available metal concentrations from the soil pH values (0-25 cm depth). Linear regression equations and parameters are indicated. Data for Pb did not fit linear distribution.

3.3. Effects of Cd on oak seedling performance

Exposure to Cd produced a decrease in root and shoot biomass of the oak seedlings (Table 7.4). At a Cd exposure of 200 mg $L^{-1}$, the shoots of 20% of the plants became dry, and the root biomass decreased to a 30%. The taproot biomass decreased with the increasing Cd exposure. The fine root biomass was reduced at the two highest exposures to Cd, but at 20 mg $L^{-1}$ there were no significant differences compared to the control treatment. Taproot length was not different among treatments, but root thickness was affected by Cd, thus resulting in thinner taproots (same length, but smaller diameter, Table 7.4). The proportion of plant biomass contributed by the roots was slightly higher in the plants exposed to Cd, as indicated by the Root Mass Ratio (RMR).

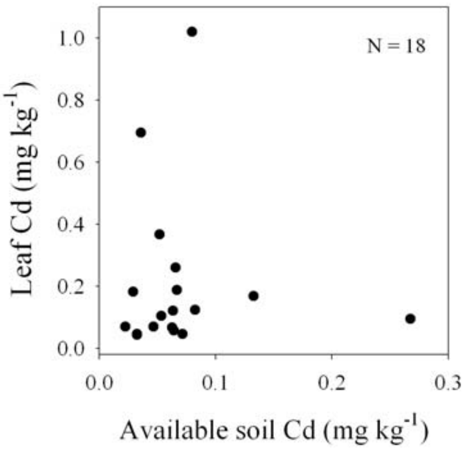


**Fig. 7.2.** Cadmium availability in soils and concentrations in Holm oak leaves.

In general, the depression of growth parameters such as root elongation, biomass production, root initiation and root hair formation are the first symptoms of heavy metal toxicity in tree species (Kahle, 1993). Different studies with woody plant seedlings exposed to Cd have reported similar or higher inhibition of root elongation (Godbold and Hütterman, 1985; Arduini et al., 1994) or inhibition of both root elongation and biomass accumulation (Kahle, 1993; Lunackova et al., 2003) at Cd doses much lower than those tested here. For Holm oak seedlings, the rapid establishment of the root system is critical for survival during the first summer periods; any factor that hampers root elongation or thickness could limit the access of the plants to the water in the deep soils during the summer, thus reducing establishment of the seedlings. At low to moderate Cd levels, the effects on root elongation are likely to be low. Other detrimental effects on the plants, such as nutritional deficiencies, may be derived from the inhibition of fine root development. However, the conditions tested in our experiment simulated extremely high contamination and maximum availability of Cd for the plants (due to the inert substrate and the soluble Cd salt used). In moderately contaminated soils, the bioavailable levels would probably be lower, especially under semi-arid conditions such as those found in the Guadiamar Valley.

The chlorophyll fluorescence data support the conclusion that the seedlings of this species have a relatively high tolerance to Cd. This variable can be a

**Table 7.4**. Root characteristics in the control and Cd-exposed seedlings, under controlled conditions (mean ± standard deviation). For each variable, significant differences among treatments are indicated by different letters ($p < 0.05$). RMR: Root Mass Ratio (root mass: total plant mass). N = 5 for Taproot and Fine root Mass; N = 10 for the rest of variables.

| Variable | Treatment | | | |
|---|---|---|---|---|
| | Control | Cd 20 mg $L^{-1}$ | Cd 80 mg $L^{-1}$ | Cd 200 mg $L^{-1}$ |
| Root Mass (g) | 4.36 ± 0.41 a | 2.83 ± 1.29 b | 0.94 ± 0.49 c | 1.29 ± 0.73 c |
| Shoot Mass (g) | 5.14 ± 1.72 a | 1.34 ± 0.32 b | 0.70 ± 0.25 b | 0.91 ± 0.41 b |
| RMR | 0.46 ± 0.07 a | 0.66 ± 0.07 b | 0.55 ± 0.12 ab | 0.57 ± 0.06 ab |
| Taproot Mass (g) | 3.75 ± 0.81 a | 1.94 ± 0.92 b | 1.04 ± 0.48 b | 1.01 ± 0.23 b |
| Fine root Mass (g) | 0.49 ± 0.26 a | 0.24 ± 0.19 ab | 0.09 ± 0.08 b | 0.11 ± 0.06 b |
| Taproot length (cm) | 38.2 ± 14.9 a | 28.5 ± 7.2 a | 31.1 ± 9.4 a | 30.3 ± 12.5 a |
| Taproot diameter (mm) | 4.94 ± 0.73 a | 3.62 ± 0.78 b | 2.17 ± 0.54 b | 2.93 ± 0.67b |

good indicator of the overall performance of the photosynthetic apparatus, and by extension, of the ability of the plants to tolerate environmental stresses (Maxwell and Johnson, 2000). In our case, Fv/Fm for the control treatment and for the exposures to 20 and 80 mg $L^{-1}$ Cd was slightly lower than the theoretical optimum (Fv/Fm = 0.83; Maxwell and Johnson, 2000) (Fig. 7.3a). At 200 mg $L^{-1}$, Fv/Fm showed a high variability because two of the six measured plants were dried, and these plants showed Fv/Fm values below 0.5.

3.4. Cadmium accumulation patterns in oak seedlings

The oak seedlings showed a high Cd retention capacity in the roots, especially in the fine roots (Fig. 7 3b). In the extremely contaminated substrate (200 mg $L^{-1}$ Cd), the fine roots accumulated up to 7 g $kg^{-1}$ of Cd. In the leaves, the lowest Cd dose (20 mg $L^{-1}$) resulted in a Cd concentration of 2.5 mg $kg^{-1}$, which is considered by some authors to be the upper limit for normal, non-phytotoxic values for higher plants (Alloway et al., 1995). For the two highest exposures, the concentrations in the leaves were well above these values (mean ± standard deviation of 28 ± 40 and 92 ± 82 mg $kg^{-1}$, respectively), and showed a high variability, as indicated by the high standard deviation. However, root-leaf TC were very low. Because of the high level of Cd accumulation in the fine roots, the maximum TC was 0.02.

The fine roots of some tree species have a special capacity for retaining Cd (Unterbrunner et al., 2007; Brunner et al., 2008). The pectins in the cell wall are the main constituents allowing metal binding due to their carboxyl groups, which have a high cation exchange capacity. Between metals, the binding preferences depend on the type of pectin, although, in general, pectins show higher affinities for $Al^{3+}$, $Cu^{2+}$ and $Pb^{2+}$ than for $Cd^{2+}$, and a higher affinity for $Cd^{2+}$ than for $Ca^{2+}$ (Franco et al., 2002). In our case, Cd was highly retained in both the fine roots and in the taproots, displacing some divalent ions such as $Ca^{2+}$ and $Mg^{2+}$. In the fine roots, Cd and Mg concentrations were negatively correlated ($r = -0.72$, $p = 0.012$). The amount of Cd in the fine roots was also negatively related to the levels of both Ca and Mg in the taproots ($r = -0.78$, $p = 0.005$, and $r = -0.61$, $p = 0.043$, respectively). Likewise, the Ca concentration in the taproot was negatively correlated with the Cd level in the leaves ($r = -0.74$, $p = 0.009$).

Under extremely high concentrations of metal in the rhizosphere, saturation of the pectins may lead to random deposition of metals in the cell wall and in the cell lumen (Brunner et al., 2008). This could explain the high variability of the Cd concentrations in the roots of the plants exposed to the highest doses of Cd.

3.5. Potential for phytostabilization of soils contaminated by Cd

For a tree species to be suitable for phytostabilization, the root system should be able to both retain and tolerate high concentrations of available metals.

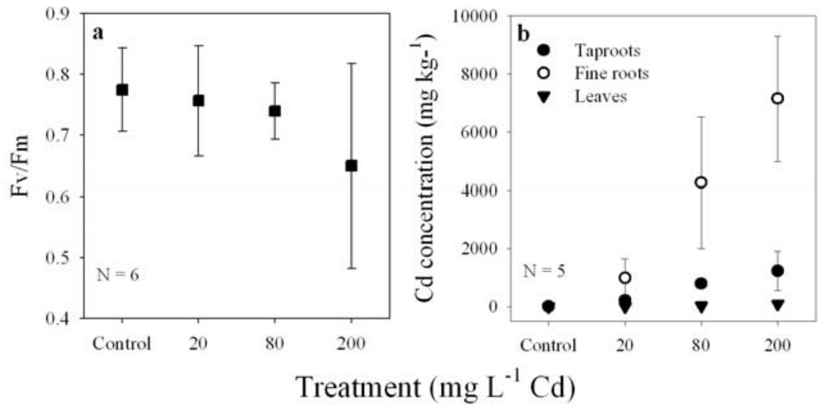


**Fig. 7.3.** Chlorophyll fluorescence (a) and Cd accumulation (b) in Holm oak seedlings exposed to different levels of Cd. Fv/Fm: maximum photochemical efficiency of the photosystem II. Mean ± standard deviation.

Under the semi-arid conditions of the study area, Cd is potentially the most available metal in the contaminated and remediated soils. The transfer of Cd to the leaves of Holm oaks growing on contaminated soil is also potentially higher than the transfer of other metals, such as Cu and Pb, although the leaves do not reflect the changes in soil availability, probably due to the high Cd retention capacity in the roots. The accumulation patterns of this species (high root retention and very low root-shoot translocation) make it very suitable for phytostabilization (Mendez and Maier, 2008). Root morphology and biomass were altered by exposure to Cd, although the seedlings showed a relatively high tolerance to the extremely high Cd accumulation in the roots and in the leaves, as observed under controlled conditions. Due to the vast root system of the Holm oak, this species may have a high potential to stabilize Cd in low and moderately polluted soils.

However, the overall resistance to simultaneous environmental stresses in the field must be also considered in afforestation programs proposing to use the Holm oak. At low and moderate contamination levels, such as the found here under the field conditions, the effect of Cd alone on plant performance is likely to be small. In our study area, the survival of this species during the first several years after afforestation was much lower than the survival of other sclerophyllous tree species, such as *Olea europaea*, due to a higher sensitivity to summer stress (Domínguez et al., 2009). The suitability of this species for phytostabilization would be enhanced if some techniques leading to an increase in summer stress resistance were considered.

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**Foto 7.1.** Juvenil de encina (*Quercus ilex* subsp. *ballota*), creciendo en una de las zonas reforestadas del Corredor Verde.

**Foto 7.2.** Ensayos con plántulas de encina, en condiciones de invernadero.


**Foto 7.3.** Realización de medidas morfológicas en las raíces de las plántulas.

# Capítulo 8

# Capítulo 8. Respuesta de las plántulas de encina a altas concentraciones de Cd y Tl en la rizosfera

Este capítulo reproduce el siguiente manuscrito:

**Domínguez, M.T., Marañón, T., Murillo, J.M. Response of Holm oak seedlings to high concentrations of Cd and Tl in the rhizosphere** (en preparación).


## Resumen

Los elementos traza pueden causar diferentes efectos negativos en las plántulas de especies de leñosas, de presentarse en altas concentraciones en la rizosfera. La inhibición del crecimiento de la raíz y la disminución de las tasas de fotosíntesis son los principales impactos de estos elementos en las plantas, los cuales pueden afectar negativamente a su crecimiento y a su establecimiento en suelos contaminados. Estos efectos dependen de la naturaleza del elemento, su concentración, y de la especie vegetal. Potencialmente, aquellos elementos altamente transferibles desde las raíces a las hojas (como Cadmio y Talio) pueden producir un mayor impacto en la fotosíntesis que aquellos que son mayoritariamente retenidos en el sistema radical. En este trabajo, estudiamos la respuesta de las plántulas de encina (*Quercus ilex* subsp. *ballota*) a altas concentraciones de Cd y Tl en la rizosfera. En condiciones de invernadero, las plántulas fueron sometidas a distintas concentraciones de Cd (20, 80 and 200 mg $L^{-1}$) y Tl (2, 10 and 20 mg $L^{-1}$) en la solución nutritiva durante 140 días. Tras 50 y 140 días de tratamiento estudiamos la morfología de raíces y vástagos, así como los patrones de alocación de biomasa y las tasas de crecimiento de las plantas. Hacia el final del ensayo realizamos medidas fisiológicas (de fluorescencia de clorofilas e intercambio gaseoso), y determinamos las concentraciones de clorofila foliar y de Cd y Tl en distintos órganos. El Cd produjo un incremento en la proporción de masa radical y una disminución del área foliar específica tras 50 días de crecimiento, respuesta que no fue observada para el Tl. La biomasa radical disminuyó con la exposición a ambos metales, aunque la longitud de la raíz principal no fue especialmente afectada. Hacia el final del ensayo el Tl provocó efectos severos en la plántulas; el tratamiento de 20 mg $L^{-1}$ registró un 30% de supervivencia. Las medidas de fluorescencia mostraron que el Cd no afectó a la eficiencia máxima del fotosistema II (FSII), mientras que el Tl provocó efectos severos tanto en los complejos antena como en los centros de reacción del FSII. Ambos elementos produjeron la disminución de las tasas de asimilación neta (hasta niveles del 20 % de las plantas control) y la conductancia estomática (hasta un 5-10 % del control). El Cadmio fue ampliamente retenido en las raíces finas (las máximas concentraciones alcanzaron los 7.2 g $kg^{-1}$), mientras que el Tl fue transportado en mayor medida a las hojas, donde se acumuló hasta niveles de 200 mg $kg^{-1}$ En general, las plántulas de encina muestran una mayor tolerancia al Cd que al Tl, debido a la alta capacidad de retención de Cd a nivel radical, aunque tales acumulaciones de Cd en la raíz podrían provocar el estrés hídrico de las plantas.

