Departamento de Química Agrícola y Bromatología
Facultad de Ciencias
Universidad Autónoma de Madrid

Biorremediación de suelos contaminados con hidrocarburos aromáticos policíclicos mediante aplicación de sustrato post-cultivo de champiñón (*Agaricus bisporus*)

Bioremediation of contaminated soils with polycyclic aromatic hydrocarbons using spent mushroom substrate (*Agaricus bisporus*)

TESIS DOCTORAL

Carlos García Delgado

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Memoria que presenta D. Carlos García Delgado para obtener el título de Doctor con Mención Europea por la Universidad Autónoma de Madrid.

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CERTIFICA:

Que D. Carlos García Delgado ha realizado bajo su dirección y en este departamento, el trabajo que lleva por título "Biorremediación de suelos contaminados con hidrocarburos aromáticos policíclicos mediante aplicación de sustrato post-cultivo de champiñón (*Agaricus bisporus*)" que constituye su Memoria de Tesis Doctoral. Dicho trabajo reúne las condiciones necesarias para su presentación y defensa.

Y para que conste a los efectos oportunos firmo el presente certificado en Madrid, a 23 de abril del 2015.

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(Siglo. XI)

A mi familia

Resumen

La presente memoria de Tesis estudia la biorremediación de suelos contaminados con hidrocarburos aromáticos policíclicos (PAH) mediante la aplicación del sustrato post-cultivo de champiñón (*Agaricus bisporus*). Los PAH son contaminantes ubicuos con propiedades hidrófobas, tóxicas y carcinogénicas. En muchos casos la contaminación de suelo por estos compuestos se encuentra acompañada de elevadas concentraciones de metales, lo que dificulta su biorremediación.

El sustrato post-cultivo de champiñón (Spent *A. bisporus* substrate, SAS) es un residuo agrícola que se genera en grandes cantidades. Históricamente ha sido tratado como un residuo y eliminado en vertederos. Esto ha generado graves problemas medioambientales en las zonas de cultivo de champiñón. Sin embargo el SAS presenta alto contenido en materia orgánica y una compleja microbiota que puede ser muy útil en procesos de biorremediación de suelo. De esa microbiota destaca la gran cantidad de micelio de *A. bisporus*. Este hongo tiene la capacidad de excretar enzimas ligninolíticas al medio. Este tipo de enzimas extracelulares y los hongos que las excretan, son capaces de degradar una gran variedad de compuestos orgánicos por lo que son muy útiles en la biorremediación de suelos contaminados.

La presente memoria se estructura en dos grandes bloques. El primero presenta los resultados de la puesta a punto de la metodología de extracción de metales y PAH en suelo y enmiendas orgánicas, así como la localización de emplazamientos contaminados. En el segundo bloque se evaluó la utilidad de SAS en procesos de biorremediación. Inicialmente se realizaron ensayos de degradación de PAH y adsorción de Cd y Pb sin suelo. Tras esta primera etapa, se pasó a realizar estudios de biorremediación con muestras de suelo real procedentes de emplazamientos anteriormente seleccionados donde se evaluaron diferentes formas de aplicación del SAS.

Para la puesta a punto de la metodología de extracción de metales de enmiendas orgánicas se compararon seis procedimientos diferentes. Se prestó especial atención a los residuos sólidos obtenidos tras la digestión en microondas.

Se observó la desaparición y formación de fases cristalinas. El uso de HF se mostró necesario para la digestión total pero en muestras con alto contenido en Ca se formaba CaF_2 y $CaAlF_5$. Finalmente se propuso la digestión en microondas con HNO_3 y HF como método de extracción total y con agua regia como extracción pseudo-total.

Para la extracción de PAH tanto de suelo como de SAS se evaluaron cuatro posibles extractantes tanto con ultrasonidos como mediante agitación. Se determinó que la extracción mediante agitación era más eficiente y entre los disolventes posibles se seleccionó acetona:hexano (1:1) por su versatilidad en la extracción de PAH en SAS húmedo y seco.

La búsqueda de suelos contaminados se realizó en los alrededores de plantas termoeléctricas de carbón, industria petroquímica, una planta metalúrgica y un campo de tiro, así como suelo de una planta de creosotado de madera. Los suelos procedentes de los dos últimos emplazamientos fueron los que mostraron las mayores concentraciones de contaminantes. Los suelos de la planta de creosotado de madera tenían las mayores concentraciones de PAH mientras que el suelo del campo de tiro mostró altas concentraciones de PAH y Pb.

En el primer paso de la evaluación del SAS como enmienda remediadora de suelos contaminados se realizaron estudios donde se comprobó su capacidad de adsorción de Cd y Pb, degradación de PAH y efecto de los metales Cd y Pb en la actividad de enzima ligninolítica mayoritaria que fue la laccasa y en el proceso de degradación de PAH que fue llevado a cabo tanto por *A. bisporus* como por el resto de la microbiota autóctona de SAS.

Se realizaron dos ensayos de biorremediación con suelo contaminado real. El primero sobre suelo contaminado con creosota, donde la contaminación se debía a PAH y el segundo sobre suelo procedente de las inmediaciones de un campo de tiro. En este último, existía contaminación tanto de PAH, especialmente de alto peso molecular (HMW-PAH), como de Pb.

En el diseño experimental de ambos ensayos se consideraron cuatro tratamientos: atenuación natural (SM, suelo sin enmendar), bioestimulación mediante la aplicación de SAS previamente esterilizado (SSAS), bioaumentación

por aplicación de SAS sin tratamiento previo (SAS) y bioaumentación por aplicación de SAS esterilizado y re-inoculado con *A. bisporus* (Abisp). La duración de los ensayos fue de 63 días.

El suelo de la planta de creosotado aumentó considerablemente su población bacteriana y fúngica con el tratamiento de bioestimulación (SSAS) aunque sólo se observó aumento de la riqueza y variedad bacteriana. Este modo de aplicación de SAS consiguió degradar PAH de bajo peso molecular (LMW-PAH) debido a la alta población y actividad bacteriana pero su impacto en los HMW-PAH fue bajo. La bioaumentación con SAS sin tratamiento previo incrementó considerablemente la población bacteriana, además de incorporar bacterias degradadoras de PAH al suelo. Los análisis por DGGE mostraron un notable aumento de la riqueza y variedad de las poblaciones fúngicas y bacterianas. A pesar del alto contenido de ergosterol de este tratamiento, la colonización del suelo por *A. bisporus* no fue completa. En este caso hubo degradación de PAH de alto y bajo peso molecular por la acción conjunta de la población bacteriana y de *A. bisporus*. La bioaumentación con el tratamiento Abisp produjo una completa colonización del suelo por *A. bisporus* aunque el contenido de ergosterol no variase a lo largo del tiempo de incubación. La población bacteriana aumentó a lo largo del ensayo y su impacto en la comunidad fúngica y bacteriana fue similar al de SSAS. Este tratamiento fue el que se mostró más eficaz en la degradación de HMW-PAH por la mayor actividad ligninolítica registrada durante el ensayo. Como resultado, los tratamientos de bioaumentación fueron los que obtuvieron una mayor mejoría de los parámetros ecotoxicológicos del suelo, especialmente Abisp.

El ensayo de biorremediación de suelo cercano al campo de tiro arrojó resultados similares al anterior. Sin embargo la mayor concentración de PAH y especialmente HMW-PAH y la presencia de Pb produjeron interesantes diferencias. En este caso la bioestimulación con SSAS también aumentó considerablemente la población bacteriana pero la degradación de LMW-PAH fue muy baja, incluso menor que en el suelo sin enmendar. Los tratamientos de bioaumentación SAS y Abisp consiguieron la degradación tanto de LMW-PAH como de HMW-PAH. En este caso *A. bisporus* sólo fue capaz de colonizar el suelo con el tratamiento Abisp. La aplicación de SAS en cualquiera de las tres formas produjo una ligera movilización

del Pb (< 1‰). Al igual que en el suelo contaminado por creosota los parámetros ecotoxicológicos mostraron una mayor mejoría en el tratamiento Abisp.

La conclusión general del trabajo es que la reutilización del sustrato post-cultivo de champiñón (*A. bisporus*) es factible para biorremediación de suelos contaminados con PAH.

Abstract

The present Doctoral Thesis aims the bioremediation of polluted soil with polycyclic aromatic hydrocarbons (PAH) using spent mushroom (*Agaricus bisporus*) substrate. PAH are ubiquitous pollutants with hydrophobic, toxic and carcinogenic properties. Many of the PAH polluted soils contain high concentrations of metals which complicate the soil bioremediation process.

The spent *A. bisporus* substrate (SAS) is an agricultural waste produced at huge amount. Historically, the management of this waste was the disposal in dump. This practice produced environmental damages in the areas of mushroom production. However, because the high organic matter content and the complex microbiota present in SAS, this waste could be useful in soil bioremediation. SAS contains high amount of *A. bisporus* mycelium. This fungus is able to produce extracellular ligninolytic enzymes. These fungi and their ligninolytic enzymes are able to degrade a wide variety of organic pollutants. Therefore they are very valuable for bioremediation of polluted soils.

This Thesis contains two parts. The first one shows the methodological development of metals and PAH extraction from organic amendments and soils, and the localization of polluted soils. The second part assesses the SAS usefulness for bioremediation. The first step of this evaluation consisted of assays about PAH degradation and Cd or Pb adsorption on SAS. Then soil bioremediation assays were carried out with the polluted soils selected in the first part. These assays assessed different SAS application methods.

Six digestion procedures were tested for the development of the method for metals extraction from organic amendments. The solid residues obtained after microwaves assisted digestions showed destruction and formation of new crystalline phases. The use of HF is mandatory for total digestion of the organic amendments. However, high Ca content produced CaF_2 and $CaAlF_5$ precipitates. Microwave assisted digestion with HF-HNO_3 is proposed for total digestion while aqua regia is proposed for pseudo-total digestion.

The PAH extraction from soil and SAS were developed by combination of four solvents with ultrasounds or orbital shaking. The best procedure for PAH extraction from soil and either fresh or dry SAS was the orbital shaking with acetone:hexane (1:1).

The selection of polluted soils took place in different locations close to thermal coal power plants, petrochemicals, metallurgic, shooting rage and creosote wood treatment plant. The soils from shooting range and creosote wood treatment plant showed the highest pollutant concentrations. Soils from creosote wood treatment plant presented very high concentrations of PAH and soil from shooting range showed high concentration of Pb and PAH simultaneously.

The first SAS evaluation for soil remediation was carried out by different assays of Cd and Pb adsorption, PAH degradation and the effect of Cd and Pb on laccase activity and PAH degradation. The PAH degradation was carried out by *A. bisporus* and other SAS autochthonous microorganisms.

Then, two bioremediation assays were performed with real polluted soils. The first assay involved the use of soil from creosote wood treatment plant where the contamination was PAH. The second one was done with the soil surrounding firing range which was polluted with PAH, mainly high molecular weight PAH (HMW-PAH) and Pb simultaneously.

Four bioremediation treatments were assessed in both soils: natural attenuation (SM), biostimulation with sterilized SAS (SSAS) and two bioaugmentation strategies, SAS without previous treatment (SAS) and sterilized SAS re-inoculated with *A. bisporus* (Abisp). The incubation period was 63 days.

Creosote polluted soil increased its bacterial and fungal population with biostimulation treatment (SSAS) nevertheless, only was observed increment of bacterial richness and evenness. This treatment degraded PAH of low molecular weight (LMW-PAH) because high bacterial population and activity. No significant HMW-PAH degradation occurred. Bioaugmentation SAS treatment increased the bacterial population and input PAH-degrading bacteria on soil. DGGE analysis demonstrated increment of bacterial and fungal richness and evenness. The *A. bisporus* soil colonization was not complete despite the high ergosterol content.

The synergist action of bacterial and fungal population achieved LMW and HMW-PAH degradation. The Abisp bioaugmentation treatment reached full soil colonization by *A. bisporus* despite the invariable ergosterol content during the incubation period. The bacterial population was increased along the assay and the DGGE results were similar to SSAS. The high ligninolytic activity in Abisp produced the highest HMW-PAH degradation rate. The two bioaugmentation treatments produced the best results of eco-toxicological tests, mainly Abisp.

The bioremediation assay of shooting range soil showed similar results. However, the higher total PAH (and specially HMW-PAH) concentration and the presence of Pb, produced interesting differences. The biostimulation (SSAS) increased the bacterial population but the LMW-PAH removal was very low, even lower than SM. The bioaugmentation treatments (SAS and Abisp) achieved LMW and HMW-PAH degradation. However *A. bisporus* was not able to colonize the soil in SAS treatment. The soil amendment with SAS irrespective the application method produce slight Pb mobilization (<1‰). Abisp showed the highest improvement of the eco-toxicological parameters.

In summary, the reutilization of spent *A. bisporus* substrate is feasible for bioremediation of PAH polluted soils.

Índice General

Capítulo 1:
Introducción

1.1. La contaminación de suelos en Europa

Las estimaciones de emplazamientos europeos potencialmente contaminados, publicadas por la Comisión Europea en 2014 (JRC EC 2014), son de 2,5 millones. De los cuales 342.000 están declarados como emplazamientos contaminados donde la contaminación del suelo ha sido confirmada y representa un riesgo potencial para la salud humana, el agua o los ecosistemas.

Las etapas de manejo y control de suelos contaminados se dividen en identificación, estudio preliminar, investigación y remediación. En ellas se ha detectado una clara dispersión de los esfuerzos individuales de cada estado miembro de la Unión. En algunos casos no se ha producido avance en programas de identificación de emplazamientos desde 2006, mientras que en otros como Bélgica, a fecha de 2014 había avances significativos en la fase final de la remediación de suelos. En el caso de España se ha observado un avance en la identificación de emplazamientos contaminados y en la investigación de los suelos identificados pero no en la fase de remediación (JRC EC 2014).

En ese informe se reporta que la mayoría de los emplazamientos contaminados están afectados por metales pesados seguido de aceites minerales y en tercer lugar por hidrocarburos aromáticos policíclicos (Polycyclic Aromatic Hydrocarbons, PAH) (Fig. 1.1).

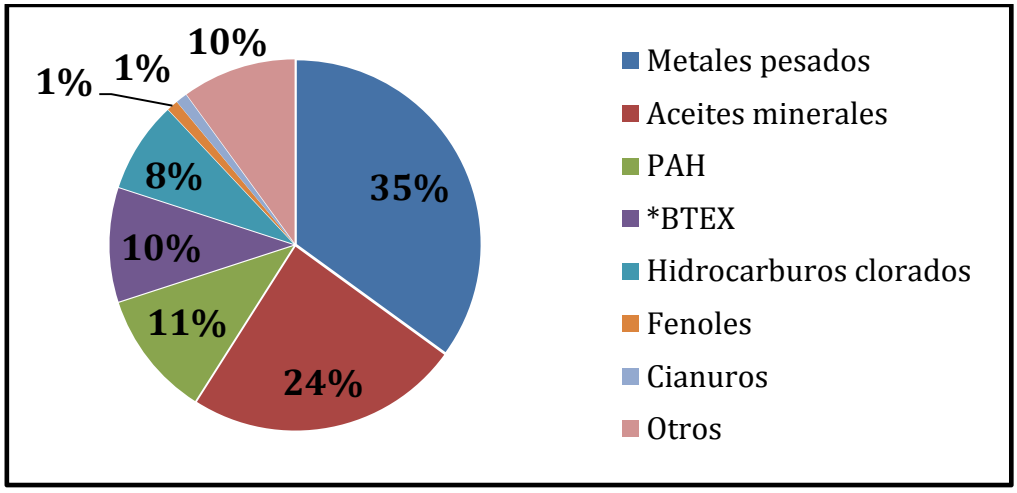

Fig. 1.1: Porcentaje de abundancia de sustancias contaminantes en suelos contaminados de Europa (JRC EC 2014).
*BTEX: benzeno, tolueno, etilbenceno y xileno.

1.1.1. Origen, toxicidad y problemática medioambiental de metales: Cd y Pb

De todos los metales y metaloides que son reportados como causantes de contaminación de suelos (Cr, Mn, Ni, Cu, Zn, As, Cd, Hg, Pb ...), el presente trabajo se centra en el estudio de Cd y Pb como modelo de dos metales tóxicos con alta (Cd) y baja (Pb) movilidad en el suelo.

Las fuentes de Cd y Pb al suelo son tanto naturales como antropogénicas, siendo estas últimas las más importantes desde el punto de vista de la dispersión de la contaminación. A continuación se detallan algunas de las fuentes más significativas.

Las actividades mineras son una conocida fuente de estos metales, tanto en el entorno de la explotación minera como a una mayor escala debido a rotura de balsas de almacenaje de lodos como los conocidos accidentes de Aznalcollar en 1998 (España) y Ajka en 2010 (Hungría).

El procesado del mineral extraído y su transformación en procesos siderúrgicos también son fuente de metales. Principalmente en la siderurgia se emiten metales a la atmósfera y estos son posteriormente depositados en el suelo. En el Registro Estatal de Emisiones y Fuentes Contaminantes (MAGRAMA 2015) se recoge esta actividad como emisora de metales a la atmósfera junto a PAH entre otros compuestos.

La producción de energía eléctrica mediante combustibles fósiles es fuente de metales a la atmósfera (Sarris et al., 2009) principalmente asociados a cenizas volantes que posteriormente pueden depositarse en el suelo. Cabe destacar que en esta actividad se emiten conjuntamente PAH a la atmósfera. Sin embargo se han implementado diversas técnicas de reducción de partículas como precipitadores electrostáticos y filtros de mangas que inciden directamente en la reducción de las emisiones de metales. Los combustibles con mayor impacto en la emisión de Cd y Pb son hulla, lignito negro y fuelóleo (Ministerio de Agricultura, 2014).

De forma análoga a la producción de energía, la combustión de diesel o gasolina en los vehículos de motor también son fuente de metales. Actualmente la emisión de Pb se ha visto reducida por la prohibición en 2001 de la

comercialización de gasolina con tetraetilo de plomo como antidetonante (BOE 2001). Sin embargo la continuada exposición de los suelos adyacentes a carreteras con intenso tráfico ha producido su contaminación por metales (Kluge and Wessolek, 2012; Werkenthin et al., 2014).

Prácticas agrícolas como la fertilización y riego son fuente de metales al suelo. A este respecto, el RD 8506/2013 (BOE 2013) legisla el contenido máximo permitido de ciertos metales en abonos y enmiendas orgánicas de uso agrícola (Tabla 1.1), así como las dosis máximas en función de la concentración de metales. Bolan et al. (2014) describe a los fertilizantes de P como fuentes de Cd al suelo y a los lodos de depuradora como una de las principales vías de adición de metales pesados al suelo. Respecto al agua de riego como fuente de metales a los suelos, destaca la reutilización de aguas residuales en la agricultura, que ha sido objeto de amplios estudios y continúa de actualidad (Almuktar et al., 2015; Farahat and Linderholm, 2015; Fatta-Kassinos et al., 2011; García-Delgado et al., 2012a).

Tabla 1.1: Límite máximo de metales pesados en abonos y enmiendas orgánicas según la clasificación de calidades del RD 506/2013 (BOE 2013).

Metal pesado	Límite de concentración (mg kg^{-1})		
	Clase A	Clase B	Clase C
Cd	0,7	2	3
Cu	70	300	400
Ni	25	90	100
Pb	45	150	200
Zn	200	500	1.000
Hg	0,4	1,5	2,5
Cr	70	250	300
Cr (VI)	0	0	0

El RD 1620/2007 (BOE 2007) legisla la reutilización de aguas depuradas y especifica los usos permitidos. En función del uso que se vaya a hacer del agua depurada se especifican los tratamientos de depuración y la calidad mínima exigida al agua depurada. Uno de los usos autorizados es la reutilización como agua de riego. Entre los parámetros de calidad legislados para uso agrícola se encuentra

la concentración máxima permitida de ciertos metales pero sorprendentemente el Pb no está entre ellos.

Las principales vías de exposición al Cd en animales y humanos son la respiratoria y la ingestión. Dependiendo de la forma química del Cd su absorción se produce en mayor o menor medida, siendo la solubilidad un parámetro clave. Compuestos solubles facilitan la absorción y los menos solubles la limitan. Una vez en el organismo, el Cd se distribuye asociado a proteínas por el torrente sanguíneo. La metalotioneína interviene en su transporte y detoxificación. Es una proteína con un elevado porcentaje de cisteína que forma complejos con metales pesados. La mayoría del Cd se localiza en los riñones e hígado asociado a metalotioneína. Su excreción se realiza principalmente por la orina. En los riñones el complejo Cd-metalotioneína es filtrado pero rápidamente reabsorbido en los tubos proximales donde se rompe el complejo metálico y el Cd termina acumulándose causando daños (IARC 2012). Por ello su excreción es baja y el tiempo de residencia del Cd en el organismo muy elevado (7-16 años) (IARC 2012). Los efectos tóxicos que produce son: enfermedades pulmonares (bronquitis, fibrosis pulmonar y destrucción del tejido alveolar), degeneración irreversible de tubos renales, hipertensión y alteraciones óseas (osteoporosis, osteomalacia y dolores óseos) fruto de la alteración del metabolismo del Ca y el aumento de su excreción por los daños renales. La IARC (2012) clasificó al Cd metal y sus compuestos como cancerígenos para humanos (Grupo I). Respecto a animales concluyó que había limitada evidencia de la carcinogenicidad de Cd metal pero suficientes evidencias de la carcinogenicidad de los compuestos de Cd. El tipo de cáncer más común asociado al Cd es el de pulmón. Se han reportado otros tipos como el de riñón o aumento del número de muertes por cáncer de próstata en varias áreas contaminadas por Cd en Japón (IARC 2012).

La absorción de Pb en humanos y animales también se realiza preferentemente por vía respiratoria e ingestión. Su distribución por el organismo se realiza por la sangre asociado a los hematíes. Se acumula preferentemente en los huesos, debido a la alta afinidad del Pb por los fosfatos, donde reside durante años y es fuente constante de Pb a la sangre. También se acumula en hígado, riñones y sistema nervioso central. La intoxicación crónica por plomo recibe el

nombre de saturnismo. Produce trastornos gastrointestinales, neuromusculares, hematológicos, renales, reproductivos y en el sistema nervioso central. Además, el Pb es capaz de atravesar la placenta afectando negativamente al peso del feto al nacer y al tiempo de gestación. Este metal también posee carácter carcinogénico según la evaluación de la IARC (2006), siendo consideradas las sales inorgánicas de Pb como probable carcinogénico para humanos (Grupo 2A). Sin embargo los compuestos orgánicos de Pb no son clasificables como carcinogénicos para humanos (Grupo 3). En el caso de animales, no hay suficientes evidencias de la carcinogenicidad de la mayoría de los compuestos de Pb y sólo alguno de ellos como el acetato, cromato y fosfato presentan evidencias suficientes de su carcinogenicidad.

El impacto negativo del Cd y Pb en el medio ambiente es bien conocido y deriva inherentemente de su toxicidad para los seres vivos. Son contaminantes ubicuos e indexados en la lista de contaminantes prioritarios de la US-EPA. Su principal transporte hasta largas distancias desde su foco de emisión se debe a la presencia de estos metales en las partículas producidas en procesos de combustión, siendo las de menor tamaño las que pueden viajar mayores distancias. En última instancia el suelo es el receptor final de estos metales. La dinámica del Cd y Pb en el suelo es muy diferente. El Pb en el suelo se caracteriza por su baja movilidad ya que es adsorbido por los minerales de la arcilla, oxihidróxidos de hierro y manganeso, carbonatos, materia orgánica, fosfatos y además su solubilidad está fuertemente influenciada por el pH, produciéndose su precipitación por encima de pH 6. El Cd también se ve influenciado por todos estos compuestos que limitan su movilidad en el suelo, sin embargo su adsorción es menor y sobre todo su solubilidad no se ve tan afectada por el pH.

La movilidad del Cd y Pb (y de los metales en general) dentro del suelo es clave en su toxicidad medioambiental. Cuanto mayor sea ésta, mayor será la capacidad de los metales para lixiviar hasta los acuíferos y contaminar masas de agua o de ser absorbidos por plantas incluyéndose en la cadena trófica (Moreno-Jiménez et al., 2009). También los metales tienen efectos en la microbiología del suelo afectando a la estructura de las poblaciones bacterianas y su actividad (Niklińska et al., 2005; Zhang et al., 2015). Esta interacción es recíproca y los

microorganismos del suelo también pueden influir notablemente en el aumento o disminución de la movilidad de los metales por lo que el potencial biotecnológico de bacteria y hongos en procesos de biorremediacón es muy prometedor y ya está implementándose comercialmente (Gadd, 2004).

1.1.2. Origen, toxicidad y problemática medioambiental de los hidrocarburos aromáticos policíclicos

Los hidrocarburos aromáticos policíclicos (Polycyclic Aromatic Hydrocarbons, PAH) son una familia de compuestos orgánicos formados exclusivamente por carbono e hidrógeno. Estructuralmente, son moléculas planas formadas por anillos de benceno fusionados. En la Fig. 1.2 se muestran las estructuras de los 16 PAH declarados como prioritarios por la EU y US-EPA y que han sido tratados en el presente trabajo.

Los PAH se forman durante la descomposición térmica de moléculas orgánicas y posterior recombinación. La combustión de materia orgánica a altas temperaturas (500 – 800 ^{o}C) o mantenimiento durante largos periodos de tiempo a bajas temperaturas (100 – 300 ^{o}C) producen este tipo de compuestos. Las mayores fuentes de PAH de origen natural son los incendios forestales y erupciones volcánicas. Las fuentes antropogénicas están relacionadas principalmente con actividades industriales que implican la quema de combustibles (madera, carbón, petróleo y sus derivados) su manipulación o transformación, unos ejemplos son:

- Generación de electricidad en plantas térmicas
- Producción coque y gasificación de carbón
- Producción de aluminio y metalurgia en general
- Plantas de gas e industria petroquímica
- Creosotado de madera
- Vehículos de transporte con motor de combustión

Derivado de la formación de PAH en estas actividades industriales, muchas de ellas son catalogadas por la IARC (2010) como actividades carcinogénicas (Grupo I) o probablemente carcinogénicas (Grupo 2A) para humanos.

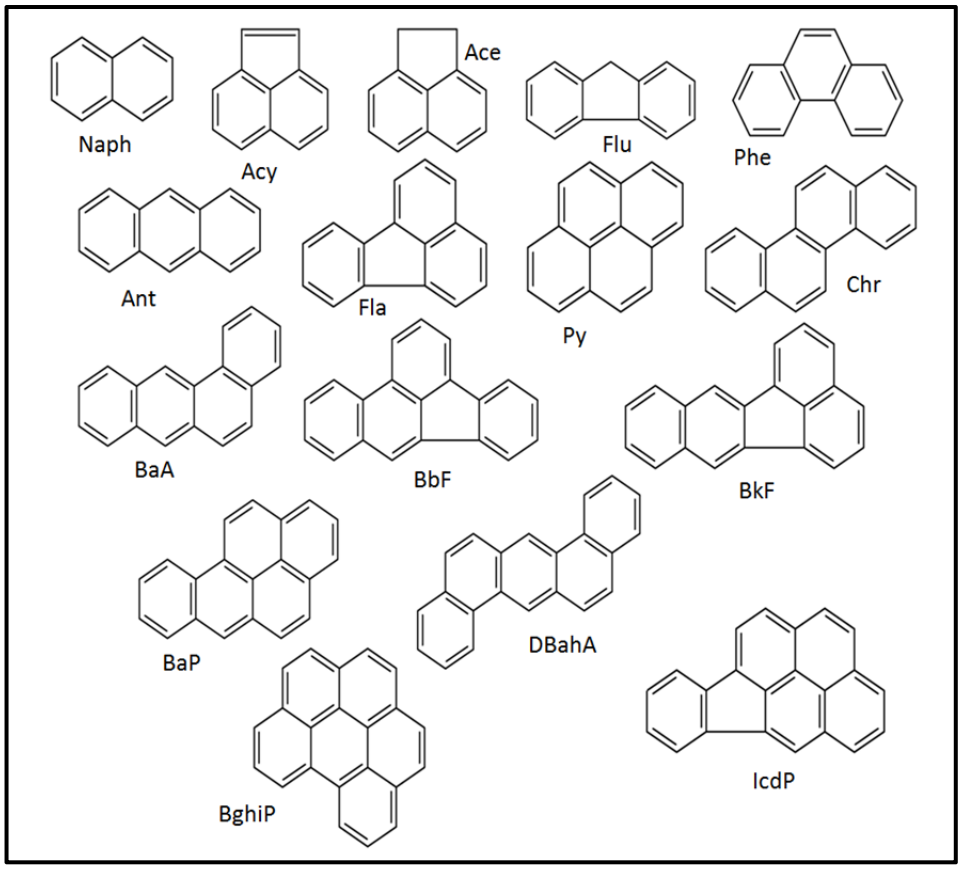

Fig. 1.2: Estructuras de los 16 UE y US-EPA PAH: Naftaleno (Naph), acenaftileno (Acy), acenafteno (Ace), fluoreno (Flu), fenantreno, (Phe), antraceno (Ant), fluoranteno (Fla), pireno (Py), benzo[a]anthraceno (BaA), criseno (Chr), benzo[b]fluoranteno (BbF), benzo[k]fluoranteno (BkF), benzo[a]pireno (BaP), dibenzo[a,h]antraceno (DBahA), benzo[g,h,i]perileno (BghiP), indeno[1,2,3-c,d]pireno (IcdP).