# Response of Holm oak seedlings to high concentrations of Cd and Tl in the rhizosphere

María T. Domínguez, Teodoro Marañón, José M. Murillo

Instituto de Recursos Naturales y Agrobiología, CSIC, P. O. Box 1052, E-41080, Seville, Spain

**Abstract**

High concentrations of trace elements may cause several detrimental effects in the performance of woody plants seedling, mainly by the impairment of root growth and photosynthetical functioning. These effects can have important consequences for their growth and establishment in contaminated sites. However, these effects are very species-specific; also the same plant species can present different degree of tolerance for different trace elements. Those elements with a high transfer from roots to shoots (such as Cadmium and Thallium) may produce a higher impact on the different processes involved in photosynthesis, than those that are mostly retained in roots. In this work, we assessed the response of Holm oak seedlings (*Quercus ilex* subsp. *ballota*) to high concentrations of Cd and Tl in the rhizosphere, under controlled conditions. A greenhouse experiment was carried out, in which seedlings were exposed to different levels of Cd (20, 80 and 200 mg $L^{-1}$) and Tl (2, 10 and 20 mg $L^{-1}$) in a growth solution during 140 days. Root and shoot morphology, biomass allocation and growth rates were studied at 50 and 140 days after the first exposure to the trace elements. Physiological variables (chlorophyll fluorescence and gas exchange), chlorophyll concentrations and Cd and Tl accumulation in different plant organs were studied after 140 days of treatment. Cadmium produced an increase in root mass ratio and a decrease in the specific leaf area of the plants after 50 growing days, while Tl did not provoked such response. Root biomass was affected by both metals, although taproot length was not specially affected. Relative growth rates were reduced by Cd and Tl, and after 140 days the plants exposed to Tl showed severe symptoms of toxicity; plants growing at 20 mg $L^{-1}$ of Tl had a 30% of survival. Cadmium did not affect the maximum efficiency of photosystem II (PSII), while Tl provoked severe damages in both antennae complexes and reaction centers of the PSII. Both elements decreased net assimilation rates (down to a 20 % of the control plants) and leaf stomatal conductance (5-10% of the values for the control plants). The patterns of Cd and Tl accumulation were very different; Cd was highly retained in fine roots (concentrations up to 7.2 g $kg^{-1}$), while Tl was highly translocated into the leaves, where it was accumulated up to 203 mg $kg^{-1}$. In general, Holm oak seedlings showed a higher tolerance for Cd than for Tl, due to a high capacity for Cd retention at the root level, although such accumulation in roots may induce water stress in the seedling exposed to Cd.

Keywords: cadmium; thallium; *Quercus ilex*; RGR; chlorophyll fluorescence; photosynthesis.

## 1. Introduction

The bioavailability of soil trace elements has increased in the last century due to the intensification of human activities (Nriagu and Pacyna, 1988; Nriagu, 1996). Some of these trace elements, such as Cu, Mn or Zn, are essential to plants, while some others, such as Cd, Tl or Pb, are non-essential; in both cases, if the uptake of the element exceeds the tolerance threshold of the plant, a toxic response will take place.

The toxic response depends on the nature and concentration of the trace element and on the physiology of plant species. Potentially, those elements which can be easily transported into the aboveground biomass would have stronger effects on the photosynthetic apparatus, decreasing assimilation rates. However, toxic response at the root level may also influence indirectly the photosynthetic rates, for example by producing a water stress status that leads to stomatal closure (Poschenrieder and Barceló, 1999).

Cadmium and Thallium are two non-essential trace elements, which are highly mobile in the soil-plant system. Although their distribution within the plant organs depends on the plant species, in general they can be easily transported from roots to leaves (Adriano, 2001). There, they may have severe effects on the different components of photosynthesis.

Cadmium is a widespread metal, released into the environment by power stations, heating systems, metal-working industries, mining activities, urban traffic and as by-product of phosphate fertilizers (Nriagu and Pacyna, 1988). In the plants, Cd can have multiple effects on the molecular, subcellular and cellular levels (reviews in Påhlsson, 1989; Sanità di Topi and Gabbrielli, 1999). Cadmium interacts with the plant water balance (Barceló and Poschenrieder, 1990; Costa and Morel, 1994), inhibits stomatal opening (Perfus-Barbeoch et al., 2002), provokes damages to the photosystems I and II (Krupa and Moniak, 1988; Küpper et al., 2007) and inhibits some of the enzimes of the Calvin cycle (Van Assche and Clijsters, 1990).

Thallium is a less abundant contaminant, although it is highly toxic for plants and animals (Heim et al., 2002). Antropogenic entries to the soils are mainly due to farming, mining, manufacturing processes, combustion of coal, and cement production (Nriagu, 1998). Very few works have analyzed the effects of Tl on photosynthesis (Bazzaz et al., 1974; Carlson et al., 1975; Gupta and Singhal, 1996a, b); some of these works indicate that this element has stronger effects on net assimilation rates and stomatal conductance than other trace elements such as Cd, Ni and Pb, although the underlying mechanisms remain unclear (Bazzaz et al., 1974; Carlson et al., 1975).

Plants have developed some strategies to resist the toxicity of trace elements (Prasad and Hagemeyer, 1999; Cobbet and Goldbrough, 2002). Most of these strategies consist of the prevention of the accumulation of these elements in the aboveground tissues, by root retention, or active efflux pumps of trace elements in the roots (Cuypers et al., 2002). Tolerant plants presenting these strategies are commonly called "excluders" (Baker, 1981). Other tolerant plants can uptake and translocate them into the shoots, where they are stored in a harmless state, e.g., by compartmentalization and complexation in the vacuole (Sanità di Topi and Gabrielli, 1999). Both types of strategies can pose an energetic cost for the detoxification of the trace elements; thus, there is a certain cost of tolerance with negative consequences for some plant processes such as growth or reproduction (Baker, 1987; Cox, 1988a, b; Hagemeyer, 1999).

In most of tree species, the tolerance to trace elements appears to be more related to plastic phenotypic responses than to specific physiological mechanisms with a genetic basis (Dickinson et al., 1991; Turner and Dickinson, 1993). However, different tree species can differs in their tolerant response to trace elements, and thus long-term changes in the abundance and relative composition of tree species can occur in contaminated forest sites (Chernenkova y Kuperman, 1999; Salemaa et al., 2001). Also, the response of a same tree species to different trace elements can be very variable (Godbold and Hüttermann, 1985; Hartley-Whitaker et al., 2000).

In the last decade, the revegetation with woody plants has emerged as a feasible strategy for the management trace element contaminated sites (Dickinson, 2000; Pulford and Dickinson, 2006). In these sites, the use of tree species that tolerate soil contaminants should be promoted. If the goal of the revegetation program is the reduction of the mobility of soil trace elements, a high capacity for the retention of these elements in the belowground biomass is also desirable. In Southern Spain, Holm oak (*Quercus ilex* subsp. *ballota* Desf. Samp) is frequently used in the afforestation of degraded sites, such as the Guadiamar Green Corridor. This area was affected by a mine spill in 1998, which contaminated soils with several trace elements, including Cd and Tl. A large-scale restoration project was launched the following years. The survival of tree species was very variable, showing Holm oak seedlings lower survival than other sclerophyllous species such as wild olive tree (*Olea europaea* var. *sylvestris*).

In this work, we assessed the response of Holm oak seedlings (*Q. ilex*) to different concentrations of Cd and Tl in the rhizosphere. We aimed to analyze the morphological and physiological changes induced by the exposure to these metals, under conditions of extremely high availability of these trace elements for a prolonged period of time (more than four months). Specifically, we studied root and shoot morphology, biomass allocation, chlorophyll content, and assimilation and relative growth rates. We combined chlorophyll fluorescence and gas exchange measurements to elucidate the effect of these elements within the photosynthetic apparatus.

## 2. Material and Methods

### 2.1. Experimental design

Acorns were collected from different trees and sites in the Guadiamar basin (Sevilla, SW Spain) during the fall 2005. Healthy acorns were selected, using the floating method to discard those infected by moth or beetle larvae (Gribko and Jones, 1995). The acorns were stored on a moist substrate in the dark at 0-4 º C until their use (Vázquez Pardo, 1998). In December, acorns were sown in forestry trays of twenty 8 x 20 cm cells, each containing 550 g of pure silica sand, and settled in a greenhouse (University of Seville). Three acorns per cell were initially sown. Previously, acorns with similar size were selected and disinfected by immersion during 10 minutes in 2% sodium hypochlorite. Trays were watered 2-3 times per week, until February 2006; then a sample of emerged seedling with similar size (N = 210) was selected for the experiment. In the selected cells, only one germinated acorn was kept, and the other two were removed.

The seedlings were exposed to seven different treatments (two trays of 15 plants for each treatment, N = 30 per treatment). The control treatment trays received a nutritive solution of 1 M $KNO_3$, 1 M $Ca(NO_3)_2$, 1 M $KH_2PO_4$, 1 M $MgSO_4$ and 0.01 M Fe-EDTA, which was renewed weekly. The other six treatments consisted of different levels of Cd or Tl, which were provided as $CdCl_2$ or TlCl added to the nutritive solution. The tested doses were 20, 80 and 200 mg $L^{-1}$ for Cd, and 2, 10 and 20 mg $L^{-1}$ for Tl. The exposure to Cd or Tl was increased gradually for the highest treatments. During the first week, these treatments received the lowest dose (20 mg $L^{-1}$ of Cd and 2 mg $L^{-1}$ of Tl, respectively), and then the dose was increased to 80 mg $L^{-1}$ of Cd and 10 mg $L^{-1}$ of Tl during the second week. The dose was increased again during the third week to 200 mg $L^{-1}$ of Cd and 20 mg $L^{-1}$ of Tl, respectively.

Plants grew in the greenhouse under the mentioned treatments over 140 days (until July 2006), with a photoperiod of 16 h and a maximum radiation of 1000 μmol m-2 $s^{-1}$. Temperature conditions were 17.07 ± 0.14 º C in winter (min. 7.1º, max. 22.5º) and 19.02 ± 0.08 º C in spring (min. 14.8º, max. 29.6º). Air relative humidity ranged from 40 to 60 %.

### 2.2. Morphometrical measurements and relative growth rates

Over the experiment, three plant harvests were conducted: at the beginning of the experiment before the exposure of the plants to Cd or Tl ($t_0$), at 50 days from the first harvest ($t_1$) and at 140 days from the first harvest ($t_2$). At each time, ten plants per treatment were collected. Plants were separated into roots, stems and leaves. Morphometric measurements of the taproots and the stems were done (total length and diameter of four sections). Leaf area was measured by image analysis of the scanned leaves (Image-Pro Plus 4.5. Media Cybernetic Inc. USA). Each part was dried at 60 ºC during at least 48 h and weighed. For each plant, the following variables were calculated: M (total plant mass, g), RMR (Root Mass Ratio, root mass : total plant mass), SMR (Stem Mass Ratio, stem mass : total plant mass), LMR (Leaf Mass Ratio, leaf mass : total plant mass), LAR (Leaf Area Ratio, leaf area : total plant mass, $m^2\ kg^{-1}$), and SLA (Specific Leaf Area, leaf area : leaf mass, $m^2\ kg^{-1}$). Relative Growth Rates (RGR) were calculated according to Hunt (1982):

$$RGR = (\ln M_t - \ln M_{t-1}) : T\ (g\ g^{-1}\ day^{-1})$$

where M = mean plant mass of each treatment at different harvests and T = days between harvests.

2.3. Chlorophyll fluorescence measurements

Chlorophyll fluorescence measurements were taken at the end of the experiment, before the last harvest, in a sample of six plants per treatment. Measurements were taken at midday, using a portable modulated fluorimeter (FMS-2, Hansatech Instruments, Ltd., UK). For each plant, three fully expanded leaves were selected. These leaves were dark-adapted for at least 20 min, using leaf clips. The minimal fluorescence value in the dark (F0) was recorded. Maximum fluorescence in the dark (Fm) was measured after applying a saturating actinic light pulse of 15000 $\mu mol\ m^{-2}\ s^{-1}$ for 0.7 s. Then F0 and Fm were used to calculated variable fluorescence (Fv = Fm- F0) and maximum efficiency of photosystem II (Fv/Fm). Dark-adapted values of Fv/Fm can be used to quantify photoinhibition, since this parameter is related to the number of functional reaction centers in the photosystem II (Maxwell and Johnson, 2000). The same leaf sections were used to measure light-adapted parameters, after adapting plants to ambient light for 30 min. Steady-state fluorescence values (Fs) were recorded. Then, the same saturating actinic light pulse was applied, which temporally inhibited the photosystem II photochemistry, and maximum fluorescence (Fm') was recorded. Fluorescence parameters in the light-adapted state were used to calculate the quantum efficiency of the photosystem II (ΦpsII) : ΦpsII = (Fm'-Fs): Fm'.