La variedad de fuentes de emisión de PAH y su facilidad de transporte atmosférico hacen de ellos contaminantes ubicuos en el medio ambiente al igual que Cd y Pb (IARC 2010).

Respecto a su toxicidad, los PAH como tal son biológicamente inertes y requieren una activación metabólica para que expresen su genotoxicidad, incluida mutagenicidad y carcinogenicidad (Fu et al., 2012).

Tabla 1.2: Principales propiedades físico-químicas de PAH, peso molecular (PM), coeficiente de sorción en materia orgánica (log Koc), hidrofobicidad (log P), solubilidad en agua (S) y potencial de ionización (PI) (Covino et al., 2010a; Eggen, 1999; Farnet et al., 2009), y clasificación según su carcinogenicidad para humanos y animales (IARC, 2010).

	PM	Log K_{oc}	Log P	S (mg l^{-1})	PI (eV)	Carcinogenicidad	
						Humanos†	Animales‡
Naph	128			31,7	8,1	n.e.	n.e.
Acy	152			3,93		n.e.	n.e.
Ace	154			3,47		3	I
Flu	166	3,88	4,18	1,90	7,88	3	I
Phe	178	4,15	4,46	1,29	8,03	3	I
Ant	178	4,47	4,54	0,045	7,43	3	I
Fla	202	4,69	5,20	0,26	7,90	3	L
Py	202	4,58	5,18	0,135	7,53	3	I
BaA	228	5,55	5,76	0,009	7,56	2B	S
Chr	228	5,49	5,81	0,002	7,59	2B	S
BbF	252	6,08	5,80	0,0015	7,65	2B	S
BkF	252	6,09	6,00	0,0008	7,48	2B	S
BaP	252	5,98	6,13	0,0016	7,12	1	S
DBahA	278	6,28	6,75	0,0006	7,38	2A	S
BghiP	276	6,20	6,63	0,0003	7,15	3	I
IcdP	276	6,54	6,70	0,0002	8,02	2B	S

† Clasificable como: 1 cancerígeno, 2A probable cancerígeno, 2B posible cancerígeno, 3 no clasificable. n.e.: no evaluado

‡ Información S suficiente, L limitada, I insuficiente.

La absorción de PAH se realiza principalmente por el tracto respiratorio, gastrointestinal o la piel. Su mecanismo de adsorción está íntimamente ligado a su alto carácter lipófilo (tabla 1.2) que facilita su difusión por los lípidos y lipoproteínas de las membranas celulares. Una vez absorbidos se distribuyen por todo el organismo pero muestran preferencia por los tejidos grasos. Esto hace que sean compuestos con tendencia a ser acumulados en la cadena trófica (Van der Oost et al., 2003). Dentro del organismo son compuestos biológicamente inertes pero para facilitar su excreción son metabolizados a compuestos más solubles (epóxidos, fenoles, dioles, fenol-dioles, diol-epóxiodos, quinonas y tetroles) y

conjugados con sulfato, glutatión o ácido glucurónico para ser finalmente excretados por las heces vía ácidos biliares y en la orina. Sin embargo la metabolización de PAH produce metabolitos electrofílicos reactivos capaces de formar aductos con el ADN. Estos daños en el ADN si no se reparan pueden generar mutaciones que a su vez pueden constituir la base para la iniciación de cáncer (Fu et al., 2012). En la Fig. 1.3 se muestran las tres posibles rutas metabólicas de activación del benzo[a]pireno (BaP): formación de diol-epóxidos, intermedios de radicales catiónicos y quinonas, que han sido descritas en animales de experimentación (IARC 2010). Fuera de los organismos vivos, también pueden ser activados por efecto de la radiación solar dando lugar al favorecimiento de la aparición de cáncer de piel (Fu et al., 2012).

Han sido reportados multitud de casos de cáncer en trabajadores de las actividades industriales que se han señalado anteriormente como productoras de PAH. Por ejemplo en plantas de gasificación de carbón y fabricación de coque se ha observado incremento de la tasa de afección de cáncer de pulmón o en las plantas de creosotado de madera un aumento en la incidencia del cáncer de piel y pulmón (IARC 2010).

Aunque la carcinogenicidad de los PAH sea la toxicidad más reportada y reconocida en la literatura, estos compuestos también poseen otros tipos de toxicidad como son (IARC 2010):

- Impacto en el desarrollo del feto humano disminuyendo el peso, longitud y tiempo de gestación.
- Disminución de la capacidad reproductora de los machos (ratas) por daños en el esperma.
- Toxicidad a altas dosis en hígado y riñón.

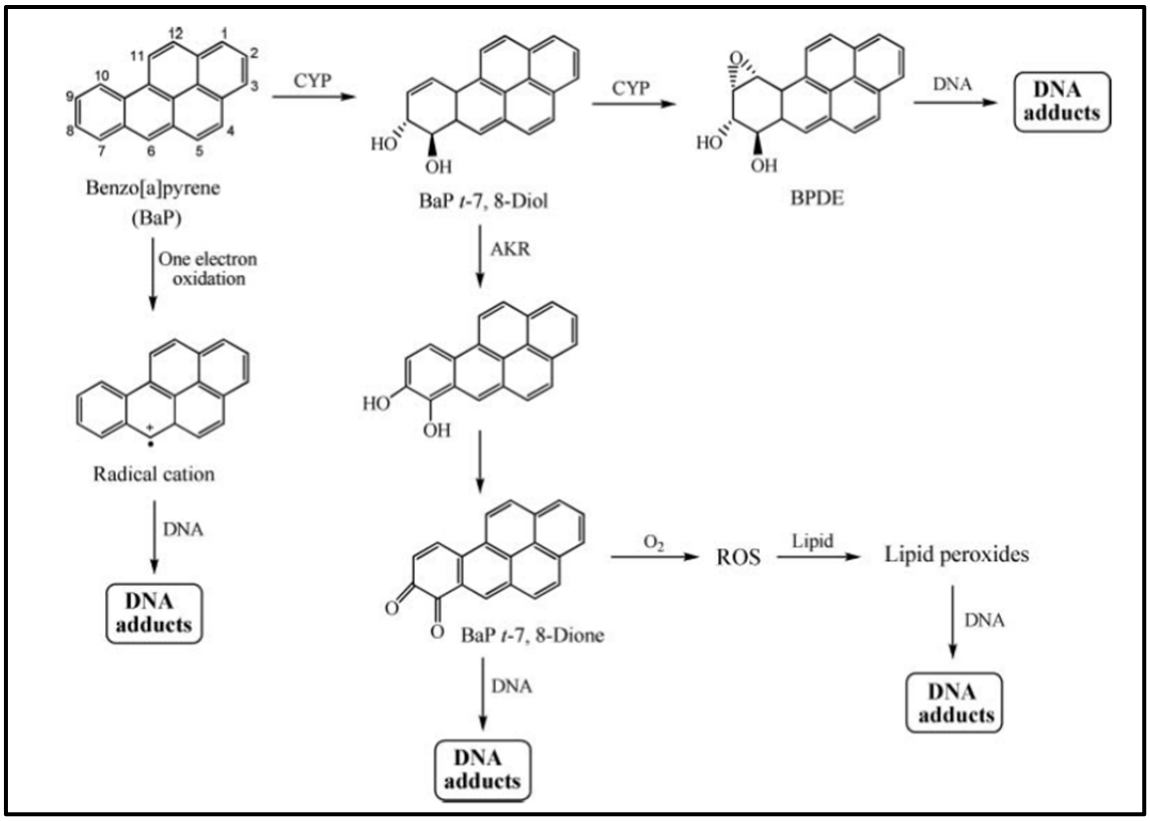

Fig. 1.3: Posibles rutas metabólicas de activación del BaP que conducen a la iniciación de cáncer (Fu et al., 2012).

Medioambientalmente existe una gran preocupación debido a su ubicuidad, resistencia a la biodegradación aumentando según lo hace su peso molecular, potencial de bioacumulación y actividad carcinogénica (Haritash and Kaushik, 2009). A pesar de que son los principales contaminantes del aire, el suelo actúa como depositario último de estos compuestos. En el suelo los PAH son retenidos por la fracción orgánica (Guo et al., 2010; Huang et al., 2003) e inorgánica como las arcillas (Hundal et al., 2001; Meleshyn and Tunega, 2011) o los oxihidróxidos de hierro (Tunega et al., 2009).

La biodegradación de PAH por los organismos del suelo suele ser lenta debido a su baja biodisponibilidad y alto potencial de ionización. Sin embargo ciertas especies de bacterias y hongos presentes en el suelo son capaces de biodegradarlos. En la revisión publicada por Fernández-Luqueño et al. (2011) puede encontrarse una relación de bacterias y hongos con potencial para degradar PAH en condiciones naturales entre las que se encuentran *Pseudomonas* como género de bacterias más característico y *Fusarium* o *Aspergillus* como hongos del suelo.

1.2. Legislación española sobre suelos contaminados

En España el Real Decreto 9/2005 (BOE 2005) establece la relación de actividades potencialmente contaminantes del suelo y los criterios y estándares para la declaración de suelos contaminados, que en el año 2011 fue ampliada y modificada parcialmente por la Ley 22/2011 (BOE 2011) de residuos y suelos contaminados. En esta legislación, se define el término suelo contaminado como aquel cuyas características han sido alteradas negativamente por la presencia de componentes químicos de carácter peligroso procedentes de la actividad humana, en concentración tal que comporte un riesgo inaceptable para la salud humana o el medio ambiente, de acuerdo con los criterios y estándares que se determinen por el Gobierno, y así se haya declarado mediante resolución expresa.

Las Comunidades Autónomas tienen la competencia de la declaración de suelos contaminados y son las encargadas de elaborar y mantener actualizado un inventario con los suelos declarados como contaminados. Todas ellas remitirán sus correspondientes inventarios al Ministerio de Agricultura, Alimentación y Medio Ambiente (MAGRAMA) que será el encargado de la realización del inventario nacional de suelos contaminados.

Un suelo será declarado como contaminado cuando se determinen riesgos inaceptables para la protección de la salud humana o, en su caso, de los ecosistemas, debido a la presencia de alguna de las sustancias contaminantes recogidas en los anexos V y VI del RD 9/2005 (BOE 2005) o de cualquier otro contaminante químico. En aquellas circunstancias en que no se disponga de la correspondiente valoración de riesgos, los órganos competentes de las comunidades autónomas podrán asumir que el riesgo es inaceptable y, en consecuencia, declarar un suelo como contaminado cuando concurra alguna de las siguientes circunstancias:

- En aquellos casos que se considere prioritaria la protección de la salud humana:
    - Que la concentración en el suelo de alguna de las sustancias recogidas en el anexo V excede 100 o más veces los niveles genéricos de referencia establecidos en él para la protección de la salud humana, de acuerdo con su uso.
    - Que la concentración en el suelo de cualquier contaminante químico no recogido en el anexo V para ese suelo excede 100 o más veces el nivel genérico de referencia calculado de acuerdo con los criterios establecidos en el anexo VII.
- En aquellos casos que se considere prioritaria la protección de los ecosistemas:
    - Que la concentración letal o efectiva media para organismos del suelo en ensayos de toxicidad es inferior a 10 mg de suelo contaminado por g de suelo.
    - Que la concentración letal o efectiva media para para organismos acuáticos en ensayos de toxicidad con lixiviados es inferior a 10 ml de lixiviado por litro de agua.

La declaración de suelo contaminado obliga a realizar las actuaciones necesarias para la limpieza y recuperación del mismo en la forma y plazo que determine la correspondiente Comunidad Autónoma. Los responsables de la descontaminación y recuperación del suelo serán los causantes de la contaminación y subsidiariamente los propietarios de los suelos contaminados y los poseedores de los mismos. El RD 9/2005 (BOE 2005) prioriza en la medida de lo posible las técnicas de tratamiento in situ que eviten la generación, traslado y eliminación de residuos. Las Comunidades Autónomas declararán que un suelo ha dejado de estar contaminado tras la comprobación de que se han realizado de forma adecuada las operaciones de descontaminación y recuperación del mismo e incluirán esta declaración en el inventario de suelos contaminados.

1.3. Técnicas de remediación de suelos contaminados

Las técnicas de remediación de suelos más habituales en Europa son las tradicionales. En concreto, el 30% de los lugares remediados ha sido por la técnica de la excavación y eliminación en vertederos lo que realmente no supone una solución definitiva del problema.

Según el RD 9/2005 (BOE 2005) sobre suelos contaminados, la declaración de un suelo como contaminado obliga a proceder a su recuperación ambiental. Las actividades que se realicen con este fin deben asegurar que la contaminación remanente si la hubiera, se traduzca en niveles de riesgo aceptables de acuerdo con el uso del suelo. En la medida de lo posible las actuaciones deben eliminar el foco de la contaminación y reducir la concentración de los contaminantes aunque si esto no es posible, también se podrán aceptar soluciones encaminadas a reducir la exposición mediante medidas de confinamiento o contención de los suelos contaminados.

Finalmente un suelo contaminado perderá esta condición cuando se realicen actuaciones de descontaminación, que en función del uso del suelo, garanticen que el suelo ha dejado se suponer un riesgo inadmisible para el objeto de protección, salud humana o ecosistemas y previa comprobación, la administración haga firme una resolución que así lo declare.

De este breve resumen de la legislación se puede deducir que se priman las actividades de recuperación por este orden:

1. Eliminación del foco de la contaminación.
2. Técnicas de descontaminación enfocadas a la reducción de la concentración de los contaminantes.
3. Técnicas de confinamiento de los contaminantes que reduzcan el nivel de riesgo para la salud humana o ecosistemas.

Y que la recuperación o descontaminación de un suelo no implica necesariamente la eliminación de la sustancia contaminante del suelo, por ello las técnicas de inmovilización de contaminantes son adecuadas siempre que reduzcan suficientemente el nivel de riesgo del suelo para su uso y el objeto de protección pertinente (salud humana o ecosistemas).

Por todo ello, las posibles técnicas de recuperación de suelos contaminados son muy variadas y la elección de una u otra depende de varios factores como pueden ser la naturaleza del contaminante/s, eliminación o confinamiento, posibilidad o imposibilidad de tratamiento *in situ*, necesidad de exhaustividad de la descontaminación en función del uso del suelo, tecnología disponible, tiempo requerido para el proceso, costo del tratamiento, etc.

Entre PAH (o contaminantes orgánicos en general) y metales pesados existen múltiples diferencias pero hay una que condiciona claramente las técnicas de remediación a aplicar en los suelos contaminados. Los metales no son susceptibles de ser degradados o destruidos mientras de los PAH sí. Por ello, la única forma de reducir la concentración de metales pesados en el suelo es mediante su extracción y posterior confinamiento en otro medio. Mientras que los PAH pueden ser extraídos o transformados en otros compuestos mediante técnicas físico-químicas o biológicas. Tradicionalmente las técnicas de remediación de suelos se clasifican en térmicas, físico-químicas y biológicas. En la tabla 1.2 se enumeran algunas de las más utilizadas (Gan et al., 2009; Khan et al., 2004; Mao et al., 2015; Mulligan et al., 2001; Tejeda-Agredano et al., 2013; Xu et al., 2015).

Tabla 1.2: Clasificación de las técnicas de remediación de suelo habituales

Clasificación	Tecnología
Fisico-química	Extracción con solventes (agua, disolventes orgánicos, surfactantes, ciclodextrinas, aceites vegetales, complejantes), extracción con fluidos supercríticos o subcríticos, extracción de vapores, remediación electrocinética, solidificación/estabilización.
Térmica	Incineración, vitrificación, inyección de aire caliente, desorción térmica, extracción de vapor asistido por calor
Química	Oxidación química con oxidantes (Reactivo Fenton, ozono, $KMnO_4$, persulfato sódico), degradación fotocatalítica
Biológica	Biodegradación (tratamientos aeróbios o anaeróbios, compostaje, laboreo, biopilas), fitorremediación, biolixiviación de metales

La aplicación de una u otra técnica de remediación no es excluyente y de hecho es común que para la recuperación de un suelo se empleen varias técnicas de forma secuencial con el objetivo de aumentar la eficacia de la descontaminación o poder actuar sobre diferentes contaminantes (Gan et al., 2009; Gong et al., 2015).

De todas estas técnicas, las biológicas son las que a priori, producen un menor impacto en el suelo (Semple et al., 2001) y por ello son las que parecen mostrarse como la mejor opción desde el punto de vista medioambiental.

1.4. Biorremediación de suelos contaminados

La remediación de suelos es un tema de actualidad debido a una creciente concienciación ambiental de la sociedad. La comunidad científica también se ha hecho eco de esta preocupación. Si atendemos al número de publicaciones científicas, la búsqueda en la colección principal de Web of Science del término "soil *remediation" arroja 20.419 resultados. Acotando la búsqueda a los últimos 5 años completos (2010 – 2014) se obtienen 8.022 resultados. Esto supone el 39% los trabajos publicados durante toda la serie histórica. Si se restringe la búsqueda al término "soil bioremediation" se obtienen 7.412 trabajos para toda la serie histórica (1988 – 2014) (Fig. 1.4) de los que 2.831 corresponden al periodo 2010 – 2014, y representan el 38% del total de trabajos de biorremediación.

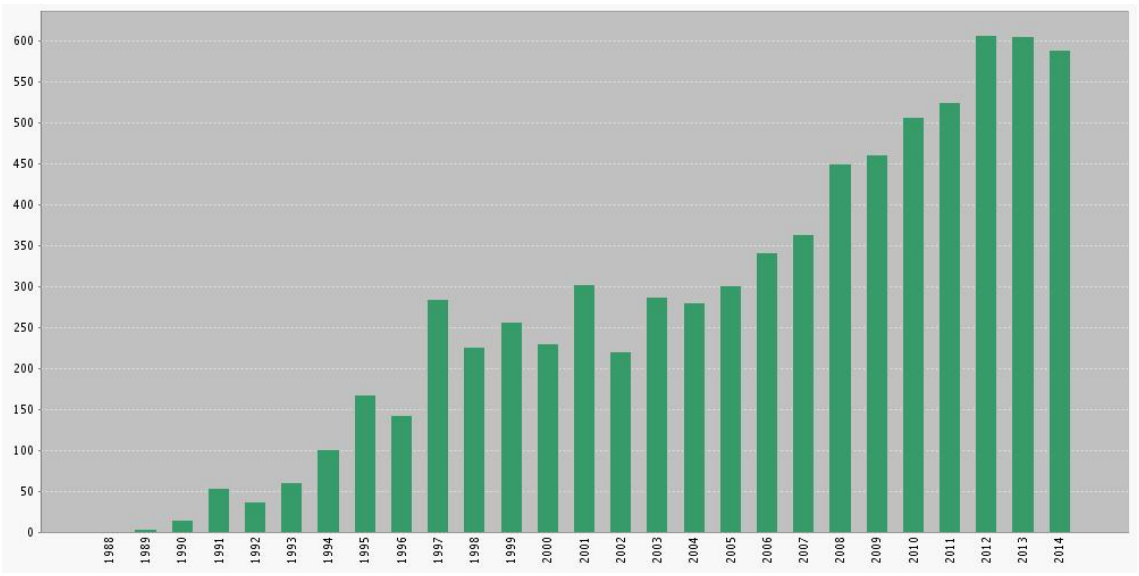

Fig. 1.4: Número de trabajos publicados e indexados en Web of Science referidos a "soil bioremediation" en el periodo comprendido entre 1988 y 2014.

Como se aprecia en la Fig. 1.4 el número de trabajos publicados anualmente está en aumento, doblándose el número de publicaciones desde 2005 hasta el 2014. Todos estos datos dan una idea de la importancia que tiene en la actualidad la remediación de suelos y más concretamente la biorremediación.

La biorremediación de suelos puede realizarse *in situ* o *ex situ*, pero en ambas modalidades de tratamiento se basan en la utilización de organismos vivos para eliminar o inmovilizar contaminantes. La eliminación de contaminantes orgánicos se realiza mediante metabolización de los compuestos por diversos organismos como bacterias, hongos, algas, plantas o consorcio de alguno de ellos (Haritash and Kaushik, 2009). A diferencia de los contaminantes orgánicos, los metales no pueden ser degradados, por lo tanto su eliminación del suelo sólo puede hacerse mediante transporte a otro lugar previa extracción. Actualmente las técnicas más populares de biorremediación para la extracción de metales de suelos contaminados son la fitoextracción y la biolixiviación con bacterias litotróficas.

En la biorremediación de PAH, tradicionalmente han sido empleadas técnicas *in situ* como la fitorremediación, compostaje y biopilas, pero con el objetivo de aumentar la eficiencia se ha recurrido también a técnicas *ex situ* como los biorreactores (Gan et al., 2009). La mayor ventaja de la biorremediación de suelos contaminados con compuestos orgánicos (PAH) es la ausencia de generación de residuos al final del proceso aunque puedan producirse intermedios tóxicos durante este (Andersson et al., 2003; Gan et al., 2009). El mayor inconveniente de esta técnica es el mayor tiempo necesario para llevar a cabo la remediación del suelo en comparación con tecnologías físicas o químicas como pueden ser el lavado con disolventes, tratamiento térmico, extracción con fluidos supercríticos y la oxidación con ozono o reactivo Fenton. Aunque para minimizar este inconveniente, la biorremediación también puede acoplarse a los tratamientos físico-químicos aumentando la efectividad del proceso y disminuyendo el tiempo de tratamiento (Haapea and Tuhkanen, 2006; Kulik et al., 2006).

Existen dos grandes grupos de posibles técnicas de biorremediación de suelos contaminados con compuestos orgánicos (incluidos PAH): bioestimulacion y bioaumentación. La biostimulación consiste en la adición al suelo de sustratos, oxígeno o nutrientes en general con la finalidad de favorecer el desarrollo de la

microbiología autóctona del suelo y su capacidad para degradar contaminantes orgánicos. La bioaumentación es el proceso que involucra la inoculación y colonización del suelo contaminado por microorganismos exógenos capaces de degradar los contaminantes presentes en el suelo.

Ciertas características del suelo, (humedad, aireación, nutrientes, co-sustratos, materia orgánica o textura), del propio contaminante (principalmente la toxicidad y solubilidad en agua) y la interacción entre suelo y contaminante (biodisponibilidad) afectan notablemente al proceso de degradación de los contaminantes orgánicos (Fernández-Luqueño et al., 2011).

Los principales organismos responsables de la biodegradación de PAH en el suelo son bacterias y hongos. Si bien su mecanismo de acción y requerimientos para una alta efectividad del proceso remediador son muy diferentes. A continuación se detalla el mecanismo de acción de bacterias y hongos en la biodegradación de PAH.

1.4.1. Degradación de PAH por bacterias

El principal mecanismo de degradación de PAH por bacterias consiste en la absorción del PAH y su posterior metabolización en el interior celular. Este requerimiento de absorción conlleva que los PAH tienen que estar en formas disponibles, es decir, en la fase acuosa del suelo o débilmente adsorbido en la fase sólida. Por ello, como norma general se asume que el número de anillos o peso molecular de los PAH es indicativo de su resistencia a la biodegradación por bacterias, siendo los PAH con más anillos los más recalcitrantes (Fernández-Luqueño et al., 2011; Haritash and Kaushik, 2009). Sin embargo, se han encontrado un limitado número de especies bacterianas capaces de desarrollarse en medios con PAH de 5-6 anillos, entre ellas se encuentran varias especies de *Pseudomonas*. La principal razón de esto, es la alta retención de este tipo de PAH en fases sólidas que ralentizan su transferencia al interior de la célula impidiendo un correcto metabolismo (Fernández-Luqueño et al., 2011).

Con el objetivo de minimizar el efecto de la baja disponibilidad de los PAH para las bacterias se han empleado diferentes surfactantes capaces de movilizar

estos contaminantes aunque se ha observado que algunos surfactantes poseen propiedades tóxicas para la microbiología del suelo y no aumentan la tasa de degradación de PAH (Lladó et al., 2013; Mao et al., 2015).

La degradación de PAH por baterías puede ser realizada en condiciones areobias o anaerobias. El mecanismo más importante de degradación aerobio de PAH por parte de bacterias es la oxidación inicial del PAH por enzimas dioxigenasas que forman *cis*-dihidrodioles. Posteriormente son deshidrogenados formando intermedios dihidroxilados que a su vez son metabolizados vía catecol hasta CO_2 y agua. En la Fig. 1.5 se muestra la ruta de degradación del naftaleno por bacterias aerobias. También existen bacterias como *Mycobacterium* sp., capaces de oxidar PAH vía monooxigenasa citocromo P-450 a *trans*-dihidrodioles.

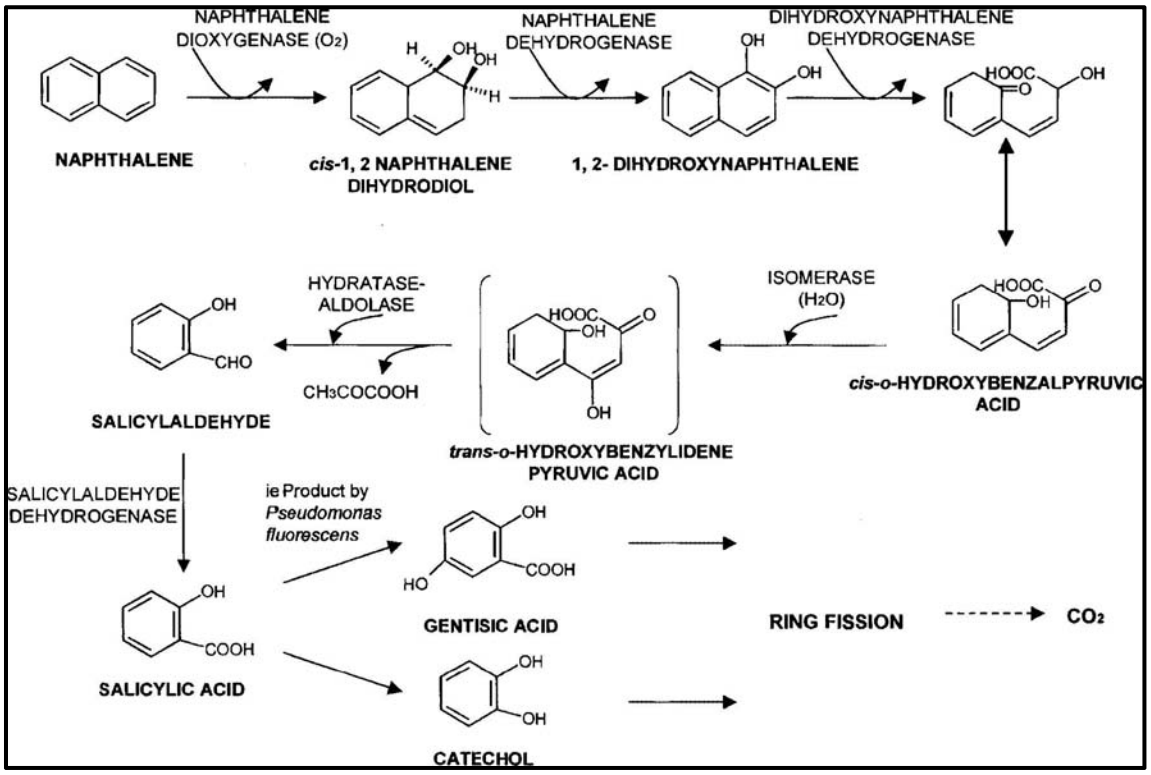

Fig. 1.5: Principal ruta de degradación de naftaleno por bacterias aerobias (Bamforth and Singleton, 2005).