2.4. Gas Exchange

Gas exchange measurements were taken at the end of the experiment, before the last harvest, in a sample of five plants per treatment. Measurements were taken in three fully expanded leaves per plant at midday in sunny days, using a portable, open-system infrared gas analyzer LCi (ADC BioScientific, Hertfordshire, UK). A 3 m height pole was connected to the $CO_2$ supply system, to keep the reference concentration of $CO_2$ constant (395 ± 1 ppm). Photosynthethic Active Radiation (PAR) was 840 ± 8 $\mu mol\ m^{-2}s^{-1}$, which can be considered as a saturating radiation for *Q. ilex*, under similar light and water availability conditions in a greenhouse (Quero et al., 2006). Leaf temperature was 31.5 ± 0.2 ºC, and atmospheric pressure was 1014 mBar. Three instantaneous measurements in each leaf were taken. Photosynthetic area in the leaf chamber was corrected by marking the leaf outline in an acetate surface and scanning and analyzing the acetate surfaces as described above for leaf area. Net photosynthetic rate (A, $\mu mol\ CO_2\ m^{-2}\ s^{-1}$), stomatal conductance (gs, mmol $H_2O\ m^{-2}\ s^{-2}$), sub-stomatal $CO_2$ (Ci, ppm) and transpiration rate (E, mmol $H_20\ m^{-2}\ s^{-1}$) were recorded. Water Use Efficiency (WUE) was calculated as the ratio between A and E.

2.5. Chlorophyll concentrations

Before the last harvest, the chlorophyll content of the leaves was analyzed, in a sample of four-five plants per treatment. In each plant, three to five leaves were selected and 2 disks of 5 mm diameter were taken per leaf. Chlorophyll was extracted with N,N-dimethylformamide, in dark conditions at 2-4 ºC during 48 h. Chlorophyll a and b were calculated from the absorbance measurements at 647 and 664 nm, according to Moran (1982).

2.6. Cadmium and Tl accumulation

The concentrations of Cd and Tl in the leaves and roots of the plants were analyzed after the morphometrical measurements in the last harvest. Five plants per treatment were selected. In the case of roots, fine roots (diameter < 2mm) were separated, and fine roots and taproots were analyzed separately. Plant material was ground using a stain-less mill and digested using concentrated $HNO_3$ in a microwave digester (ETHOS D, Milestone, Italy). Cadmium and Tl concentrations were determined by ICP-MS (Inductively Coupled Plasma Mass Spectroscopy; Perkin Elmer, Sciex-Elan 5000). For each element, the translocation coefficients (TC) from roots to leaves were calculated as the ratio between the concentrations in leaves and roots: TC = Cleaf : Croot (Adriano, 2001).

2.7. Data analyses

For each element separately, differences in the studied variables among treatments were analyzed by factorial analyses. For the morphometrical variables, one way ANOVAs and Tukey post-hoc tests were used; previously, data were log-transformed to meet normality. The physiological variables (fluorescence and gas exchange parameters), as well as the chlorophyll and metal concentrations showed a high heterogeneity in the variance among treatments. Therefore, non parametric tests (Kruskal-Wallis and Mann-Whitney U test) were used to assess the differences among treatments.

Relationships between the gas exchange parameters (average values per leaves) were explored by bivariate homogeneity-of-slopes regression models. These models were used to assess if the relationships between pairs of physiological variables (continuous variables) were constant across the different treatments (categorical factor). If the models showed that the treatment factor significantly influenced those relationships, correlation parameters were calculated separately for each treatment.

Linear and exponential correlations were used to explore the relationships between the structural and the physiological studied variables, with the average values of each treatment. Weighted regressions were used for those variables that showed heteroscedasticity among treatments. In those cases, the inverse of the variance was used as weighting variable.

All these analyses were performed with Statistica 6.0 (Statsoft Inc., Tulsa, USA).

## 3. Results

3.1. Growth and biomass allocation

The exposure to Cd and Tl produced a detrimental effect on the growth of the oak seedlings, although the morphological and physiological changes were different depending on the time of exposure and on the element. In general, Tl provoked more severe effects than Cd at prolonged times of exposition. By the end of the experiment, changes in the pigmentation of the leaves and signs of water stress could be observed for up to 87 % of the plants exposed to Tl. At 20 mg $L^{-1}$ of Tl, 70 % of the plants showed dried shoots (Table 8.1). By this time, at the highest exposure to Cd, 20 % of the plants showed water stress and leaf senescence.

After 50 growing days, very few morphological changes were observed in the plants. Root lenght was not affected, and just slight decreases in the thickness of the stems were observed in some of the treatments (Table 8.2). After 140 growing days, root length remained unaffected, but root diameter was decreased both by Cd and Tl. The stems showed the most important morphological changes; in general plants exposed to Cd or Tl had shorter and thinner stems than the control plants.

Cd provoked changes in the investment in shoot biomass at 50 growing days. Total biomass of the plants treated with Cd was not significantly different than those in the control treatment, but the plants showed a decrease in the production of leaves under the influence of Cd. Therefore, there was an important decrease in the leaf area, LMR, LAR and SLA, and an increase in the RMR (Table 8.3). Thallium did not induce such changes in the biomass allocation in the plants. After 140 growing days, root biomass was highly reduced by Cd and Tl. Leaf area decreased in

**Table 8.1**. Percentage of plants with dried shoots at 50 ($t_1$) and 140 ($t_2$) days from the beginning of the treatments.

| Treatment | Dried Plants (%) | |
|---|---|---|
| | $t_1$ | $t_2$ |
| *Control* | 0 | 0 |
| *Cd* | | |
| 20 mg $L^{-1}$ | 0 | 0 |
| 80 mg $L^{-1}$ | 0 | 0 |
| 200 mg $L^{-1}$ | 0 | 20 |
| *Tl* | | |
| 2 mg $L^{-1}$ | 0 | 0 |
| 10 mg $L^{-1}$ | 0 | 27 |
| 20 mg $L^{-1}$ | 0 | 70 |

all the Cd treatments, and in the two highest treatments of Tl.

The evolution of the RGRs illustrates this time-dependent effect of Tl. For the first period (from $t_0$ to $t_1$), the RGRs of the plants exposed to Tl (15-20 mg $g^{-1}$ $day^{-1}$) were even higher than those of the control plants, while the RGRs of the plants exposed to Cd were lower (Fig. 8.1). During the second period (from $t_1$ to $t_2$) a generalized decrease in the RGRs was observed, both for Cd and Tl. The plants at 20 mg $L^{-1}$ of Tl showed around 80% lower RGR than the control plants. The plants at 200 mg $L^{-1}$ of Cd had negative growth rates, due to loss of some of the leaves during this period, which were not accounted for the measurement of final plant biomass.

3.2. Chlorophyll concentrations

Chlorophyll total content (a + b) was severely reduced in the highest exposures of Cd and Tl. At the lowest doses of Cd and Tl, the total content was only slightly lower than 3 mg $g^{-1}$ (mean value for the control treatment, data not shown). Chlorophyll a was more influenced by Cd and Tl than chlorophyll b, and showed a decreasing pattern with increasing exposures to metals (Fig. 8.2).

3.3. Chlorophyll fluorescence

Plants exposed to Cd and Tl showed different patterns of fluorescence emission (Fig. 8.3). For Cd, F0 did not decrease, but increased at 80 mg $L^{-1}$. Variable fluorescence in the dark-adapted state (Fv) slightly decreased at 200 mg $L^{-1}$ of Cd. On average, the maximum photochemical efficiency of photosystem II (Fv/Fm) did not decrease significantly with the exposure to Cd, although the plants showed a higher variability in this parameter than the control plants. The quantum efficiency of the photosystem II decreased at 80 and 200 mg $L^{-1}$, but not at 20 mg $L^{-1}$.

In contrast, F0 and Fv decreased with the exposure to Tl. Maximum photochemical efficiency was highly reduced; at 20 mg $L^{-1}$ of Tl, the average value of Fv/Fm was lower than 0.4. Quantum efficiency also decreased significantly at 20 mg $L^{-1}$ of Tl.

3.4. Gas exchange

Assimilation rates (A) were highly reduced by the exposure to Cd, down to a 20% of the rates of the control plants (Fig. 8.4). The plants showed important reductions in stomatal conductance: control plants had average gs values of 190 mmol $m^{-2}$ $s^{-1}$, while all the plants exposed to Cd had gs values lower than 40 mmol $m^{-2}$ $s^{-1}$. Water use efficiency increased at the lowest dose of Cd 20 mg $L^{-1}$, in relation to the control treatment.

Thallium provoked a similar decrease in assimilation rates (A) than Cd (Fig. 8.4). Reductions in stomatal conductance were significant for 10 and 20 mg $L^{-1}$. In contrast, water use efficiency (WUE) was reduced (not increased as for Cd) at the lowest dose of Tl (2 mg $L^{-1}$).

In the control plants A was positively correlated with gs and Ci (Fig. 8.5). Likewise, stomatal conductance and Ci were positively correlated in the control plants (not shown). Some of these patterns of correlations changed in the plants exposed to Cd or Tl. In the plants exposed to Tl, the linear correlation between gs and A persited and was constant across all the Tl-treatment (homogeneity of slopes-test, gs x treatment $p > 0.05$); however the slope of this relationship in Tl-plants was different than that in control plants (Fig. 8.5). Sub-stomatal $CO_2$ (Ci) was related neither to gs nor to A. In the Cd-treated plants, A and gs were neither correlated. In these plants, a treatment-dependent and negative relationship between A and Ci was observed; at 80 and 200

**Table 8.2.** Morphometrical measurements (mean ± standard error) of the roots and the stems of the seedlings, at 50 ($t_1$) and 140 ($t_2$) days from the beginning of the treatments. For each variable and element, values followed by the same letters did not show significant differences (Tukey test, $p < 0.05$).

| | Treatment | Taproot | | Stem | |
|---|---|---|---|---|---|
| | | Length (cm) | Thickness (mm) | Length (cm) | Thickness (mm) |
| $t_1$ | | | | | |
| | *Control* | 27 ± 3 a | 2.8 ± 0.2 a | 22 ± 2 a | 2.1 ± 0.1 a |
| | *Cd* | | | | |
| | 20 mg L$^{-1}$ | 30 ± 3a | 2.9 ± 0.2 a | 18 ± 1 b | 1.8 ± 0.1 a |
| | 80 mg L$^{-1}$ | 30 ± 2 a | 2.7 ± 0.2 a | 16 ± 1 a | 1.5 ± 0.1 b |
| | 200 mg L$^{-1}$ | 31 ± 3 a | 2.4 ± 0.2 a | 16 ± 1 b | 1.4 ± 0.1 b |
| | *Tl* | | | | |
| | 2 mg L$^{-1}$ | 31 ± 3 a | 3.0 ± 0.1 a | 20 ± 1 a | 1.9 ± 0.1 a |
| | 10 mg L$^{-1}$ | 34 ± 4 a | 2.8 ± 0.3 a | 18 ± 1 a | 1.7 ± 0.1 b |
| | 20 mg L$^{-1}$ | 30 ± 2 a | 2.7 ± 0.2 a | 16 ± 1 a | 1.7 ± 0.1 b |
| $t_2$ | | | | | |
| | *Control* | 38 ± 5 a | 4.9 ± 0.2 a | 38 ± 5 a | 3.2 ± 0.2 a |
| | *Cd* | | | | |
| | 20 mg L$^{-1}$ | 28 ± 2 a | 3.6 ± 0.2 b | 14 ± 1 b | 2.2 ± 0.1 b |
| | 80 mg L$^{-1}$ | 31 ± 3 a | 2.1 ± 0.2 b | 13 ± 1 b | 1.5 ± 0.1 c |
| | 200 mg L$^{-1}$ | 30 ± 4 a | 2.9 ± 0.2 b | 12 ± 1 b | 1.6 ± 0.1 c |
| | *Tl* | | | | |
| | 2 mg L$^{-1}$ | 33 ± 3 a | 3.9 ± 0.4 a | 29 ± 2 a | 2.6 ± 0.1 b |
| | 10 mg L$^{-1}$ | 31 ± 4 a | 3.3 ± 0.3 b | 18 ± 1 b | 2.0 ± 0.1 c |
| | 20 mg L$^{-1}$ | 27 ± 1 a | 3.5 ± 0.2 b | 17 ± 2 b | 1.8 ± 0.1 c |

mg L$^{-1}$ of Cd this correlation existed, while at 20 mg L$^{-1}$ it was not significant (Fig. 8.5).

3.5. Relationships between morphological and physiological variables

Considering the average values for each treatment, some significant relationships between structural and physiological variables were observed. Relative growth rate (RGR) was influenced by assimilation rate (A) and stomatal conductance (gs) (Fig. 8.6). These two variables (A and gs) were related to the total leaf area of the plants. Assimilation rate was highly correlated with the chlorophyll a content (Chla), by an exponential function. Water use efficiency (WUE) and root allocation (RMR) were positively correlated. For the rest of the variables, there were no significant correlations.

3.6. Cadmium and Tl accumulation patterns

Cadmium and Tl differed in their patterns of allocation in the different plant organs. Both elements showed increasing concentrations in roots with increasing concentrations in the substrate, but the distribution between different types of roots was different for each element. Cadmium was mostly retained in fine roots. At 200 mg L$^{-1}$ of Cd, fine roots accumulated up to 7.2 ± 1.7 g kg$^{-1}$, while taproots had 1.2 ± 0.2 g kg$^{-1}$1 of Cd (Fig. 8.7). In constrast, the concentrations of Tl in fine and taproots showed a similar pattern, and even the taproots showed higher concentrations than fine roots at the lower dose (2 mg L$^{-1}$). Maximum concentrations reached up to 0.7 g kg$^{-1}$ in both types of roots of the plants exposed to 20 mg L$^{-1}$.

The translocation of these elements to the leaves was also different. Again, Cd was highly retained in the root system. Leaf Cd concentrations at 20, 80 and 200 mg L$^{-1}$ were, on average, 2.5, 28 and 92 mg kg$^{-1}$, respectively. Traslocation coefficients from roots to leaves were low, with a maximum value of 0.08 (Table 8.4). In contrast, Tl was much more transport-

**Table 8.3**. Main structural variables of the seedlings (mean ± standard error) at 50 ($t_1$) and 140 ($t_2$) days from the beginning of the treatments. For each variable and element, values followed by the same letters did not show significant differences (Tukey test, $p < 0.05$). M: mass; RMR: Root Mass Ratio; SMR: Stem Mass Ratio; LMR: Leaf Mass Ratio; LA: Leaf Area; LAR: Leaf Area Ratio; SLA: Specific Leaf Area.

| | Treatment | $M_{total}$ (g) | $M_{root}$ (g) | $M_{shoot}$ (g) | RMR | SMR | LMR | LA ($cm^2$) | LAR ($m^2$ $kg^{-1}$) | SLA ($m^2$ $kg^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|
| $t_1$ | | | | | | | | | | |
| | ***Control*** | 2.65 ± 0.18 a | 1.10 ± 0.12 a | 1.51 ± 0.13 a | 0.42 ± 0.04 a | 0.142 ± 0.009 a | 0.46 ± 0.03 a | 116.9 ± 11.8 a | 4.43 ± 0.36 a | 10.13 ± 0.27 a |
| | ***Cd*** | | | | | | | | | |
| | 20 mg $L^{-1}$ | 2.08 ± 0.20 b | 1.14 ± 0.14 a | 0.93 ± 0.09 b | 0.54 ± 0.03 b | 0.132 ± 0.009 a | 0.32 ± 0.02 b | 64.0 ± 10.5 b | 3.14 ± 0.27 b | 9.64 ± 0.24 b |
| | 80 mg $L^{-1}$ | 1.98 ± 0.22 b | 0.97 ± 0.10 a | 1.01 ± 0.14 b | 0.50 ± 0.02 a | 0.137 ± 0.005 a | 0.36 ± 0.02 b | 69.6 ± 10.5 b | 3.44 ± 0.23 b | 9.36 ± 0.24 b |
| | 200 mg $L^{-1}$ | 2.01 ± 0.24 b | 1.08 ± 0.15 a | 0.93 ± 0.10 b | 0.52 ± 0.03 a | 0.134 ± 0.007 a | 0.35 ± 0.02 b | 61.1 ± 6.3 b | 3.25 ± 0.24 b | 9.32 ± 0.26 b |
| | ***Tl*** | | | | | | | | | |
| | 2 mg $L^{-1}$ | 2.73 ± 0.27 a | 1.26 ± 0.13 a | 1.47 ± 0.16 a | 0.46 ± 0.02 a | 0.131 ± 0.006 a | 0.41 ± 0.02 a | 110.2 ± 12.3 a | 4.05 ± 0.21 a | 9.88 ± 0.28 a |
| | 10 mg $L^{-1}$ | 2.42 ± 0.28 a | 1.17 ± 0.16 a | 1.25 ± 0.16 a | 0.48 ± 0.03 a | 0.115 ± 0.007 a | 0.41 ± 0.03 a | 93.1 ± 11.8 a | 3.94 ± 0.28 a | 9.63 ± 0.24 a |
| | 20 mg $L^{-1}$ | 2.55 ± 0.23 a | 1.17 ± 0.15 a | 1.33 ± 0.12 a | 0.45 ± 0.03 a | 0.131 ± 0.022 a | 0.43 ± 0.02 a | 97.0 ± 7.4 a | 3.87 ± 0.15 a | 9.26 ± 0.24 a |
| $t_2$ | | | | | | | | | | |
| | ***Control*** | 9.50 ± 0.89 a | 4.36 ± 0.18 a | 5.14 ± 0.77 a | 0.46 ± 0.03 a | 0.235 ± 0.029 a | 0.30 ± 0.01 a | 203.6 ± 22.0 a | 2.13 ± 0.10 ab | 7.25 ± 0.18 a |
| | ***Cd*** | | | | | | | | | |
| | 20 mg $L^{-1}$ | 4.18 ± 0.64 b | 2.83 ± 0.53 b | 1.34 ± 0.13 b | 0.66 ± 0.03 b | 0.099 ± 0.019 b | 0.24 ± 0.02 a | 67.4 ± 4.5 b | 1.74 ± 0.18 a | 7.28 ± 0.25 a |
| | 80 mg $L^{-1}$ | 1.64 ± 0.20 c | 0.94 ± 0.15 c | 0.70 ± 0.08 b | 0.55 ± 0.04 ab | 0.120 ± 0.015 b | 0.33 ± 0.03 a | 43.3 ± 5.6 b | 2.63 ± 0.25 b | 8.34 ± 0.20 b |
| | 200 mg $L^{-1}$ | 2.20 ± 0.50 bc | 1.29 ± 0.32 c | 0.91 ± 0.18 b | 0.57 ± 0.02 ab | 0.116 ± 0.017 b | 0.31 ± 0.01 a | 55.1 ± 10.4 b | 2.57 ± 0.11 ab | 8.29 ± 0.26 b |
| | ***Tl*** | | | | | | | | | |
| | 2 mg $L^{-1}$ | 6.72 ± 2.31 b | 2.80 ± 0.94 ab | 3.19 ± 0.32 b | 0.39 ± 0.12 a | 0.167 ± 0.015 a | 0.39 ± 0.06 a | 221.8 ± 35.9 a | 3.53 ± 0.68 a | 8.08 ± 0.33 a |
| | 10 mg $L^{-1}$ | 3.47 ± 0.40 b | 1.50 ± 0.30 b | 1.82 ± 0.28 b | 0.49 ± 0.08 a | 0.193 ± 0.035 a | 0.32 ± 0.05 a | 86.2 ± 13.7 b | 2.53 ± 0.45 a | 7.93 ± 0.57 a |
| | 20 mg $L^{-1}$ | 3.19 ± 0.49 b | 1.91 ± 0.25 b | 1.27 ± 0.37 b | 0.55 ± 0.13 a | 0.147 ± 0.025 a | 0.23 ± 0.05 a | 52.6 ± 20.0 b | 1.52 ± 0.38 a | 6.40 ± 0.70 a |

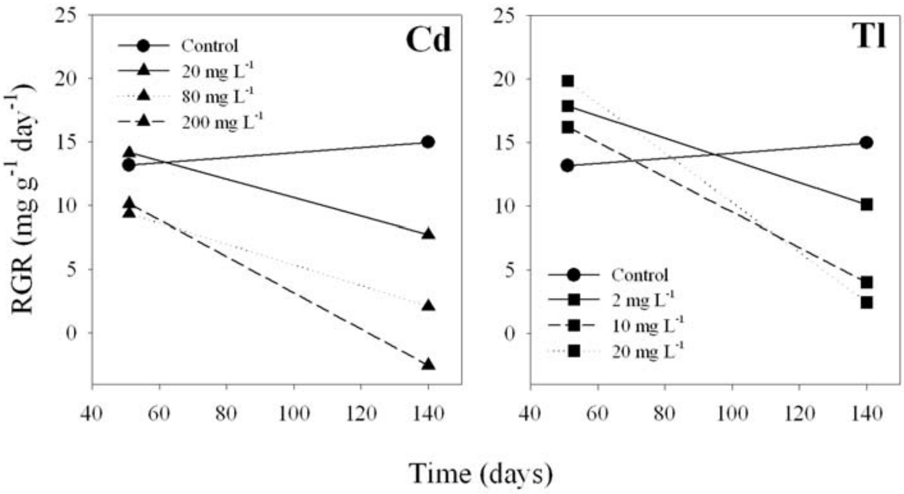


**Fig. 8.1**. Relative growth rates (RGR) of the oak seedlings under different exposures to Cd (left) and Tl (right).

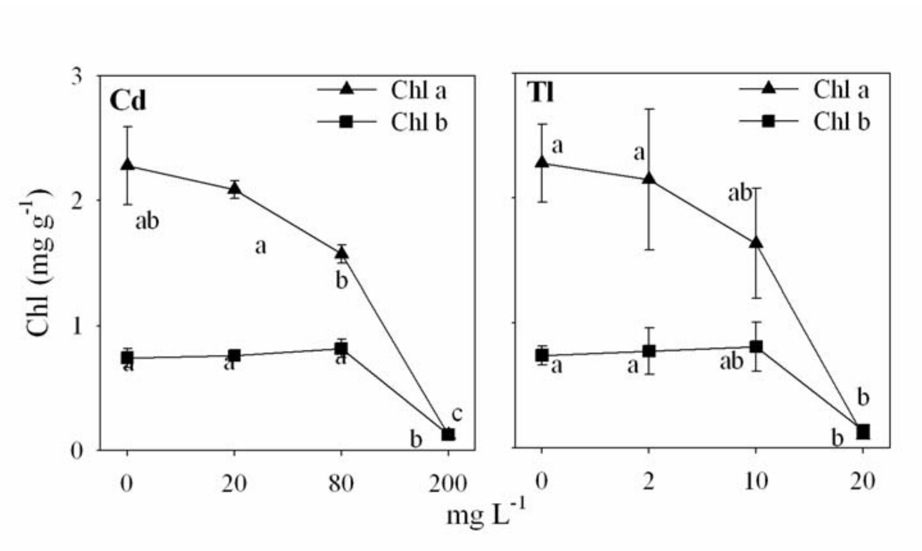


**Fig. 8.2**. Chlorophyll concentrations (mean ± SE) in the leaves of oak seedlings. For each type of chlorophyll, values with the same letter did not show significant differences (Mann-Whitney-U test; $p < 0.05$).

ed into the leaves than Cd. Leaf concentrations at 2, 10 and 20 and mg $L^{-1}$ of Tl in the substrate were 66, 116 and 203 mg $kg^{-1}$, respectively. Translocation coefficients were much higher than for Cd: minimum TC was 0.1 and maximum TC was close to 1, considering the fine roots concentrations of the plants growing at 2 mg $L^{-1}$ of Tl.