En las revisiones realizadas por Juhasz y Naidu (2000), Haritash y Kaushik (2009) y Fernández-Luqueño et al. (2011) pueden encontrarse amplias relaciones de bacterias degradadoras de PAH y cientos de estudios en los que mediante la acción de bacterias, tanto autóctonas como alóctonas, se obtuvo degradación de PAH. Sin embargo, la mera presencia de bacterias capaces de degradar PAH no

aseguran adecuadas tasas de degradación de PAH, siendo necesario una población mínima que se estima en 10^5 UFC g^{-1} (Liu et al., 2010). Tanto en descontaminación con bacterias autóctonas como alóctonas, se recurre a la adición de enmiendas orgánicas o nutrientes con el objetivo de asegurar una alta población bacteriana durante la remediación del suelo.

En el caso de suelos con reducida porosidad y permeabilidad donde el oxigeno es limitado, la degradación bacteriana anaerobia es una buena alternativa. En este caso la degradación bacteriana de PAH se produce mediante el uso de nitrato, sulfato, hierro, manganeso o dióxido de carbono como aceptores finales de electrones obteniendo dióxido de carbono o metano como productos finales (Gan et al., 2009; Tomei and Daugulis, 2013). El mecanismo de degradación no parece estar muy claro en estas condiciones pero puede dividirse en dos etapas. En la etapa inicial los PAH son parcialmente degradados en condiciones sulfato y nitrato reductoras con la formación de ácidos orgánicos de bajo peso molecular como intermedios que a su vez actúan como complejantes de Fe(III) facilitando su disolución. Finalmente el Fe(III) movilizado se encuentra disponible para las bacterias reductoras de Fe que intensifican la degradación de PAH (Haritash and Kaushik, 2009).

1.4.2. Degradación de PAH por hongos

La degradación de PAH por hongos tiene lugar por dos posible rutas en función de si se trata de un hongo ligninolítico o no ligninolítico. La mayoría de los hongos son no ligninolíticos (carecen de enzimas ligninolíticas). Su principal ruta de degradación es la oxidación de anillo aromático vía citocromo P-450 formando en primer lugar un epóxido que es hidratado dando lugar a *trans*-dihidrodiol. Estos dioles, así como posibles fenoles formados pueden ser finalmente sulfonados, metilados o conjugados con glucosa, xilosa o ácido glucónico. El resultado es un compuesto menos tóxico, más polar y por lo tanto soluble en agua que el PAH de partida. Algunos ejemplos de hongos que siguen este ruta son: *Chrysospirum pannorum*, *Cunninghamella elegans* y *Aspergillus niger* (Bamforth and Singleton, 2005).

Los hongos ligninolíticos, también llamados hongos de podredumbre blanca, han sido ampliamente estudiados para biorremediación de suelos. Las especies más utilizadas en estos estudios de biorremediación han sido *Phanerochaete chrysosporium, Bjerkandera adusta, Trametes versicolor* y *Pleurotus ostreatus* aunque muchos otros han sido estudiados con éxito para este fin como pueden ser *Irpex lacteus, P. eryngii, Anthracophyllum discolor* o *Lentinus tigrinus* entre otros (Acevedo et al., 2011; Covino et al., 2010a; Haritash and Kaushik, 2009; Novotný et al., 2009; Stajić et al., 2009).

Estos hongos se caracterizan por segregar enzimas ligninolíticas cuya función es degradar la lignina presente en la madera y otros tipos de materia orgánica vía formación de radicales. Las principales enzimas ligninolíticas son peroxidasas (Mn-Peroxidasa y lignina peroxidasa) y fenol oxidasas (lacassa). Poseen baja especificidad enzima-sustrato, alto potencial de ionización, y capacidad para difundir en la fase sólida del suelo por lo que no necesitan que el compuesto sobre el que van a actuar se encuentre en disolución. Sus características hacen de las enzimas ligninolíticas excelentes candidatas para la oxidación de contaminantes orgánicos, incluidos PAH.

La enzima laccasa (EC 1.10.3.2) es una proteína extracelular excretada por hongos, aunque también se ha encontrado en plantas, cuya finalidad es degradar lignina. Es una enzima excretada por la práctica totalidad de los hongos ligninolíticos. Oxida una amplia variedad de sustratos incluidos mono-, di- o polifenoles, aminofenoles, metoxifenoles, y aminas aromáticas usando oxígeno como aceptor final de electrones. La enzima está formada por unos 500 – 520 aminoácidos y en su centro activo se encuentran cuatro átomos de cobre responsables de la transferencia electrónica, con diferentes características de absorción en UV y paramagnetismo. Se denominan cobre tipo 1 (T1) (fuerte absorción a 600 nm, responsable del color azul de la enzima y detectable en resonancia electrónica paramagnética (EPR)), T2 (incoloro pero detectable en EPR) y dos átomos de cobre T3 (absorbancia débil UV y no señal en EPR). Los tres átomos de cobre T2 y T3 forman un cluster donde tiene lugar la reducción del O_2 a H_2O mediante intermedio epóxido. La oxidación del sustrato tiene lugar en el átomo de cobre T1 y es el que desencadena la reacción (Fernández-Fernández et

al., 2013). Sin embargo su potencial de ionización (IP) es relativamente bajo (IP ≤ 7,45 eV) (Haritash and Kaushik, 2009) comparado con otras enzimas ligninolíticas, lo que limita su potencial oxidativo. Esta limitación puede ser suplida mediante la utilización de mediadores redox como el ABTS que reacciona con la enzima laccasa produciendo un intermedio con alto potencial oxidativo capaz de oxidar el sustrato de alto IP. Estos mediadores han sido utilizados con éxito para degradar ciertos PAH o tintes sintéticos (Fernández-Fernández et al., 2013). Entre los PAH susceptibles de ser degradados por la laccasa se encuentra el BaP que es oxidado a diversas quinonas entre las que se identificaron 6,12-BaQ, 3,6-BaQ y 1,6-BaQ (Xie et al., 2010).

La enzima Mn-Peroxidasa (MnP; E.C. 1.11.1.13) es una hemo proteína glicosilada con peso molecular entre 38 y 62,5 kDa capaz de oxidar el Mn^{2+} a Mn^{3+}. A diferencia de la laccasa solo algunos basidiomicetos poseen los genes que la codifican (Janusz et al., 2013). El ciclo catalítico (Fig. 1.6) comienza con unión de H_2O_2 o un peróxido orgánico al hierro del grupo hemo formando un complejo Fe-peróxido. La rotura del enlace O-O del peróxido requiere la transferencia de 2 electrones desde el grupo hemo con lo que se forma el complejo MnP I (Fe IV). Después se rompe el enlace O-O del peróxido produciendo agua. El Mn^{2+} actúa como donador de un electrón y es oxidado a Mn^{3+}. En ese momento se forma el complejo MnP II que de forma similar vuelve a oxidar a un ion Mn^{2+} a Mn^{3+} para completar el ciclo catalítico. Ésta última oxidación de Mn^{2+} es específica para este ion e imprescindible para que el ciclo catalítico se complete. El Mn^{3+} es una especie muy oxidante pero a su vez altamente inestable por lo que los hongos segregan oxalato, malonato o tartrato para complejarlo. El complejo de Mn^{3+} es el que difunde en el medio y oxida a la lignina (Hofrichter, 2002).

La lignina peroxidasa (LiP; E.C. 1.11.1.14) está formada por 343 – 345 aminoácidos (Janusz et al., 2013), estructuralmente es muy similar a la MnP y su mecanismo de reacción sólo difiere en que el complejo II, en este caso puede reaccionar con sustratos diferentes al Mn^{2+} como fenoles (Hofrichter, 2002).

Todas estas enzimas poseen múltiples isoenzimas, por ejemplo se han descrito 16 isoenzimas de LiP para *T. veriscolor*, 11 isoformas de MnP en

Ceriporiopsis subvermispora y 8 en el caso de laccasa para *P. ostreatus* (Janusz et al., 2013).

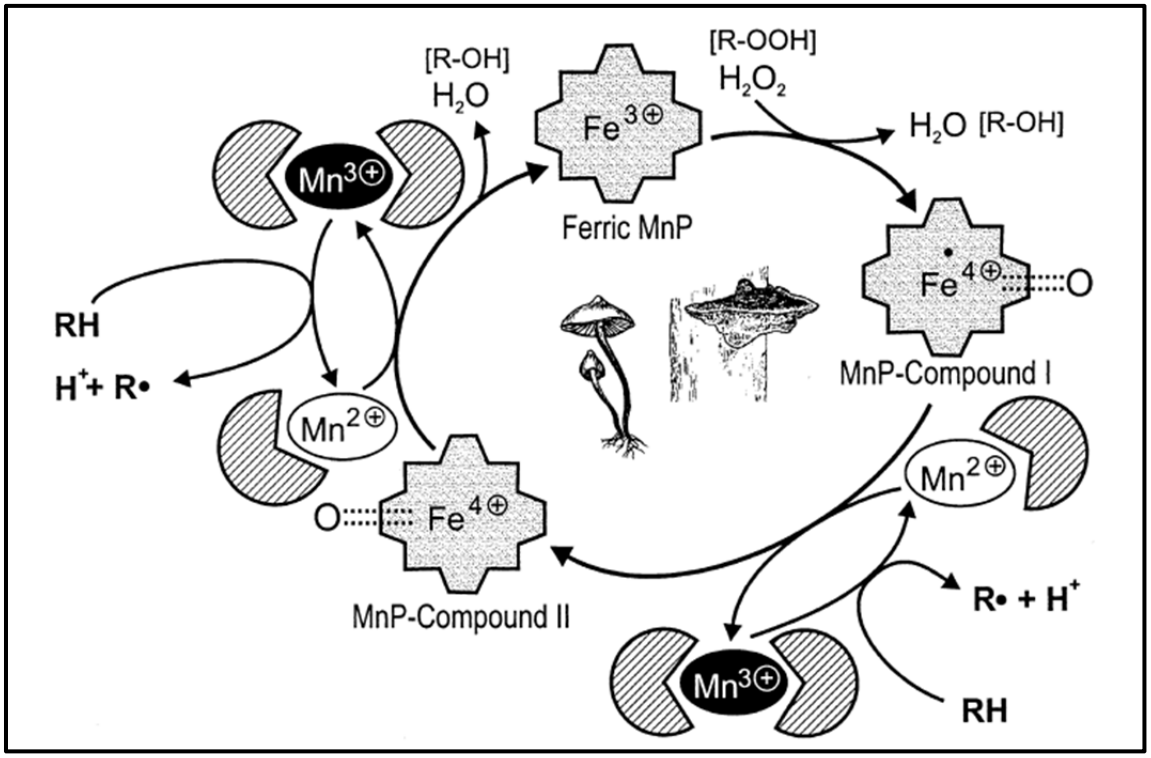

Fig. 1.6: Ciclo catalítico de la enzima Mn-Peroxidasa (Hofrichter, 2002)

La alta inespecificidad enzima – sustrato que poseen se debe en gran medida a que la lignina posee estructuras muy variadas y múltiples grupos funcionales sobre los que estas enzimas tienen que actuar. Como consecuencia las enzimas ligninolíticas pueden actuar sobre multitud de compuestos orgánicos y sus potenciales uso biotecnológicos son muy prometedores (Fernández-Fernández et al., 2013; Hofrichter, 2002). Entre estas aplicaciones destaca la degradación de contaminantes orgánicos. Se ha descrito su capacidad de degradar clorofenoles, tintes, bisfenol A, PCBs, antibióticos y PAH entre otros contaminantes orgánicos (Ba et al., 2013; Chang et al., 2014; Čvančarová et al., 2015; Haritash and Kaushik, 2009; Novotný et al., 2004; Yang et al., 2013).

De forma general, la degradación de PAH mediante enzimas ligninolíticas se basa en la producción de radicales libres. En la Fig 1.7 se muestran posibles rutas de degradación de antraceno por *I. lacteus*. El resultado de una primera oxidación

es la formación de quinonas (metabolito 5) que pueden volver a ser oxidadas por estas enzimas o degradadas en el interior del hongo vía citocromo P-450.

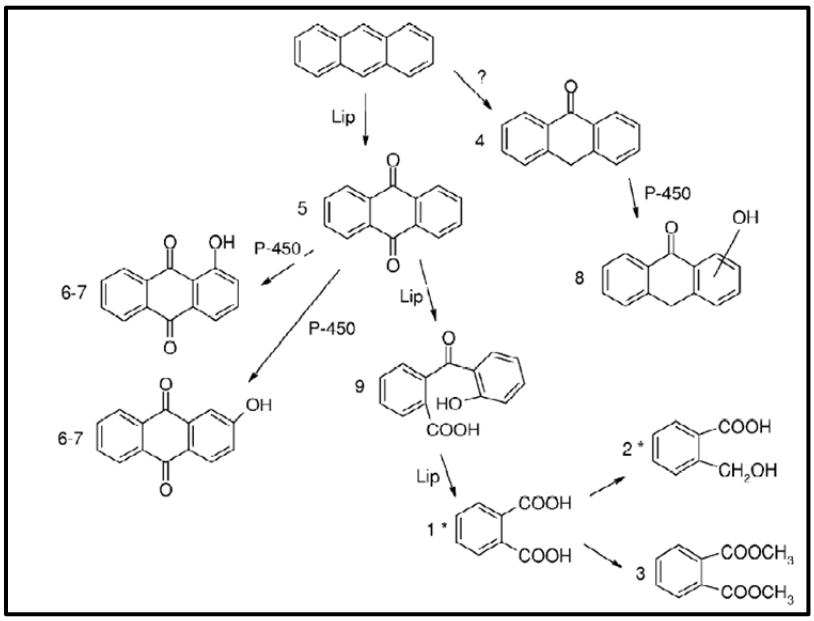

Fig. 1.7: Ruta de degradación de antraceno por *I. lacteus* (Cajthaml et al., 2002).

Sin embargo parece ser que la degradación de PAH mediante hongos ligninolíticos no se limita a su sistema ligninolítico porque se ha observado una falta de correlación entre degradación de PAH y actividad ligninolítica (Haritash and Kaushik, 2009). Este tipo de hongos también son capaces de biodegradar PAH en el interior del organismo mediante la acción del citocromo P-450 (Bhattacharya et al., 2013; Covino et al., 2010c). Incluso durante la fase de crecimiento del hongo y en ausencia de la necesidad de enzimas extracelulares, la degradación de PAH se realiza mayoritariamente por absorción de PAH y degradación en el interior del hongo (Haritash and Kaushik, 2009).

La inoculación de hongos en el suelo se realiza mediante sustratos orgánicos que sirven de soporte y alimento al hongo. Se han propuesto multitud de materiales ligninolíticos para este fin como pueden ser paja de trigo o maíz, pellets de paja de trigo, mazorcas de maíz, grano de trigo y sustratos de cultivo de hongos (Borràs et al., 2010; Covino et al., 2010a, 2010b; D'Annibale et al., 2005; Leonardi et al., 2008; Li et al., 2012; Lladó et al., 2013; Márquez-Rocha et al., 2000). Algunos autores también han propuesto la utilización de sustrato post-cultivo de hongos como inóculo, concretamente para *P. ostreatus* (Eggen, 1999; Mauricio-Gutiérrez et al., 2014).

Estos materiales orgánicos se emplean como fuente de nutrientes y soporte para el hongo que se inocula, sin embargo debido a sus características producen efectos sobre el resto de la microbiología del suelo o sobre los propios contaminantes.

1.4.3. Efectos de las enmiendas orgánicas sobre la microbiología del suelo y los contaminantes

Históricamente se ha aplicado materia orgánica al suelo como fertilizante con el objetivo final de incrementar la producción agrícola. Sin embargo, ahora se sabe que la aplicación de materia orgánica en el suelo produce cambios mucho más profundos afectando tanto a sus características físico-químicas como a la microbiología del suelo.

La utilización de enmiendas orgánicas con fines medioambientales está ampliamente implantada. Se han utilizado con éxito en suelos contaminados tanto con metales pesados como con contaminantes orgánicos o combinación de ambos para su biorremediación por inmovilización de los contaminantes o como promotor de la degradación de compuestos orgánicos entre los que se encuentran los PAH (Beesley et al., 2010; Galende et al., 2014; Karami et al., 2011; Manzano et al., 2014; Marín-Benito et al., 2014).

El número y naturaleza de las enmiendas orgánicas es muy variado. Como generalidad suelen ser producidas con subproductos o residuos agrícolas, ganaderos, forestales o urbanos. El procesamiento más habitual previo a su utilización en el suelo es el compostaje aunque en la actualidad el tratamiento térmico mediante pirolisis para la producción de biochar se está popularizando (Beesley et al., 2011). Este material es capaz de adsorber tanto metales como compuestos orgánicos en el suelo de forma que se limita su movilidad aunque se ha observado que la composición inicial del material orgánico y la temperatura de pirolisis varían notablemente su capacidad de adsorción (Brennan et al., 2014; Zhang et al., 2013).

Los principales efectos beneficiosos de la aplicación de enmiendas orgánicas son: mejora del estado nutricional del suelo, mejora de la porosidad y estructura e

incremento de la actividad microbiológica del suelo (Park et al., 2011). Estos beneficios tienen repercusiones directas en el proceso de biodegradación de PAH (o contaminantes orgánicos en general) por la mejora de la habitabilidad del suelo para los microorganismos y plantas. Se ha reportado que la aplicación de enmiendas orgánicas al suelo contaminado incrementa la población bacteriana y fúngica (biostimulación) y como consecuencia aumenta la tasa de degradación de contaminates (Federici et al., 2012; Li et al., 2012; Lladó et al., 2013, 2012; Wu et al., 2013). Además en función de su composición y propiedades físico-químicas será capaz de adsorber PAH en mayor o menor medida modificando la disponibilidad de los mismos (Guo et al., 2010; Yang et al., 2010). De acuerdo a Marín-Benito et al. (2012b), los procesos de adsorción de contaminantes orgánicos en la materia orgánica se encuentra controlado por la polaridad y los grupos funcionales del contaminante y el tipo de materia orgánica.

De este parámetro, no sólo depende la movilización o inmovilización de PAH, también influencia fuertemente su degradación. Sayara et al. (2010a, 2010b) realizó estudios de compostaje de suelo contaminado con PAH como técnica de remediación, usando compost de diferentes grados de estabilidad y observó como la aplicación de materia orgánica más estable produce mayor degradación de PAH a pesar de que la materia orgánica menos estable producía mayor actividad microbiológica. Este hecho puede ser explicado por la formación de ácidos húmicos durante el proceso de compostaje de la materia orgánica que pierde carácter alifático aumentando su polaridad, como resultado se produce una disminución de la capacidad de adsorción de PAH, que facilita la disponibilidad de PAH a los microorganismos favoreciendo su degradación (Plaza et al., 2009).

La aplicación de enmiendas orgánicas sobre suelos contaminados por metales pesados suele estar enfocada a su inmovilización, sin embargo dependiendo de las características de la enmienda utilizada, como puede ser su pH y contenido en carbono orgánico soluble, el efecto puede ser el contrario (Herrero-Hernández et al., 2011).

El uso de enmiendas orgánicas también se encuentra relacionado con la fitorremediación siendo su uso conjunto una práctica muy habitual. En este escenario, la enmienda orgánica mejora las características físico-químicas del

suelo, su estado nutricional y detoxifica el suelo mediante inmovilización de metales pesados favoreciendo el enraizamiento y desarrollo de la planta (Frutos, 2009). Pardo et al. (2014) combinaron la aplicación de materia orgánica con fitoestabilización en suelos contaminados por actividades mineras. Los resultados fueron la disminución de la toxicidad del suelo y estimulación de su microbiología, como consecuencia de ello se obtuvo una mejora de la habitabilidad del suelo y la reactivación de los ciclos biogeoquímicos.

Según la revisión realizada por Park et al. (2011) los principales mecanismos de biorremediación de metales con enmiendas orgánicas son inmovilización, reducción y volatilización.

- La inmovilización de metales por aplicación de enmiendas orgánicas se debe a fenómenos de adsorción tanto en la materia orgánica como en la fracción inorgánica (fosfatos y óxidos de Al, Fe, Mn). Además, si la enmienda es básica se produce precipitación de metales en forma de hidróxidos debido al incremento del pH del suelo.
- La reducción de ciertos metales o metaloides con propiedades redox (Cr, Hg, Se, As) con enmiendas orgánicas se produce tanto por reacción entre los metal(oid)es y la materia orgánica, como por acción biológica. Por ejemplo el Cr(IV) es reducido por enmiendas orgánicas a Cr(III) que es menos móvil y tóxico.
- La mayoría de metal(oid)es no pueden ser volatilizados desde el suelo pero As, Se y Hg pueden serlo tras procesos de reducción (Hg) o metalización (As, Se). Estas transformaciones son llevadas a cabo por microorganismos que ven incrementada su actividad con aplicaciones de enmiendas orgánicas como compost, estiércol o residuos vegetales.

1.5. El cultivo de champiñón y otras setas

1.5.1. Producción en España e importancia económica

El cultivo de champiñón (*Agaricus bisporus*) surgió en Francia durante el siglo XVIII. Actualmente es uno de los hongos cultivados más importantes desde el punto de vista económico y su cultivo está ampliamente distribuido por todo el mundo. En España su consumo es muy popular y su producción ha ido aumentando a lo largo de los años. En la Fig. 1.7 se puede ver que la producción española de champiñón ha aumentado significativamente desde finales de los años 90 y se ha mantenido en el entorno de las 125.000 toneladas en lo que llevamos de siglo XXI (MAGRAMA 2013).

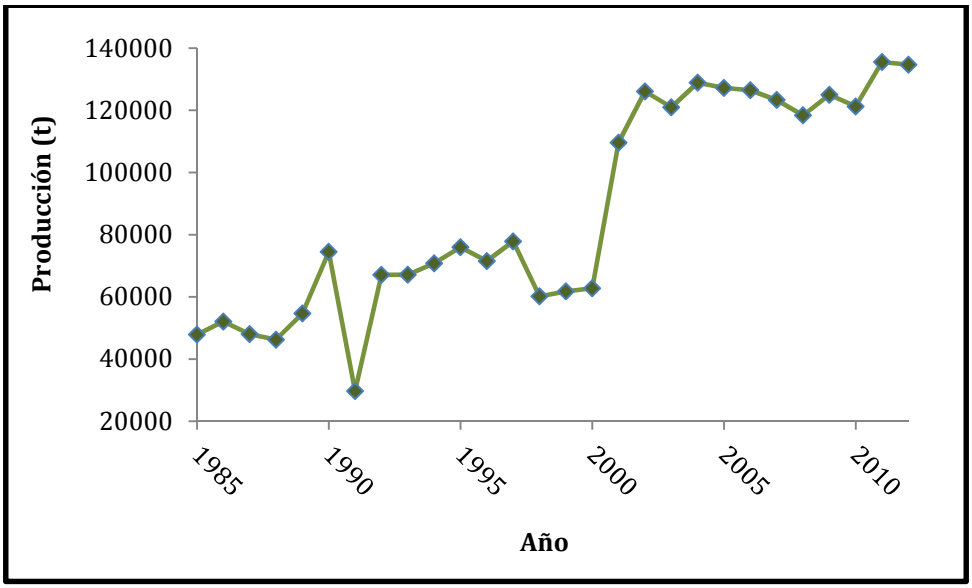

Fig. 1.7: Producción anual de champiñón en España 1985-2012 (MAGRAMA 2013)

Su cultivo en España, así como el de otras setas, está centralizado en dos zonas, La Rioja y la comarca de la Manchuela en Castilla – La Mancha. En estas dos áreas se produce aproximadamente el 95% de la producción española de champiñón. La importancia económica del cultivo de champiñón es alta, sobre todo en las dos principales zonas de cultivo. El precio medio percibido por los agricultores en el periodo 2002 - 2012 es de 104,49 €/100 kg, sobrepasando el valor de la cosecha los 100 millones de € al año. En el último año (2012) con datos disponibles del Ministerio de Agricultura, Alimentación y Medio Ambiente el valor de la cosecha se estimó en 152,88 millones de € (MAGRAMA 2013).

La producción de otras setas es mucho menor que la producción de champiñón. Según datos del Ministerio de Agricultura, Alimentación y Medio Ambiente la producción total de otras setas diferentes al champiñón fue de 12.764 t en el año 2012 (MAGRAMA 2013). Esto equivale a algo menos de 9,5% de la producción de champiñón. Entre las setas cultivadas que se engloban en esta categoría se encuentran *P. ostreatus*, *P. eryngii* y *L. edodes*. Siendo *P. ostreatus* la especie con mayor importancia.

1.5.2. Proceso del cultivo y generación de sustrato post-cultivo

El cultivo de champiñón se realiza en condiciones controladas en naves de cultivo donde se monitoriza la temperatura, humedad y concentración de CO_2. El proceso de crecimiento y fructificación del champiñón se realiza en un sustrato selectivo para este hongo. Su cultivo es relativamente complejo comparado con otros hongos y requiere varias etapas.

El sustrato para el cultivo de champiñón se elabora a partir de paja de trigo, gallinaza, urea, yeso y agua hasta el 70% de humedad. Los componentes se mezclan y se procede a su fermentación en dos fases (Fig. 1.8) El compostaje fase I (fermentación libre) se basa en un corto proceso de compostaje en pilas durante 15 -20 días con volteos cada 4 – 5 días. El compostaje en fase II (pasteurización y acondicionamiento) se lleva a cabo en túneles de pasteurización donde la fermentación del compost se realiza de forma controlada y con inyección de aire. Se incrementa la temperatura del compost gradualmente durante 2 - 3 días hasta los 60 ^{o}C, después se baja la temperatura gradualmente hasta 48 ^{o}C, durante 4 – 6 días en el túnel. La fase II completa dura entre 6 y 8 días. El sustrato se saca del túnel y cuando su temperatura desciende hasta 20 – 24 ^{o}C se inocula (siembra) con micelio de *A. bisporus* desarrollado sobre grano de trigo a razón de 100 – 150 g por 25 kg de sustrato y finalmente se empaqueta con una película plástica de polietileno. En este momento las balas de sustrato con el hongo inoculado se llevan a las naves de cultivo.

Fig. 1.8: Detalle de la fase I y II del proceso de compostaje del sustrato de cultivo de champiñón.

Una vez en las naves, el control de temperatura, humedad y concentración de CO_2 será imprescindible para un correcto desarrollo del hongo (Fig. 1.9). Los paquetes de sustrato son colocados en estantes dentro de la nave. Para favorecer la colonización del sustrato por *A. bisporus* se mantiene la temperatura del sustrato entre 25 y 27 ^oC, humedad alta y se limitará la ventilación de la nave para aumentar el nivel de CO_2 hasta el 3%. Esta fase de germinación llevará unos 15 - 20 días hasta conseguir la correcta y completa colonización del sustrato.

En la siguiente etapa se distribuye en la superficie del sustrato una capa de cobertura necesaria para que se produzca la floración del champiñón. El material de la cobertura en el pasado era una mezcla de suelo calizo y turba rubia pero los cultivadores han ido derivando hacia una mezcla de turba negra y carbonato cálcico por su mejor capacidad de retención de agua y calidad en el producto final. Actualmente es la cobertura más utilizada. Tras la aportación de la cobertura, el sustrato se mantiene a 24 ^oC con alta concentración de CO_2 en la nave y se produce la colonización de la cobertura por el hongo. Es muy importante que la cobertura tenga humedad suficiente y no se seque por lo que se aconsejan riegos ligeros pero frecuentes. La operación de rascado acelera la colonización de la capa de cobertura por arrastre de micelio a la superficie.

La siguiente fase en la producción de champiñón es la inducción de la floración. Esta operación consiste en una bajada brusca de la temperatura a 14 – 16 ^oC y ventilación de la nave para disminuir el CO_2 a la mínima concentración. Con esta operación se induce la formación de primordios que posteriormente generarán el cuerpo fructífero. En esta etapa los riegos son mínimos porque dañan

la floración así que las reservas de agua en la capa de cobertura son muy importantes. Al cabo de 16 - 20 días tras la cobertura, se produce la primera cosecha (oleada, Fig. 1.9). Normalmente se producen tres cosechas espaciadas unos 7 – 10 días una de la otra.