## 4. Discussion

4.1. Effects of Cd and Tl on biomass allocation and growth rates

Non-essential trace elements may interact with multiple physiological processes resulting in alterations in the seedling growth rates and the biomass alloca-

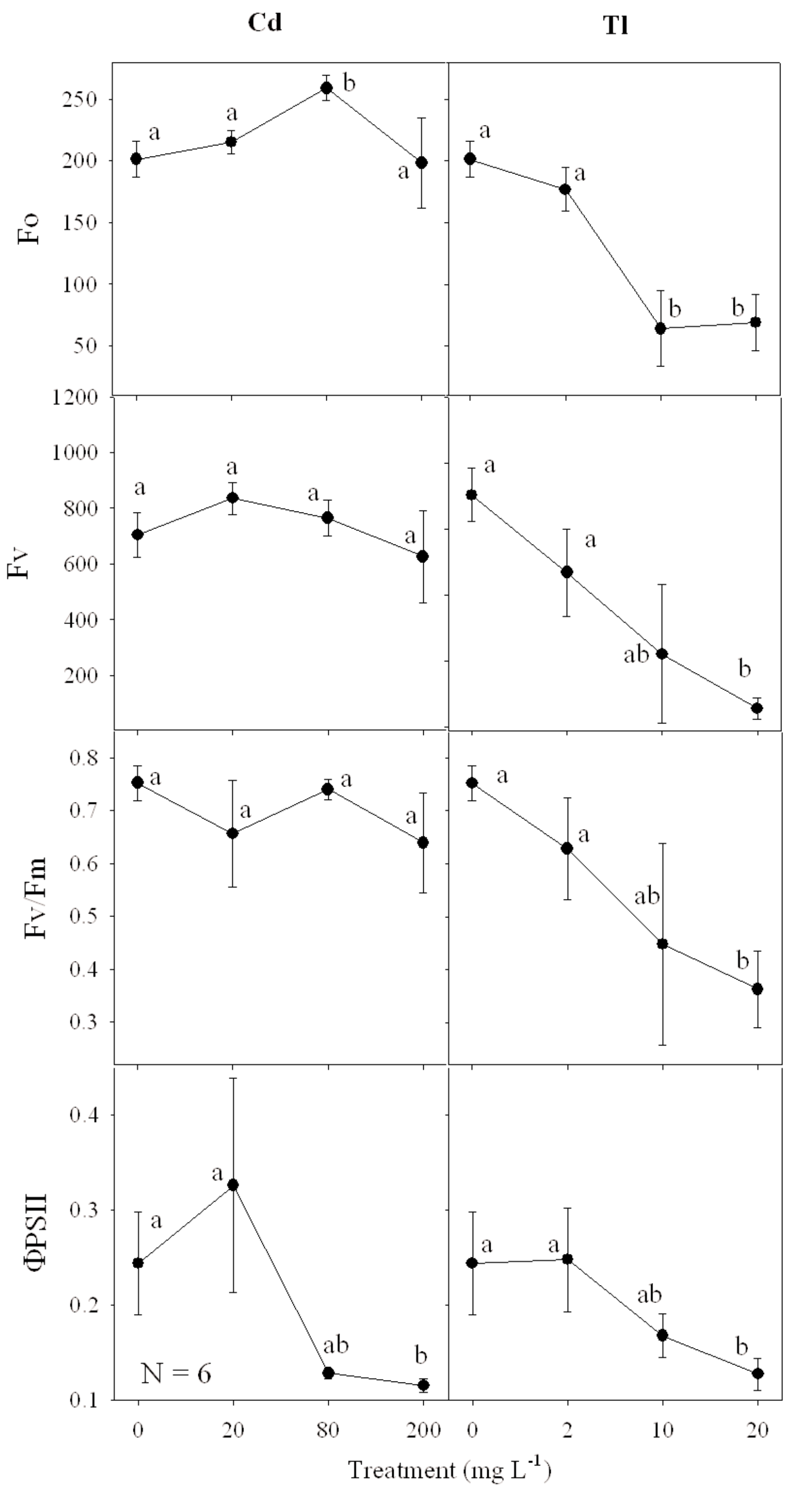


**Fig. 8.3**. Effects of cadmium (left column) and thallium (right column) on chlorophyll fluorescence parameters (mean ± SE) of oak seedlings: minimal fluorescence (Fo), maximum efficiency of PSII (Fv/Fm) and quantum efficiency of PSII (ΦPSII). For each variable, values with the same letters did not show significant differences (Mann-Whitney-U test, $p < 0.05$)

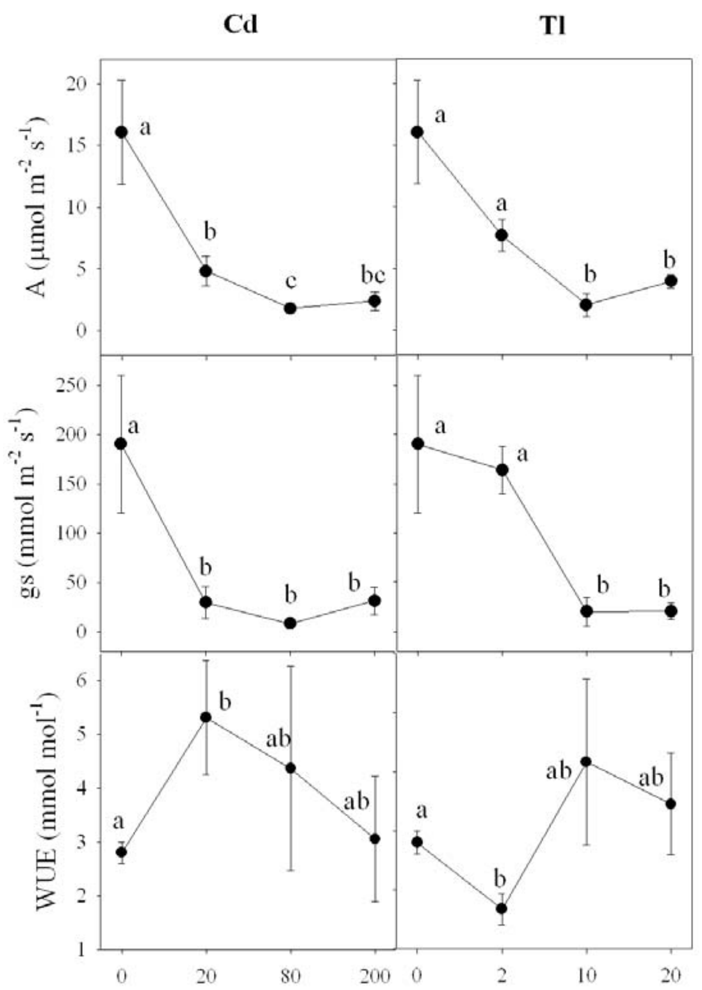


**Fig. 8.4**. Effects of Cd (left column) and Tl (right column) treatments on gas exchange parameters (mean ± SE) of oak seedlings: photosynthetic rate per area (A), stomatal conductance per area (gs) and water use efficiency (WUE). For each variable, values with the same letters did not show significant differences (Mann-Whitney-U test, $p < 0.05$).

tion patterns. These effects usually depend on the nature and concentration of the element, and on the plant species. In this work, we have assessed the eco-physiological response of Holm oak seedlings to two non-essential trace elements, with a potential high mobility in the soil-plant system. We have found that, despite both elements negatively affected plant growth, the underlying mechanisms of interaction were different, at the morphological and the physio-logical levels. In general, the decrease in growth was higher at longer exposure to the metals, and Tl provoked more severe effects than Cd.

Root system alteration is considered as the first sign of toxicity to high concentrations of trace elements in the soil (Kahle, 1993), and in particular to Cd (Sanità di Toppi and Gabbrielli., 1999; Chen et al., 2003). Different studies with woody plant seedlings have shown that root elongation rates (Arduini et al., 1994; Hartley et al., 1999, Reichman et al., 2001), or root biomass (Lunackova et al., 2003a; Wisniewski and Dickinson, 2003; Fuentes et al., 2007) decrease with increasing trace element concentrations in the growing medium. In this study, taproot length was not especially affected. During the first growing period (50 days of exposure to the metals) neither taproot measures (length or thickness), nor total root

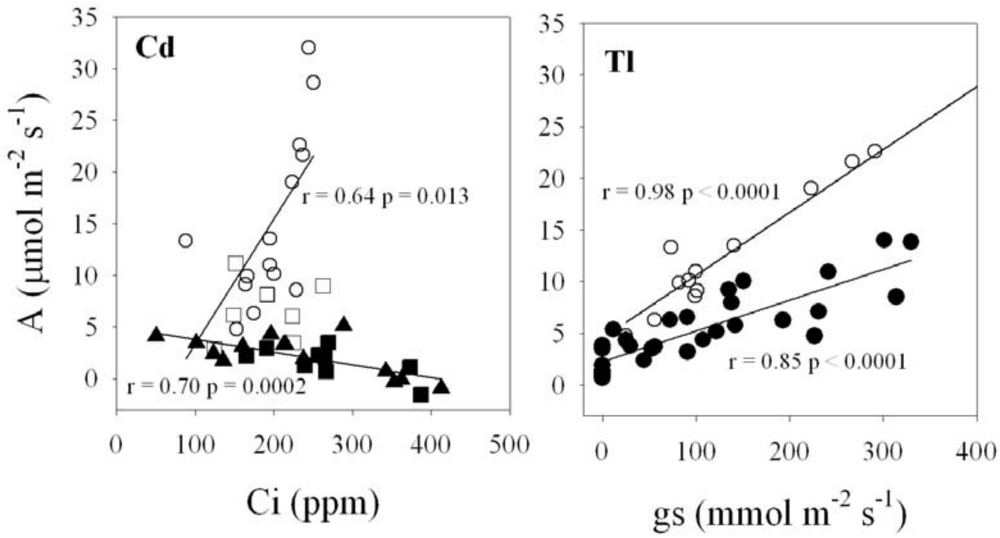


**Fig. 8.4**. Relationships between assimilation rates (A), stomatal conductance (gs) and sub-stomatal $CO_2$ (Ci) in the control plants (○) and in the plants exposed to Cd (different symbols for each treatment) and Tl (●). For Cd-plants, the correlation coefficient and line correspond to the 80 (■) and 200 (▲) mg $L^{-1}$ treatments. For the 20 mg $L^{-1}$ treatment (□) there was no significant relation between Ci and A.

biomass were significantly reduced by Cd or Tl. During the early stage of growth oak seedlings are very dependent of the maternal seed reserve and in consequence relatively independent of environmental conditions (Quero et al. 2007).

At the end of the second growing period (140 days), taproot thickness and biomass decreased, especially in the plants exposed to Cd, but length was not altered. Elongation of fine lateral roots may be more sensitive than taproot elongation (Gussarsson, 1994; Greger, 2004). Thus, if morphometrical measurements of fine roots had been taken, possibly the effect of Cd and Tl on the root system would have been detected earlier. Some studies indicate that stem elongation can be more sensitive to Cd exposure than root elongation (Aidid and Okamoto, 1992; Gussarsson et al., 1996). We have found that shoot biomass was more severely reduced by Cd than root biomass, resulting in an increase in the RMR of the Cd-exposed plants, while Tl did not provoke changes in the patterns of biomass allocation. The increase in the root:shoot ratio in the presence of Cd has been been previously reported for other tree species (Kahle, 1993; Österås et al., 2000).

This increase in the RMR and other structural changes, such as the increase in the SLA and the decrease in the LA in the Cd-treated plants, could be the related to a possible water stress status induced by Cd. Under low water availability, increased root mass fraction and decreased SLA are common responses of evergreen Mediterranean oak seedlings (Valladares et al., 2008). In this study, the Cd and, to a lesser extent, the Tl exposure, produced a reduction of gs, and gs was positively correlated to LA. This relationship has been previously observed in Holm oak seedlings, in relation to drough tolerance (Leiva and Fernández-Ales, 1998). Water use efficiency was also positively correlated with RMR. Metal toxicity can affect plant water relations at multiple levels, by impairing the water transport into root cells and through roots, or by altering stomatal conductance (review in Poschenrieder and Barceló, 1999). The reduced production of aboveground biomass may be also related to a higher demand for resources in the roots for the detoxification of the accumulated Cd. An increase in the root respiration rates is usually associated to the tolerance of plant roots to Cd

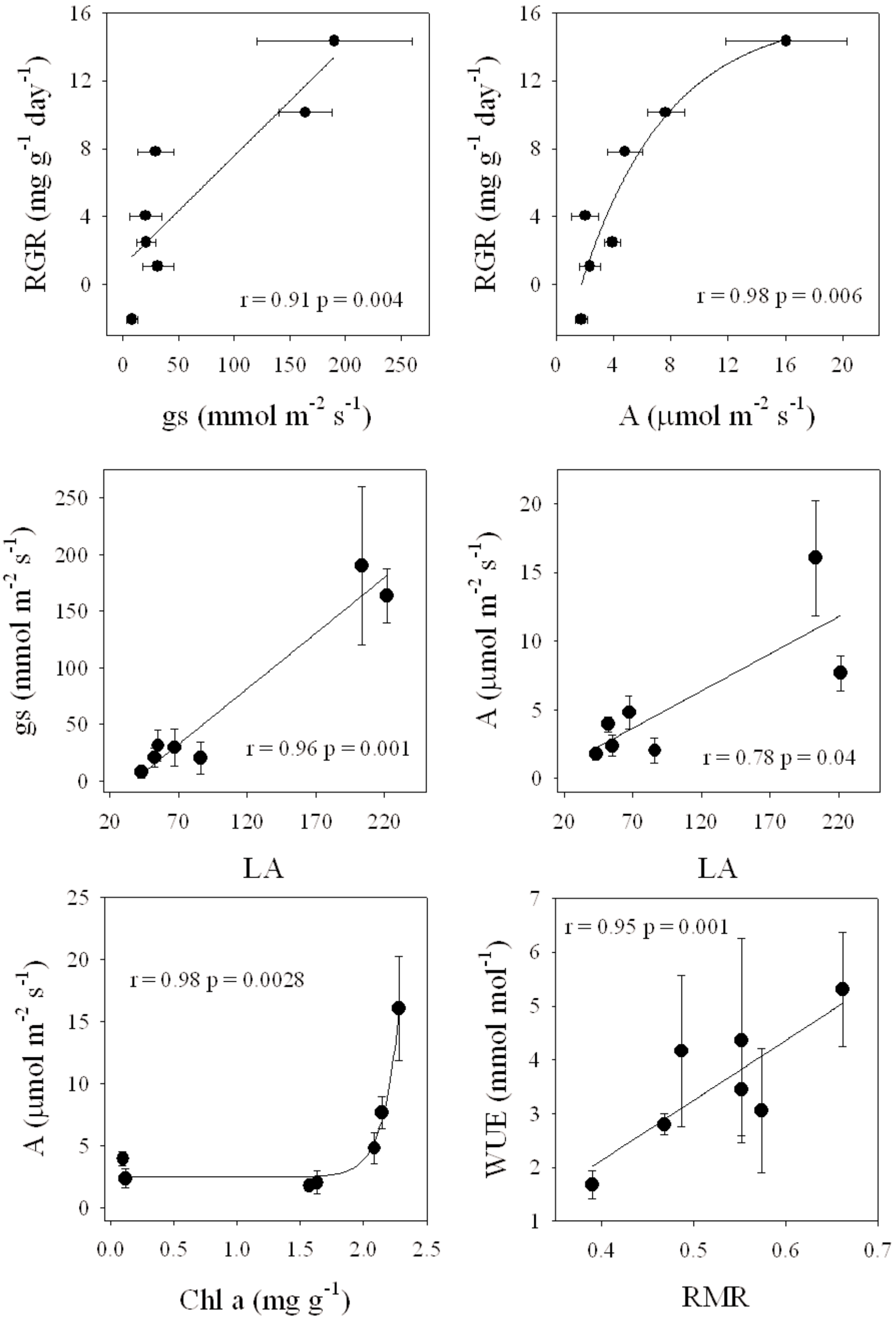


**Fig. 8.5**. Significant relationships between physiological and morphological variables for the combination of the two types of treatment. A: Assimilation Rate; Chl a: Chlorophylla concentration; gs: stomatal conductance; LA: Leaf Area; RGR: Relative Growth Rate; RMR: Root Mass Ratio; WUE: Water Use Efficiency.

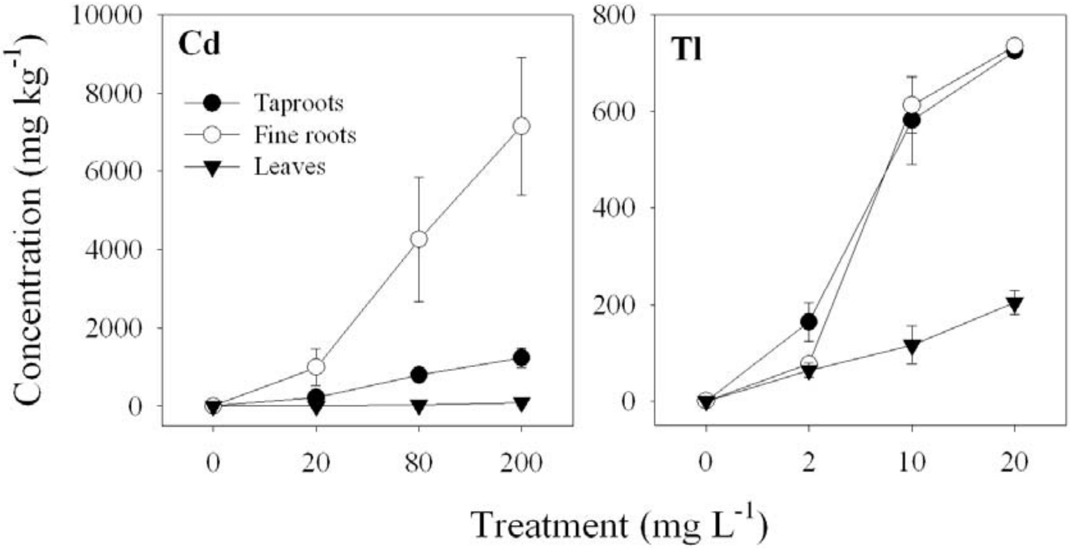


**Fig. 8. 7**. Concentration of Cd (left) and Tl (right) in different parts of the seedlings (mean ± SE).

**Table 8.4**. Translocation coefficients (TC) of Cd or Tl to the leaves, calculated from the concentrations in the fine or in the taproots. Mean ± standar error**.**

| Treatment | Fine roots-leaves TC | Taproots-leaves TC |
|---|---|---|
| *Cd* | | |
| 20 mg $l^{-1}$ | 0.003 ± 0.002 | 0.009 ± 0.003 |
| 80 mg $l^{-1}$ | 0.02 ± 0.02 | 0.06 ± 0.05 |
| 200 mg $l^{-1}$ | 0.02 ± 0.01 | 0.08 ± 0.04 |
| *Tl* | | |
| 2 mg $l^{-1}$ | 1.04 ± 0.07 | 0.54 ± 0.14 |
| 10 mg $l^{-1}$ | 0.10 ± 0.06 | 0.29 ± 0.15 |
| 20 mg $l^{-1}$ | 0.24 ± 0.02 | 0.26 ± 0.15 |

and other trace elements (Lösch and Köhl, 1999; Lunackova et al., 2003b).

Although gas exchange variables were more highly related to RGR than morphological variables, the morphological changes may also contribute to the reduced growth rates of the plants exposed to Cd. A high proportion of leaf mass favours high growth rates, while a high root mass proportion negatively correlates with RGR (Cornelissen et al., 1996; Reich et al., 1998; Antúnez et al., 2001). This could explain why during the first growing period the plants exposed to Cd showed alterations in biomass allocation and a reduced RGR, in contrast to Tl-plants, which even had higher RGR than control plants.

4.2. Effects on photosynthesis

Both elements reduced net assimilation rates, although they showed contrasted mechanisms of interactions with the different components of the photosyntethic apparatus.

The reduction of the Chlorophyll content was similar for Cd and Tl. However, the effects on the photosystem II functioning were very different, as revealed by the chlorophyll fluorescence measurements.

For Cd-treated plants, the fluorescence parameters in the dark state were similar to the control plants. Although a higher variability in the Fv/Fm was observed at 200 mg $L^{-1}$, in all the treatments the Fv/Fm was slightly below 0.83, which is the theoretical optimum (Maxwel and Johnson, 2000). F0 increased at 80 mg $L^{-1}$, reflecting a reduction in the transfer of energy from the collecting antennae of the PSII to the reaction centers, which is a common response to metal-stress in plants (Mateos-Naranjo et al., 2008a, b). In contrast, Tl reduced Fv/Fm values, down to 0.4 in the plants exposed to 20 mg $L^{-1}$, which indicates severe damage in the PSII. Both F0 and Fv were reduced in Tl-treated plants, although the decrease in Fv/Fm was due to a higher decrease in Fv. The decrease in F0 could be due to structural alterations in the antenna complexes. Under metal stress the substitution of Mg-ions by metal ions in the chlorophyll of the light harvesting complexes (LHCII) of the PSII can take place (Küpper et al., 1996, 2002). Substitution of Mg occurs preferentially under low irradiance and dark conditions (shade reaction), while under high irradiance conditions direct damage to the reaction centers are more probable (sun reaction). The metal-Chlorophylls have different wavelength of fluorescence emission than Mg-Chlorophyll, or do not emit fluorescence (Küpper et al., 1996, 2002). By Mg substitution in the LHCII, the functionality of PSII can be much reduced. The strong reduction in the Fv in the dark state also suggests direct damages in the reaction center. Thus, plants exposed to Tl had reduced quantum efficiency under light conditions (ΦPSII).

Although the PSII in Cd-treated plants was relatively unaltered, these plants also showed reduced values of ΦPSII. This could be due to alterations in the carbon assimilation, producing a down-regulation of the photochemical conversion of the energy. The low carbon fixation could be due to a decrease in the internal $CO_2$ concentrations by the low stomatal conductance (Perfus-Barbeoch et al., 2002; Linger et al., 2005), or due to alterations in some of the enzymes involved in the carbon metabolism (Krupa and Moniak, 1998; Burzynski and Klobus, 2004). In the plants growing under extremely high Cd concentrations (80 and 200 mg $L^{-1}$), assimilation rates and sub-stomatal $CO_2$ (Ci) were negatively correlated. Thus, $CO_2$ limitation is not likely to be the main reason for the reduced assimilation rates. The increase in Ci could be due to the impairement of carbon assimilation in the dark phase of photosynthesis, as has been reported for many plant species under Cd stress (Krupa et al., 1998; Burzynski and Klobus, 2004). In contrast, such negative relationship between A and Ci was not observed for Tl-treated plants, for which a highest correlation between A and gs was observed.

The reduction of stomatal conductance was an important effect of both Cd and Tl. In the case of Cd, the low conductance can be provoked directly by interferences in the movement of $K^+$ and $Ca^{2+}$ in the guard cells (Barceló et al., 1986; Perfus-Barbeoch et al., 2002), or indirectly by the toxic effects at the root level, which produce water stress in the plant. In particular, Cd can decrease the hydraulic conductivity of the root cells and obstruct the xylematic transport, which activate the mechanisms of stomatal closure (Poschenrieder and Barceló, 1999). Thallium appears to interfere in the $K^+$ movement between guard and subsidiary cells (Pallaghy, 1972).

4.2. Cadmiun and Tl accumulation patterns

The different physiological responses of oak seedlings to Cd versus Tl can be related to the different patterns of translocation of these elements to the shoots. Cadmium was highly retained in fine roots, and scarcely translocated into the leaves, while Tl was highly transported to leaves. The high capacity of Holm oak roots for retaining Cd had already been described for aqueous solution by Prasad and Freitas (2000), who reported that root material can filter up to 60 % of the Cd in the solution. Such Cd accumulation could provoke toxic effects, leading to water stress, as exposed above. The threshold of phytotoxic concentrations of a given metal can be very variable depending on the plant species. For instance, concentrations of 42 mg $kg^{-1}$ of Cd in roots of *Cannabis sativa* reduced total plant biomass to 50% (Linger et al., 2005), while the same reduction was observed in *Arabidopsis thaliana* at root concentrations of 100 mg $kg^{-1}$ Cd (Perfus-Barbeoch et al., 2002); concentrations higher than 4000 mg $kg^{-1}$ Cd in the roots of different *Salix* and *Populus* species reduced dry mass in a 36%, but they did not produce changes in the assimilation rates or chlorophyll content (Lunackova et al., 2003a).

For Tl, there is very scarce information about phytotoxic levels at the roots. At the leaf level, in all the treatments the concentrations were well above 20 mg $g^{-1}$, which is considered as the lower limit of the phytotoxic range for Tl (Kabata-Pendias and Pendias, 2001). The translocation coefficients were much higher for Tl than for Cd; considering the concentrations in the fine roots, maximum TC of Cd was 0.02, while for Tl this maximum TC was around 1, in the plants exposed to 2 mg $L^{-1}$. Thus, the possible mechanisms of trace element retention in roots are much more effective for Cd than for Tl. Cadmium could be retained by the pectins of the cell walls, (Zornoza et al., 2002; Vázquez et al., 2006) where $Cd^{2+}$ could displace some other divalent cations, such as $Ca^{2+}$ and $Mg^{2+}$ (Brünner et al., 2008; Domínguez et al., 2009a). In the plants exposed to Tl, the symptoms of damages in the photosynthetic components suggest that there are no effective mechanisms of detoxification of Tl in the leaves of the oaks, at the tested doses.

4.3. Tolerance of Holm oak seedlings to Cd and Tl

Both elements, Cd and Tl, affected negatively growth rates and photosynthesis, but Tl had a stronger effect on the performance of the seedlings. There are evidences that Holm oak seedlings are much more resistant to Cd than to Tl. Firstly, the plants exposed to Cd showed the typical structural changes that favours drought tolerance in Holm oak seedlings, as a possibly plastic reponse to the water stress status provoked by Cd accumulation in the roots. This response was not observed for Tl, despite the stomatal conductance also showed important reductions in the Tl plants. Secondly, PSII in the Cd-treated plants was practically unaltered, while in Tl-treated plants severe damages to the PSII were observed. Finally, Cd was highly retained in fine roots, with a very restricted transport to the leaves, while Tl was highly transported to the leaves provoking the mentioned damages, and posing a higher risk of transfer for the food chain. For Cd, there could be a trade-off between growth and resistance from short-time expositions to the metal, which was not observed for Tl. The exclusion of metal transport (for instances, by cell-wall binding at the root levels) and the accumulation of metals in detoxified forms are two basic strategies of metal tolerance (Baker, 1981). Therefore, we can say that Holm oak has a relatively good tolerance to Cd, given the extremely high concentrations tested in this work.