Fig. 1.9: Detalle de una nave de cultivo de champiñón (*A. bisporus*) durante la fase de fructificación.

Tras la tercera cosecha, el sustrato ha agotado sus nutrientes para la generación de una cuarta económicamente sostenible, por lo que ésta se descarta y se procede al vaciado de la nave de producción. Es en este momento cuando el sustrato de cultivo se considera un residuo y pasa a denominarse sustrato agotado o sustrato post-cultivo (spent *A. bisporus* substrate, SAS) (Fig. 1.10).

Fig. 1.10: Pilas de sustrato post-cultivo de champiñón.

1.5.3. Problemática medioambiental del sustrato post-cultivo de champiñón y antecedentes de su reutilización

Se estima que en el cultivo de champiñón se generan 5 kg de SAS por cada kg de champiñón producido. Por lo tanto si la producción española de champiñón se estima en 125.000 t año^{-1}, la generación de SAS puede estimarse en el entorno de las 600.000 t año^{-1}. Según estimaciones de 2008 la cantidad de SAS producido en Europa asciende a 3,5 millones de toneladas al año (Pardo-Giménez and Pardo-González, 2008). Si se comparan los datos de producción de champiñón con la producción de otras setas (12.700 t año^{-1}) la cantidad total de residuos de sustrato post-cultivo de otras setas puede estimarse en 63.500 t. Por lo tanto la problemática medioambiental del cultivo de champiñón es mucho mayor que la de otros hongos por la mayor generación de sustratos post-cultivo

Tradicionalmente el SAS se desechaba en vertederos produciendo contaminación del suelo y las aguas subterráneas por la lixiviación de sales y carbono orgánico soluble (Guo and Chorover, 2004; Guo et al., 2001). Ante los

problemas medioambientales generados y la rápida colmatación de los vertederos se han buscado diversas utilidades a este residuo.

Dentro del propio cultivo de champiñón el SAS puede ser reutilizado en diversas formas. Se ha aplicado como ingrediente de la cobertura sustituyendo en parte a la turba con buenos resultados (Pardo-Giménez et al., 2011). Esta reutilización del SAS, por otra parte reduce el uso de turba como cobertura de forma que minimiza el impacto ambiental de este cultivo por dos vías, reutilización de un residuo agrícola como es el SAS y reducción de la extracción de turba. Otra forma de reutilizar el SAS en el propio cultivo de champiñón consiste en la generación de té de compost. Este té se obtiene mediante suspensión de SAS en agua durante 24h. El extracto obtenido (té de compost) actúa como plaguicida contra *Lecanicillium fungicola* (Gea et al., 2013) que produce la enfermedad conocida como mole seca y que está volviéndose resistente a procloraz, principio activo utilizado en la actualidad contra este patógeno.

Medina et al., (2009) propuso su utilización como componente de sustratos de cultivo aunque desaconseja su utilización en solitario por su elevada salinidad. Por otro lado la reutilización de SAS en sustratos de cultivo reduciría la extracción de turba con lo que el beneficio medioambiental vuelve a ser doble.

La aplicación al suelo del SAS está ampliamente extendida, siendo su uso en agricultura como enmienda y abono orgánico la clásica reutilización de un residuo orgánico aprovechando su alto contenido en materia orgánica y nutrientes (Medina et al., 2012). Puede utilizarse directamente o tras un proceso de re-compostaje. Como mejora a su aplicación directa se ha propuesto y está en uso la peletización del compost. Esta alternativa facilita el transporte, por la menor humedad del SAS, y su aplicación ya que puede ser distribuido en campo con máquinas abonadoras tradicionales para abonos químicos. En La Rioja, la reutilización de SAS como abono orgánico para viñedo (González-Marcos et al., 2014; Peregrina et al., 2012) está siendo potenciado por ser el cultivo más extendido en la zona. Las dosis recomendadas para aplicaciones en fresco, re-compostado o peletizado son de 21, 13 y 9 t ha^{-1}respectivamente suponiendo un aporte de nitrógeno de 50 kg ha^{-1}. Por otra parte se ha observado que la aplicación en suelos de viñedo tiene efectos positivos en la degradación (Marín-Benito et al.,

2014, 2012a) y adsorción (Marín-Benito et al., 2012b) de diferentes plaguicidas usados en este cultivo, así como efectos en la movilidad del Cu aportado como fungicida (Herrero-Hernández et al., 2011).

Una alternativa de reutilización de SAS íntimamente ligada a los trabajos descritos anteriormente en viñedo y al presente trabajo es su aplicación a la remediación de suelos contaminados con metales. Frutos (2009) demostró la utilidad de SAS para la remediación de suelos ácidos de mina mediante la inmovilización de metales y minimización de su lixiviación, subida de pH, mejora de la fertilidad del suelo y revegetación con *Atriplex halimus*. Tapia et al., (2010) observó la capacidad del SAS recompostado junto con el sustrato post-cultivo de *P. ostreatus* para inmovilizar Cd, principalmente en su fracción orgánica y minimizar su lixiviación.

También se han abordado estrategias de reutilización del SAS atendiendo a la naturaleza ligninolítica de *A. bisporus*. De esta forma se ha propuesto al SAS como una buena fuente de la enzima ligninolítica laccasa que a su vez puede ser empleada para biodegradación de ciertos PAH o compuestos fenólicos (Li et al., 2010; Mayolo-Deloisa et al., 2011, 2009; Trejo-Hernández et al., 2001). Hasta el momento no se han realizado estudios de remediación de suelo mediante aplicación de SAS como inóculo del hongo o fuente de enzimas ligninolíticas. Sin embargo, en la búsqueda de un sustratos baratos que sirva de inóculo de hongos ligninolíticos existen antecedentes de la reutilización del sustrato post-cultivo de *P. ostreatus,* hongo con probada capacidad de producir enzimas ligninolíticas tras la colonización del suelo (Lang, 1998; Lang et al., 1997) y con capacidad de remediar suelos contaminados con PAH (Eggen and Majcherczyk, 1998; Novotný et al., 1999), como inóculo del hongo para la remediación de suelos contaminados con PAH (Eggen, 1999; Mauricio-Gutiérrez et al., 2014).

Por todo ello, la presente Memoria de Tesis expone y discute los resultados obtenidos en estudios de biorremediación de suelos afectados por PAH, en presencia o ausencia de metales, mediante diversos métodos de aplicación de sustrato post-cultivo de champiñón (*Agaricus bisporus*).

Capítulo 2:
Objetivos / Objectives

El objetivo general de la presente Tesis consiste en determinar la capacidad del sustrato post-cultivo de champiñón (*Agaricus bisporus*), SAS, para biorremediar suelos contaminados con hidrocarburos aromáticos policíclicos (PAH) tanto en presencia como ausencia de metales pesados (MP), con el fin de mejorar la habitabilidad y función biológica del suelo.

Para la consecución del objetivo principal se establecieron los siguientes objetivos específicos:

- ✓ Puesta a punto de metodologías analíticas para la extracción y determinación de PAH y MP.
- ✓ Localización de suelos contaminados con PAH y/o metales
- ✓ Determinar el potencial degradador de PAH por el SAS y diferenciar el papel de su población bacteriana y el de *A. bisporus* en el proceso de biodegradación.
- ✓ Optimización de la forma de aplicación de SAS como inóculo de *A. bisporus* en suelos contaminados.
- ✓ Estudiar la utilidad del SAS en bioestimulación de la microbiología nativa de suelos contaminados para degradar PAH.
- ✓ Estudiar los efectos del SAS sobre la movilidad de MP y PAH en el suelo.
- ✓ Evaluar la posibilidad de mejora de la habitabilidad del suelo tras los procesos de biorremediación.

The main objective is the assessment of the ability of spent *Agaricus bisporus* substrate (SAS) to bioremediate polluted soils with polycyclic aromatic hydrocarbons alone or in combination with heavy metals. The aim of the bioremediation process is to improve the soil habitability and health.

To achieve the global objective, partial objectives were researched.

- ✓ To develop analytical procedures for PAH and trace elements extraction and quantification.
- ✓ To locate potential polluted soils with PAH and/or metals
- ✓ To determine the ability of SAS to degrade PAH and identify the role of the inherent bacterial population and *A. bisporus* respectively in the biodegradation process.
- ✓ To optimize the method of SAS application to polluted soils as *A. bisporus* inoculum carrier in bioaugmentation strategy.
- ✓ To assess the ability of SAS to the biostimulation process of the autochthonous microbial population from polluted soils which is able to degrade PAH.
- ✓ To determine the effects produced by SAS on PAH and metals mobility.
- ✓ To evaluate the improvement of the soil habitability and health after the bioremediation process.

Bloque I:
Optimización de metodologías de extracción de metales y PAH y selección de suelos contaminados

En este bloque de capítulos, se presentan los resultados de los trabajos de optimización de la metodología de extracción de metales de enmiendas orgánicas y PAH del SAS y suelo, así como la concentración de estos compuestos en los suelos muestreados durante la localización y selección de suelos contaminados. La puesta a punto de las metodologías analíticas se realizó como paso previo a la localización de emplazamientos potencialmente contaminados y a los ensayos de remediación.

La decisión de optimizar la extracción total/pseudototal de metales en enmiendas orgánicas se basó en la falta de referencias claras en la bibliografía para este tipo de materiales ya que históricamente se han tratado de la misma forma que los suelos o materiales completamente orgánicos. En el presente trabajo se pretendió una interpretación más amplia de la matriz, atendiendo tanto a su fracción orgánica como a la inorgánica.

El desarrollo de la metodología de extracción de PAH de suelo y SAS se debe a la necesidad de implementar en el laboratorio un método de extracción y análisis de PAH que permita el análisis de un gran número de muestras en un tiempo razonable.

Por último, para la elección de suelos reales contaminados con PAH y/o metales pesados con los que se trabajaría a lo largo de la Tesis, se realizaron muestreos de suelo en emplazamientos sospechosos de encontrarse contaminados. El principal criterio para la elección del emplazamiento fue la posibilidad de encontrar multicontaminación en la zona (PAH + metales). Por ello se recurrió a emplazamientos cercanos a centrales térmicas de carbón, industria metalúrgica, industria del creosotado de la madera y campo de tiro. De las muestras recogidas se realizaron extracciones totales de PAH y pseudo-totales de metales conforme a las metodologías desarrolladas en los capítulos 3 y 4.

Parte de los trabajos incluidos en este bloque han sido publicados bajo los siguientes títulos:

- "Influence of chemical and mineralogical properties of organic amendments on the selection of an adequate analytical procedure for trace elements determination." Talanta (2012) 88: 375-384.
  DOI: 10.1016/j.talanta.2011.11.003
- "Methodology for polycyclic aromatic hydrocarbons extraction from either fresh or dry spent mushroom compost and quantification by high-performance liquid chromatography–photodiode array detection." Communications in Soil Science and Plant Analysis (2013) 44: 817-825. DOI: 10.1080/00103624.2013.749439

Capítulo 3:

Influence of chemical and mineralogical properties of organic amendments on the selection of an adequate analytical procedure for trace elements determination.

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Abstract

Six digestion procedures were tested to improve extraction methods for determination of trace elements in various organic amendments with high inorganic fractions. These procedures were tested in terms of pH, $CaCO_3$, organic matter, elemental analysis, BCR sequential extraction and X-ray diffraction analysis. Aqua regia extraction (ISO 11466), total digestion HF-HNO_3-$HClO_4$ and four microwave digestions (i.e., HNO_3, HCl-HNO_3, HNO_3-HF and HCl-HNO_3-HF) were used. The effect of acid mixtures on microwaves-assisted digestion of mineral fractions was assessed by Si and Al analysis and X-ray diffraction in the solid residues obtained. Microwave HF acid mixtures obtained highest trace element recoveries for all tested metals except Al. CaF_2 and $CaAlF_5$ precipitates were also detected using X-ray diffraction in the residues after microwave digestions with HF acid mixtures of amendments with high calcium content. A decision flowchart was suggested to determine the best acid mixture according to the amendment and the metals to be analyzed.

3.1. Introduction

Composted organic wastes are commonly used as organic amendment, fertilizer or growing media in agriculture and degraded soil recovery. The composts supply organic matter, nutrients and microorganisms, which improve the physico-chemical and microbiological soil properties and the fertility status. However, organic wastes can be an important source of trace elements in soils. During the stabilization process of organic matter and nitrogen, trace elements are concentrated in the compost matrix due to mass loss (Tandy et al., 2009). A major limitation of compost application to soil is thus the potential for high trace elements content.

The incorporation of trace elements after repeated applications of organic amendments in soils can have phytotoxic effects and also affect soil microorganisms (Schramel et al., 2000). In addition, once trace elements are applied, they have very long residence times in soil (Smith, 2009). An exhaustive control of trace elements in agricultural soils is therefore necessary to prevent soil degradation and trace elements incorporation in the food chain. Fast, repeatable and robust methods for trace elements analysis are necessary to control organic amendments samples with different compositions and from multiple origins.

Regulations and guidelines for compost applications are currently based on total metal loadings. Traditionally, the literature and international directives have described compost as materials based in organic components, so analytical procedures to digest them and determine trace elements content are usually focused on the total oxidation of the organic matter. However, organic amendments can be composed of diverse organic wastes and inorganic materials including soils, sediments and inorganic residues, such as lime, gypsum, clay and silica.

The inorganic fraction may be relatively large in some organic amendments. In numerous countries, composted and co-composted wastes are being generated with appreciably high mineral fractions. For example, sewage sludge produced as a by-product of municipal wastewater treatment is composed of approximately 50% organic and 50% inorganic material (Haynes et al., 2009). Because the analysis of the trace elements concentrations in these waste materials is crucial for

monitoring and risk assessment, the methods of sample mineralization must be able to liberate the trace elements associated with the inorganic fractions of compost.