The possible implications of the metal stress for the overall resistance to simultaneous environmental stresses in the field should be taken into account. In particular, the effects on plant water relations are of important concern. In the polluted soils of the Guadiamar River basin, the survival of this species during the first several years after afforestation was much lower than the survival of other sclerophyllous tree species, such as *Olea europaea*, due to a higher sensitivity to summer stress (Domínguez et al., 2009b). However the conditions tested here are of extremely high contamination, with a high bioavailibilty of trace elements, which is not likely to occur under field conditions. The high capacity of Cd retention in roots by oak seedlings is an interesting feature for their use in the phytostabilization of polluted soils.

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**Foto 8.1.** Separación de raíces, tallos y hojas de las plántulas de encina, para su análisis de crecimiento.

**Foto 8.2.** Detalle de una de las medidas de fluorescencia (medidas de luz) en una de las plántulas tratadas con Tl.


**Foto 8.3.** Realización de medidas de intercambio gaseoso.

**Foto 8.4.** Detalle de los síntomas visuales de toxicidad en las hojas de plantas expuestas a Tl.

# Capítulo 9

# Discusión

# Capítulo 9

## Discusión General

La contaminación del suelo es un aspecto muy importante a considerar en la restauración de zonas degradadas. En primer lugar, la contaminación puede influir negativamente en el establecimiento de la cubierta vegetal, por los posibles efectos negativos sobre el crecimiento y establecimiento del sistema radical, la asimilación de nutrientes o la reproducción. En segundo lugar, en las zonas contaminadas existe la necesidad de controlar los flujos de contaminantes, que pueden ser movilizados y translocados desde el suelo mediante erosión, lixiviación, y absorción y acumulación por las plantas. En el caso de los elementos traza (ET), la reforestación de zonas contaminadas con especies leñosas es una práctica en auge, que se plantea como alternativa técnica y económicamente más viable que otras prácticas que incluyen el tratamiento mecánico o químico del suelo (Dickinson, 2000; Pulford y Dickinson, 2006), con beneficios ecológicos adicionales. En esta Tesis hemos considerado distintos aspectos de la dinámica de ET en el sistema suelo-planta, preferentemente de especies leñosas mediterráneas, haciendo énfasis en los aspectos más relevantes para la restauración y gestión de zonas degradadas.

La reforestación puede tener una amplia influencia en los flujos de ET, dependiendo de los patrones de acumulación de las especies seleccionadas. Si el objetivo es la extracción de los ET del suelo, deben seleccionarse especies que acumulen estos elementos en partes cosechables de la planta, favoreciendo el flujo de los mismos a través del sistema suelo-planta. Si por el contrario, el objetivo es la estabilización de la contaminación en el suelo, este flujo de ET debe minimizarse, seleccionando plantas con una baja translocación de los mismos desde las raíces a las partes aéreas.

Las especies leñosas parecen ofrecer más ventajas para la estabilización que para la extracción de los ET del suelo (Pulford y Watson, 2003; Mertens et al., 2004; van Nevel et al., 2007). En la práctica, bajo condiciones reales de campo, la extracción de ET mediante plantas no parece ser totalmente viable, al requerir tiempos de extracción excesivamente largos, durante los que se generan ciertos riesgos derivados de la movilización de los ET del suelo por las plantas (Audet y Charest, 2007; van Nevel et al., 2007).

En el **Capítulo 4** hemos estudiado los patrones de acumulación de ET en la biomasa aérea de la comunidad de especies leñosas del Corredor Verde del Guadiamar, a lo largo de un gradiente de contaminación y sobre un rango amplio de propiedades del suelo. Los suelos, a pesar de las labores de retirada de lodos, siguen contaminados por ET, principalmente As, Cd, Cu, Pb, Tl y Zn. Aunque las concentraciones totales son mayores en las capas superficiales (0-25 cm) que en las subsuperficiales (25-40 cm), se observó, en general, una penetración de los ET en el suelo hasta los 40 cm de profundidad. A pesar de las altas concentraciones de ET en el suelo, hemos encontrado que, de manera general, la transferencia de ET a las hojas de las especies leñosas mediterráneas es baja, y está escasamente influenciada por los factores edáficos. La excepción es el álamo blanco (*Populus alba*), que puede acumular Cd y Zn en las hojas a concentraciones superiores a las presentes en el suelo, como indican los coeficientes de transferencia suelo-planta, mayores que 1 (Fig. 4.3.), frente al intervalo de coeficientes de 0.35-0.13 obtenido para estos dos elementos en las otras dos especies arbóreas predominantes *(Q. ilex* y *O. europea*). La capacidad de acumular Cd y Zn es una característica de las especies de *Populus* y otras salicáceas. Así, especies de los géneros *Populus y Salix* son actualmente objeto de múltiples estudios para optimizar su uso en la fitoextracción de Cd y Zn de suelos contaminados (Kòmarek et al., 2008; Jensen et al., 2009; Krpata et al., 2009). Sin embargo, este patrón de acumulación de ET no parece conveniente en el contexto de una zona extensa como el Corredor Verde del Guadiamar. En primer lugar, la movilización de Cd y Zn del suelo hasta las hojas puede suponer un mayor riesgo de transferencia de estos elementos a los herbívoros y a otros componentes de la cadena trófica. En segundo lugar, el desfronde puede provocar el transporte de Cd y Zn a zonas adyacentes, así como la acumulación de estos elementos en los horizontes superficiales del suelo donde, debido a la producción de ácidos orgánicos durante la descomposición de la hojarasca, pueden presentarse en formas altamente biodisponibles (Perronet et al., 2000; Mertens et al., 2007). Algunos estudios muestran que, a medio plazo, la plantación de especies acumuladoras de ET causa una redistribución de los estos elementos en el suelo, aumentando su disponibilidad en las capas más superficiales (Mertens et al., 2007).

El riesgo real que supone para los herbívoros la acumulación de ET en una especie determinada es difícil de evaluar. Existen algunos modelos que predicen el número de especies que podrían verse negativamente afectadas por la ingestión de un rango de concentraciones de ET en la dieta. En el caso del Cd, la ingestión de un material vegetal con 3.13 mg $Kg^{-1}$ de Cd (media de la concentraciones en las hojas de *P. alba* encontradas en el Corredor Verde), produciría efectos tóxicos en cerca del diez por ciento de las especies de aves y mamíferos que consumieran dicho material (van Nevel et al., 2007). Sin embargo, estas estimas están frecuentemente sobredimensionadas, ya que asumen que la dieta de dichas especies de herbívoros está basada únicamente en el material vegetal contaminado, situación que no tiene porqué representar las condiciones reales en el campo.

La ingesta potencial de ET por los herbívoros en una zona contaminada puede condicionar el manejo de dicha zona, en particular en lo referente al uso ganadero. En el caso del Corredor Verde, se prohibió el pastoreo, y cualquier otra actividad agrícola o cinegética, desde el primer momento, aunque paulatinamente se ha ido introduciendo ganado, sobre todo equino, de manera no controlada. Actualmente, en la zona se desarrollan amplios pastizales que suponen un alto riesgo de incendio, y su control mecánico es costoso. Estos pastizales compiten con los plantones por el agua y los nutrientes; algunos estudios han demostrado que en plantaciones jóvenes de *Quercus*, la competencia con las herbáceas influye de manera importante en la supervivencia y crecimiento de los plantones (Rey-Benayas et al., 2003; 2005). Bajo las condiciones del Corredor Verde, hemos podido comprobar que la competencia de las herbáceas (indicada por la cantidad de biomasa de herbáceas producida por unidad de superficie) tiene cierta influencia negativa en el crecimiento de plántulas de *Q. ilex*, emergidas a partir de siembras experimentales en distintos micrositios (zonas protegidas por matorral y zonas abiertas, Domínguez et al., en preparación). En el **Capítulo 5** hemos estudiado los patrones de acumulación de ET en los pastizales del Corredor Verde y la ingesta potencial de estos elementos por parte del ganado, como primer paso para evaluar la viabilidad del pastoreo selectivo como medida de control de los herbazales. La contaminación del suelo parece no influir especialmente en la composición florística de los pastos. Sin embargo, la composición florística sí influyó en las concentraciones de ET del pastizal; pastizales con predominio de gramíneas mostraron menores concentraciones de ET debido a la mayor biomasa que alcanzan las especies de esta familia presentes en el Corredor, que produce un efecto de dilución de los ET y que hace disminuir la cantidad de suelo adherido, bastante contaminado, en el material vegetal.

Hubo diferencias estacionales en la ingesta potencial de ET por parte del ganado, con un aumento de las cantidades potencialmente ingeridas en otoño, debido a la mayor concentración de ET en los pastos poco desarrollados. A pesar de ello, la ingesta potencial de ET por el ganado fue tolerable en términos generales, circunstancia corroborada por el análisis de la crin de algunos caballos del Corredor. Se comprobó además (mediante análisis de las heces) que se produce una excreta preferencial de elementos no esenciales, como As, Cd, Pb y Tl, respecto de los esenciales para el ganado (Cu y Zn). El pastoreo en la zona sería pues posible, siempre que se controlen las situaciones de mayor riesgo de ingesta directa de suelo contaminado por parte del ganado, en pastizales de otoño poco desarrollados. En cualquier caso, serían necesarios otros estudios para evaluar el posible riesgo que supondría el consumo de dietas basadas en especies distintas de las gramíneas (por ejemplo, asteráceas) y, sobre todo, monitorizaciones a más largo plazo con el fin de detectar la posible aparición de efectos crónicos debido a una ingestión continuada de ET.

Aunque la translocación de ET a las partes aéreas de las plantas sea baja, la presencia de altas concentraciones de ET en la rizosfera puede afectar a ciertos procesos a nivel de raíz, como la absorción de nutrientes. La composición química de la hoja puede ser indicativa de procesos de competencia y antagonismo entre nutrientes y elementos no esenciales a nivel de raíz. En el **Capítulo 6** hemos analizado la influencia de la contaminación del suelo en el estado nutricional de las principales especies de árboles del Corredor Verde (*O. europaea*, *P. alba* y *Quercus ilex*), en un amplio rango de condiciones de suelo. Se observaron algunas deficiencias nutricionales, sobre todo de fósforo, en las especies estudiadas. Estas deficiencias de P fueron más evidentes para *O. europaea*, y estuvieron acentuadas bajo condiciones de acidez (Fig. 6. 6). En estos suelos ácidos, la asimilación de otros nutrientes como Mg y S por parte de *P. alba* aumentó, posiblemente como respuesta a la liberación de cationes del complejo de cambio en estos suelos ácidos y a la alta disponibilidad de sulfatos debido a la oxidación de los restos de polisulfuros metálicos del suelo. *Populus alba*, al ser una especie caducifolia de crecimiento rápido, podría responder más rápidamente a esta liberación de nutrientes que *O. europaea* o *Q. ilex*. La acidificación del suelo, asociada a la contaminación, parece ser uno de los factores más importantes para la nutrición de las especies estudiadas. A largo plazo, la acidificación podría ocasionar la disminución de la disponibilidad de bases cambiables en el suelo (Likens et al., 1996; Tomlinson, 2003), provocando mayores desequilibrios nutricionales de los detectados hasta ahora en los árboles.

En los suelos ácidos el riesgo de toxicidad por ET puede ser mayor, ya que el pH del suelo es uno de los factores que más influyen en la solubilidad y biodisponibilidad de la mayoría de los ET. En el **Capítulo 7** hemos profundizado en los factores que condicionan la biodisponibilidad de ET en los suelos del Corredor Verde, y en la respuesta foliar de la encina, una de las especies más utilizadas en las reforestaciones, a cambios en los niveles de biodisponibilidad. El pH del suelo fue el factor de mayor influencia en la disponibilidad de Cd, Cu, Pb y Zn (Fig. 7. 1), mientras que otros factores como el contenido en materia orgánica, la textura o la capacidad de intercambio catiónico tuvieron escasa influencia. La disponibilidad fue altamente variable, debido a la heterogeneidad de los valores de pH del suelo. En los suelos extremadamente ácidos ($pH < 4$), la disponibilidad de Cd y Zn fue relativamente alta. La distribución de las concentraciones totales de elementos traza a lo largo de la cuenca del Guadiamar es consecuencia de los niveles de disponibilidad de los distintos elementos: Cd y Zn, elementos más solubles, parecen haber sido transportados con mayor facilidad desde las zonas ácidas de la parte central hasta el sur de la cuenca, mientras que elementos menos móviles, como Cu y Pb, experimentaron un transporte menor desde las zonas centrales. Cadmio fue el elemento potencialmente más lábil, aunque en condiciones de campo fue escasamente translocado desde la raíz a las hojas de la encina. Se demostró experimentalmente, bajo condiciones controladas en invernadero, que esta especie presenta una alta capacidad para retener Cd en las raíces finas (Fig 7. 3b).

Debido a la importancia del pH en la biodisponibilidad de ET y en la nutrición de las especies de árboles, la monitorización y, en su caso, corrección del pH es uno de los puntos más importantes a considerar en la gestión del Corredor Verde del Guadiamar. Tras la retirada de los lodos y la capa superficial de suelo contaminada, se añadieron distintos tipos de enmiendas con la finalidad de aumentar el pH, inmovilizar a los ET catiónicos del suelo, y mejorar la fertilidad del suelo. A partir de los datos recogidos en esta Tesis hemos podido comprobar que, casi diez años después de las tareas de limpieza de los suelos, todavía existen algunas zonas muy contaminadas, donde el efecto de estas enmiendas sobre el pH del suelo puede haberse atenuado, debido a la oxidación de los sulfuros presentes en los restos de lodos que aún persisten en el suelo. Sin embargo, las concentraciones altas de algunos nutrientes en el suelo, principalmente P, todavía reflejan, en algunos casos, la composición química de las enmiendas añadidas (la espuma de azucarera es extremadamente rica en P).

Finalmente, en el **Capítulo 8** hemos analizado la respuesta ecofisiológica de plántulas de *Q. ilex* a exposiciones altas de cadmio (Cd) y talio (Tl), dos elementos traza no esenciales y potencialmente muy móviles en el sistema suelo-planta. A las dosis tan altas utilizadas, los dos elementos provocaron efectos adversos en las tasas de crecimiento y las tasas de asimilación, aunque los mecanismos responsables de estos efectos fueron distintos para los dos elementos. Para el Cd, la tolerancia de las plántulas de encina fue relativamente buena; el fotosistema II permaneció relativamente inalterado, aunque las tasas de fotosíntesis neta disminuyeron, probablemente por una inhibición en la fijación del carbono inducida por el Cd. Además, las plantas mostraron síntomas de estrés hídrico. El Tl, sin embargo, provocó efectos más severos, incluso letales en las plántulas, con una fuerte inhibición del fotosistema II, disminución de las tasas de asimilación y conductancia estomática. Los patrones de transporte y acumulación de los dos elementos en la planta fueron bastante distintos: para el Cd existen mecanismos efectivos de retención a nivel de raíz, principalmente en las raíces finas, mientras que el Tl es retenido en menor medida en la raíz y ampliamente transportado a las hojas donde, a juzgar por la respuesta tóxica de las plantas, no hay mecanismos efectivos de tolerancia (Fig. 8.7).

La tolerancia de las plántulas de encina a dosis moderadas de Cd y su alta capacidad para retener este metal en el sistema radical le confieren un alto potencial para la estabilización de suelos contaminados con Cd. Posiblemente, otros ET también sean altamente retenidos en la raíz de *Q. ilex*, como indican las bajas concentraciones de ET en las hojas en condiciones de campo, y la nula correlación entre estas concentraciones foliares y las concentraciones de ET disponibles en el suelo. Además, en el campo hemos podido comprobar que, en lo que a estado nutricional se refiere, no es la especie más sensible a la acidificación del suelo. Sin embargo, la supervivencia de los plantones de *Q. ilex* durante los primeros años posteriores a la plantación fue muy baja, inferior a la de otras especies esclerófilas como *O. europaea*, probablemente debido a una mayor sensibilidad a la sequía estival (Domínguez et al., 2009). El potencial de *Q. ilex* para la fitoestabilización de suelos contaminados con Cd y otros ET podría aumentar si durante el diseño de las plantaciones se tuvieran en cuenta medidas encaminadas a aumentar la supervivencia de los plantones durante los primeros veranos posteriores a la plantación. En la última década, se han desarrollado algunas técnicas encaminadas a mejorar la supervivencia de especies de *Quercus* bajo condiciones de clima mediterráneo seco y semiárido (Cortina et al., 2004; Pausas et al., 2004; Rey-Benayas et al., 2005). Estas técnicas incluyen el endurecimiento previo de los plantones a las condiciones de campo, el establecimiento de sistemas colectores de la escorrentía o el uso de umbráculos para mejorar las condiciones microclimáticas o mejorar la resistencia de las plantas a la sequía.

Por otra parte, la heterogeneidad espacial a pequeña escala puede tener mucha influencia en la supervivencia y el desarrollo de los plantones. En los últimos años, se ha generado bastante información sobre la aplicación de las interacciones positivas planta-planta en la restauración ecológica en ambientes mediterráneos (Maestre et al., 2001; Castro et al., 2004; Gómez-Aparicio et al., 2004). Las condiciones microclimáticas asociadas a las manchas de vegetación existentes pueden ser más favorables para el establecimiento de especies leñosas. Así, en siembras experimentales realizadas en el Corredor Verde, (Domínguez et al., en preparación), hemos comprobado que las condiciones ambientales bajo especies de matorral como *Retama sphaerocarpa* y *Phillyrea angustifolia* son mucho más favorables para el establecimiento de plántulas de encina que las de zonas abiertas, desprovistas de vegetación leñosa, donde la competencia con las herbáceas es mucho mayor.

**Perspectivas de investigación: efectos a largo plazo de la reforestación de zonas contaminadas**

El efecto a largo plazo del crecimiento de los árboles sobre la dinámica de los elementos traza es un aspecto actualmente en discusión, en el ámbito de la restauración de zonas contaminadas. Durante los últimos años, se ha generado una valiosa información en el Corredor Verde del Guadiamar, que complementada con estudios futuros podría servir para esclarecer algunos aspectos de la dinámica temporal, a largo plazo, de los ET en los sistemas forestales. Las distintas especies leñosas pueden tener distintos efectos sobre las propiedades del suelo y los procesos en el ecosistema; los cambios que inducen en el suelo pueden a su vez afectar directamente a la misma planta, estableciéndose retroalimentaciones en el sistema planta-suelo, que pueden llegar a ser bastante complejas (Eviner y Chapin, 2003; Hobbie et al., 2006). En el caso de antiguos terrenos agrícolas reforestados, como es el caso del Corredor Verde, la reforestación suele provocar la acidificación del suelo, aunque el nivel de acidificación depende de la especie plantada. Al tratarse de una zona contaminada, donde el pH juega un papel fundamental en la biodisponibilidad de ET, sería interesante estudiar a largo plazo el efecto de las distintas especies de árboles sobre la disponibilidad de ET del suelo. Asimismo, sería interesante estudiar la calidad y cantidad de los aportes de materia orgánica de cada especie en el desfronde, y su posible influencia en la dinámica de los ET del suelo.

La diferente composición química de la hoja de las especies introducidas en el Corredor Verde puede tener una gran influencia en la redistribución de los ET del suelo, y en otros procesos a nivel ecosistémico, como el reciclado de nutrientes. Al estar presentes especies que acumulan de manera diferencial los ET en sus hojas (especies acumuladoras como *Populus alba* y las especies del género *Salix* vs. especies exclusoras, como *Quercus ilex*), sería interesante estudiar la dinámica de los ET bajo el dosel de cada especie, siendo esperable una redistribución de Cd y Zn hacia los horizontes orgánicos de los suelos bajo salicáceas. En estos horizontes sería interesante estudiar cómo afecta la acumulación de ET en la hojarasca a procesos como su propia descomposición, mineralización de nutrientes, y si esta redistribución diferencial de los ET tiene alguna influencia sobre la dinámica de los ecosistemas del Corredor Verde del Guadiamar.

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# Capítulo 10

# Conclusiones

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## Conclusiones

1. Los suelos del Corredor Verde del Guadiamar siguen estando contaminados por elementos traza (ET), fundamentalmente por As, Cd, Cu, Pb, Tl y Zn. El arsénico es el elemento cuyas concentraciones totales han aumentado más en relación a los suelos no contaminados. Un 86% de los suelos analizados tenían concentraciones de As mayores que 40 mg $kg^{-1}$ (limite superior del rango para suelos normales). Se ha observado en general una penetración significativa de la contaminación en el perfil del suelo hasta, al menos, 40 cm de profundidad.

2. La disponibilidad de los elementos traza catiónicos, es decir Cd, Cu, Pb y Zn, es muy variable en el Corredor Verde. El pH del suelo es el factor con mayor influencia en la disponibilidad de Cd, Cu y Zn, analizada mediante extracción con nitrato amónico, de forma que se puede predecir la disponibilidad del elemento a partir de los valores de pH. Otros factores como el contenido en materia orgánica, la textura o la capacidad de intercambio catiónico tuvieron escasa influencia en la disponibilidad de estos elementos. En términos generales, el cadmio fue el elemento traza más móvil, con una media del 14% del total extraíble con nitrato amónico.

3. Existen en el Corredor suelos extremadamente ácidos ($pH < 4$), debido a la oxidación de los sulfuros de los restos de lodos del suelo, donde la disponibilidad de los ET catiónicos es comparativamente alta, existiendo un mayor riesgo de lixiviación y de toxicidad para las plantas.

4. A pesar de las altas concentraciones totales de elementos traza en el suelo contaminado, su transferencia a las hojas de las especies leñosas mediterráneas estudiadas fue muy baja, con coeficientes de transferencia inferiores a 0.03 para As, Bi, Pb, Sb y Tl, y superiores a 0.1 para Cd, Cu y Zn. Para estos tres elementos, hubo mayores diferencias interespecíficas.

5. El álamo blanco (*Populus alba*) fue una excepción, siendo capaz de acumular en sus hojas concentraciones mayores de 3 mg $kg^{-1}$ de Cd y 400 mg $kg^{-1}$ de Zn. Fue la única especie estudia-

da con coeficientes de transferencia mayores que 1 para los dos elementos. En consecuencia se pueden alcanzar en sus hojas concentraciones de Cd y Zn que son tóxicas para los herbívoros. La abundancia de álamos blancos en el Corredor Verde del Guadiamar, tanto naturales como reforestados, supone un riesgo de transferencia de Cd y Zn en la red trófica, y de concentración en los horizontes superficiales del suelo mediante la acumulación y descomposición de la hojarasca.

6. Las concentraciones de elementos traza en los pastizales del Corredor Verde del Guadiamar dependen de la estación y de la composición florística del pasto. En otoño, los pastizales poco desarrollados contienen en mayor proporción suelo adherido, altamente contaminado. En primavera, la mayor biomasa, especialmente de las gramíneas, reduce la proporción de material vegetal expuesto a la contaminación con suelo, y diluye las concentraciones de ET absorbidas por la raíz. Por esta razón, los pastos con predominio de gramíneas presentan menores concentraciones de ET.

7. En consecuencia, la ingesta potencial de ET por parte del ganado equino, muy selectivo en su dieta, a base de gramíneas, se encuentra dentro de límites tolerables, siendo los ET no esenciales excretados preferentemente. A la hora de considerar el pastoreo selectivo como medida de control de los herbazales de la zona, sería recomendable evitar aquellos pastizales otoñales donde la contaminación del pasto con suelo pueda ser alta. En cualquier caso, hay que tener en cuenta los posibles efectos crónicos por la ingestión prolongada de ET en la dieta.

8. Las especies de árboles dominantes en el Corredor Verde: acebuche (*Olea europaea*) álamo blanco (*Populus alba*) y encina (*Quercus ilex* subsp. *ballota*) mostraron, en ciertas zonas, concentraciones deficitarias de nutrientes, sobre todo de fósforo en sus hojas. La contaminación asociada a la acidificación potencial del suelo, influyó en las concentraciones foliares de varios nutrientes. Las mayores interacciones negativas entre contaminación y nutrición se observaron para el fósforo en las hojas de acebuche (*O. europaea*). Por el contrario, para el álamo blanco la contaminación influyó de manera positiva en las concentraciones de algunos nutrientes, como magnesio y azufre, probablemente como respuesta a una mayor disponibilidad de estos nutrientes en los suelos acidificados.

9. La encina (*Quercus ilex* subsp. *ballota*) es una especie exclusora de elementos traza, con

una baja transferencia de estos elementos a las hojas en condiciones de campo, que no está influida por los niveles de biodisponibilidad en el suelo. Este patrón se debe a la alta capacidad de retención de elementos traza en la raíz, como ha quedado demostrado para el caso del cadmio en plántulas de encina cultivadas bajo condiciones controladas en invernadero.

10. La respuesta de las plántulas de encina a altas concentraciones de cadmio o talio en el sustrato fue bastante distinta. El talio produjo efectos más severos, incluso letales, a periodos de exposición prolongados (5 meses). Ambos elementos redujeron las tasas de fotosíntesis neta y los valores de conductancia estomática de las plantas, aunque los mecanismos responsables fueron distintos para cada elemento.

11. El talio produjo importantes daños en el fotosistema II, mientras que el Cd no produjo alteraciones en el funcionamiento de este componente fotosintético. Para el Cd, la reducción en las tasas de asimilación parece estar más relacionada con la inhibición en la fijación del carbono. En general, las plántulas de encina muestran mayor tolerancia al Cd que al Tl, posiblemente debido a la retención efectiva del Cd en las raíces finas de las plantas. Por el contrario, el Tl es ampliamente transportado hasta las hojas.

12. La buena tolerancia al cadmio mostrada por la encina en condiciones experimentales, así como su capacidad de retenerlo a nivel de raíz le confiere un alto potencial para la fitoestabilización de suelos contaminados con este elemento. El uso de la encina en la restauración del Corredor Verde y otras zonas contaminadas debe tener en cuenta además medidas encaminadas a mejorar su resistencia a otras condiciones ambientales adversas, en particular la sequía estival.