In the literature, various methods for compost digestion have been described: dry ashing (Zheljazkov and Warman, 2002), heated mixture of acids (Hseu, 2004), microwave wet digestion (Florian et al., 1998) and various acid mixtures for wet digestions (e.g., HNO_3-HCl (Fuentes et al., 2004; ISO, 1995; US-EPA, 2007), HNO_3-$HClO_4$ (Alburquerque et al., 2011; Hullebusch et al., 2005; Sastre et al., 2002), HNO_3, (Sandroni and Smith, 2002; Sastre et al., 2002; US-EPA, 2007), HNO_3-HF-H_2O (Sandroni et al., 2003), HNO_3-H_2O_2 (Veschetti et al., 2000), and HNO_3-HF-$HClO_4$ (Sastre et al., 2002). Not all acid mixtures completely attack the inorganic fraction, so hidrofluorhidric acid is sometimes necessary for complete digestion. Hseu (2004) and Sandroni and Smith (2002) reported that HNO_3 produced the best recoveries for metal analysis except Al, which required HF.

Microwave-assisted acid extraction has proven to be a suitable method for the digestion of complex matrices, such as soils, sediments, biosolids and compost (Ivanova et al., 2001; Marin et al., 2008; Sandroni and Smith, 2002; Zhou et al., 1995). This procedure presents great advantages over traditional wet digestion, such as reduced duration of digestion, smaller quantities of acids, improved detection limits, less frequent contamination and loss of volatile analytes and increased reproducibility and accuracy.

Sequential extraction procedures have been used to determine the chemical forms of trace elements present in sediments and soils, and also in organics amendments, such as green waste, biosolids and municipal solid waste composts (Aparicio et al., 2009; Farrell and Jones, 2009; Fuentes et al., 2004; Smith, 2009; Tandy et al., 2009). Such information is traditionally considered to be valuable for predicting metal mobility, bioavailability and leaching rates when composts are applied to soils (Flyhammar, 1998). Information from sequential extraction can also be considered in the selection of the optimal method for the extraction of trace elements present in composts. However, metal fractionation using sequential extractions is largely operational because the reagents are not completely selective and re-adsorption and redistribution of metal ions after release can occur (Pueyo

et al., 2008). For this reason, better understanding of trace elements speciation in the amendments can be obtained by combining different analytical methods, such as basic characterization, sequential extraction and mineralogical analysis, to better understand the chemical forms of trace elements and the mineralogical composition of organic amendments for selecting the most adequate analytical procedures for trace elements determination.

The purposes of this work were as follows. (1) To evaluate the Cr, Mn, Fe, Ni, Cu, Zn, Cd and Pb content in four organic amendments with high inorganic fractions using different digestion methods. These methods included the conventional aqua regia extraction ISO 11466 (ISO, 1995), an open-vessel HF-HNO_3-$HClO_4$ total digestion, and four microwave-assisted methods (i.e., HNO_3 and HCl-HNO_3 as acid extractable and HNO_3-HF and HCl-HNO_3-HF as total digestion). (2) To select the most appropriate microwave-assisted acid digestion method for trace elements in amendments according to their chemical and organic compositions, sequential extraction data and mineralogical changes in residues after microwave-assisted acid digestion.

3.2. Experimental

3.2.1. Samples and characterization

The following organic amendments were used:

1) Spent mushroom compost (SMC). Initial compost is produced from wheat straw (55%), grape marc (15%) and poultry litter (30%) (m/m); this is commonly used as a mushroom (*Agaricus bisporus*) cultivation medium. After cultivation, waste is composted for 3 months. 2) Green waste + sewage sludge (3:1 m/m) (GWS). This mix is composted in tunnels with forced air for 14 days and matured in the open air. Composting is completed in approximately 6 months. 3) Natural black peat (BP), obtained from northern Spain. 4) Horticultural waste compost (HWC), obtained from greenhouse production, sieved at < 25 mm and composted in the open air for 90 days.

Accuracy of analysis was evaluated using a Certified Reference Material: sewage sludge LGC6181 from LGC Standards (UK). The extractable metal content refers to metals soluble in hot aqua regia using methods based on ISO 11466 (ISO, 1995).

All samples were air-dried for 14 days, and electrical conductivity (EC) and pH were determined in water extracts (1:5 v/v) using a conductivity meter (Crison CM 2200, Barcelona, Spain) and a pH electrode (Orion 720A, Beverly, MA, USA), respectively. Organic matter content was determined by mass loss-on-ignition at 450 ºC over 4 hours. Dry samples were milled and sieved to 100 µm. $CaCO_3$ content was analyzed by calcimetry. Total carbon, nitrogen, hydrogen and sulfur were analyzed using combustion (LECO CHNS-932 analyzer, USA).

A mineralogical characterization of the raw amendments samples and their solid residues remaining after microwave digestion was carried out by X-ray diffraction (Panalytical X´Pert Pro, Almelo, The Netherlands). The configuration of this apparatus was θ/2θ with an X´Celerator detector. Diffraction profiles were analyzed using X'Pert High Score Plus software and PDF-4 of the International Center for Diffraction Data for crystalline identification.

3.2.2. Reagents

All reagents used in sample digestion were better than analytical grade and supplied by Panreac (Spain), including Hiperpur HNO_3 (69%), Hiperpur HCl (35%), Hiperpur HF (48%) and Hiperpur $HClO_4$ (70%). Reagents used for sequential extraction were of analytical grade or better and supplied by Merck (Germany): acetic acid glacial, ammonium acetate, hydroxylammonium chloride, Suprapur hydrogen peroxide (30%). Ultrapure water was obtained from a Milli-Q water purification system (Millipore Corporation, Spain) and used throughout the work. All glassware and plasticware used were washed with 5% (v/v) nitric acid and rinsed with ultrapure water.

3.2.3. BCR sequential extraction procedure

The optimized BCR sequential extraction procedure (Pueyo et al., 2008) was applied to the organic amendment samples. This extraction procedure consists of three steps: Step 1 (exchangeable and weak acid soluble fractions) with acetic acid (0.11 M, 16 h). Step 2 (reducible fraction; Fe-Mn oxides), with hydroxylammonium chloride (0.5 M, pH 1.5, 16 h). Step 3 (oxidizable fraction; organic matter and sulfides) with H_2O_2 (8.8 M, 2 x 1 h, 85 ºC) followed by extraction with 1.0 M ammonium acetate. Additionally, a fourth step was added to dissolve the final residue. The residual resistant fraction represented metals that were strongly associated with crystalline structures of minerals and which were unlikely to be released under conditions that are normally encountered in the environment. This residual fraction is typically extracted with aqua regia (ISO 11466), but HF-HNO_3-$HClO_4$ total digestion was performed in this work instead.

3.2.4. Aqua regia extraction (ISO 11466)

The aqua regia extraction was based on the ISO 11466 (ISO, 1995) procedure. Briefly, 3 g (± 0.1 mg) of sample was placed in a 250 mL Pyrex digestion tube; 21 ml of 35% HCl and 7 mL of 69% HNO_3 were added, then the sample was covered with a watch glass and pre-digested at room temperature for 16 h. Subsequently, the suspension was digested for 2 h under reflux conditions. After cooling, the suspension was filtered through an ashless Whatman 42 filter, diluted to 50 ml with 0.5 M HNO_3, and stored in polyethylene bottles at 4 ºC for analyses. Blanks were also treated using the same procedure.

3.2.5. Total digestion (HF-HNO_3-$HClO_4$)

For this method, 1 g (± 0.1 mg) of sample was placed into a Teflon open vessel. A 10 mL volume of 48% HF was then added and pre-digested at room temperature over 16 h. Next, the suspension was heated until dryness on a sand bath; 10 mL of 69% HNO_3 and 5 mL of 37% $HClO_4$ were then added and the reaction was heated until approximately 0.5 mL final volume remained. The final volume was then made up to 50 mL with 0.5 M HNO_3 and stored in polyethylene

bottles at 4 ºC for later analyses. Blank digestions were also performed using the same protocol.

3.2.6. Microwave-assisted acid digestion

A pressurized closed-vessel microwave system (CEM Mars X Press, USA) was used to digest the samples. Microwave polyfluoroacethylene (PFA)-teflon vessels were cleaned before each digestion using 12 mL of 69% HNO_3, heated for 20 min at 200 ºC and then rinsing with ultrapure water. After the microwave digestion procedure was optimized, the following conditions were employed: samples were accurately weighed to 250 mg (± 0.1 mg) in microwave vessels. They were then subjected to four different digestion procedures using various acid combinations (Table 3.1) using the same solid sample/reagent volume ratio (250 mg to 12 mL). The digestion program consisted of a 15-minute gradual increase to 200 ºC, a 15-minute digestion step at 200 ºC and 1200 W and then a cooling stage. Acid mixtures used were selected according to previous reports for soil and compost trace elements analysis (Marin et al., 2008; Nemati et al., 2010; Sastre et al., 2002; US-EPA, 2007). Blanks were processed in a method identical to the samples.

Table 3.1: Acid volumes (mL) used in microwave-assisted digestion procedures.

	HCl	HNO_3	HF
Mixture AR	9.00	3.00	-
Mixture ARF	8.25	2.75	1.00
Mixture N	-	12.00	-
Mixture NF	-	11.00	1.00

3.2.7. Trace and major elements analysis

Analyses were conducted on a ICP-MS spectrometer (Perkin Elmer Sciex Elan 6000) equipped with an autosampler (AS 91, Canada). The isotopes used for metals analyses were ^{52}Cr, ^{55}Mn, ^{56}Fe, ^{58}Ni, ^{63}Cu, ^{64}Zn, ^{114}Cd and ^{208}Pb. In addition, Al and Si (isotopes measured ^{27}Al and ^{28}Si) were analyzed for monitoring microwave digestion of silicates and aluminosilicates. Limits of quantitation were calculated over 10 measurements of the chemical blank from each acid mixture of microwave digestion as $10\sigma/b$, where σ is signal standard deviation and b is linear coefficient of the calibration graph.

3.2.8. Statistical analysis and chemical speciation software

SPSS 15.0 software was used for statistical analysis. Significant differences between different microwave-assisted acid digestions, aqua regia extraction (ISO 11466) and HF-HNO_3-$HClO_4$ total digestion were compared using 1-way ANOVA with Tukey´s post-hoc test. Statistical significance was defined as $p < 0.05$. The V MINTEQ v.3.0 software was used to confirm Ca chemical species in microwave HF acid mixtures.

3.3. Results and discussion

3.3.1. Basic characterization of organic amendments

After a first approach based on basic chemical properties (Table 3.2), the selected organic amendments could be classified into three types:

1) SMC and HWC are composted agricultural wastes with high $CaCO_3$ and EC (10.4 and 11.6 dS m^{-1}), very low organic matter and C content (close to threshold values for organic amendments, as required by Spanish law RD 824/2005), and C/N values of approximately 10. These two materials have similar percentages of S.

2) GWS and LGC1681 present sewage sludge in their composition. They have remarkably high organic matter (50-60%), with almost 25% of their content

coming from C. They also have several interesting similarities in pH, lack of carbonates and high EC. An important difference between these materials is elemental composition, as sewage sludge LGC6181 has higher values for N (low C/N) and S.

3) Natural black peat (BP), which has a lower pH value (4.86) and a low EC, but the highest organic matter (81%), C content and C/N.

Table 3.2: Basic chemical characteristics and elemental analysis of organic amendments.

	pH	$CaCO_3$ %	EC[a] (dSm^{-1})	OM[b]	C	N	H	S	C/N
						%			
SMC	7.02	28.1	10.4	25.2	12.4	1.37	1.73	0.21	10.7
GWS	7.19	-	2.00	58.1	26.2	1.41	3.49	0.04	23.9
BP	4.86	-	0.6	80.8	37.5	0.87	4.68	0.06	53.9
HWC	9.00	18.1	11.6	32.6	19.2	1.82	2.47	0.17	10.4
LGC1681	6.57	-	6.0	53.0	24.6	3.54	4.14	1.08	8.7

[a] Electrical Conductivity
[b] Organic Matter

3.3.2. Mineralogical characterization

All organic amendments were found to have quartz as the main mineral through X-Ray diffractometry (Fig. 3.1). In addition to quartz, SMC showed calcite and gypsum. HWC showed calcite, dolomite, mica, anorthite and whewellite (CaC_2O_4* H_2O) as secondary minerals. Whewellite also appeared in GWS, as did mica and anorthite. The presence of whewellite in HWC and GWS indicated the vegetal origin of these composts, as many plants contain calcium oxalate phytolites in their leaves, bark and wood as monoclinic whewellite crystals (Nakata, 2003).

The secondary mineralogical composition of the reference standard material LGC1681 was gypsum, aluminum hydroxides and iron oxide. This was not surprising, as gypsum has been reported to precipitate during wastewater and sludge treatment (Essington and Mattigod, 1991). In contrast, BP showed mica as a secondary mineral. Poorly crystalline iron hydroxides, such a ferrihydrite, might also have been present in SMC and BP due to the presence of a weak and broad XRD peak at 2.58 Å, but this was difficult to identify in bulk XRD analysis.

3.3.3. Distribution of trace elements in organic amendments according to the BCR procedure

The BCR extraction results are shown in Fig. 3.2. As can be seen, Cr, Fe, Ni and Pb were mainly associated with the residual fraction, suggesting that these metals were primarily contained in silicates and other resistant minerals. Other works obtained similar results for sewage sludge (Alonso et al., 2009), municipal solid waste compost (Farrell and Jones, 2009), and deinking paper fiber and green waste compost (Tandy et al., 2009). A particularity of LGC6181, and sewage sludge in general, was the great mobility of Ni compared to other trace elements (Alonso et al., 2009; Smith, 2009; Tandy et al., 2009)(Alonso et al., 2009; Smith, 2009; Tandy et al., 2009). The majority of Fe in the amendments was associated with the residual fraction (26% to 84%), followed by the oxidizable fraction (9% to 47%). This observation implied that Fe occurred in silicates and organic matter as sulfides with very little in the poorly crystalline hydroxides. Alonso et al. (2009) found similar distribution patterns of Fe in sewage sludges.

Mn appeared in all four fractions with no preference for any particular one. In addition, most of the Cu was released by H_2O_2 extraction (70-85%), implying a great tendency to be bound in the organic fraction. Furthermore, a significant proportion of Cu (approximately 25%) was present in the residual fraction, suggesting a presence in resistant minerals. The majority of Cd was released during hydroxylamine-HCl extraction (17-71%), suggesting that Cd was primarily contained in reducible hydroxides and oxides. Very little Cd was present in the residual fraction (2-10%), except for HWC (24%). Finally, Zn was mainly associated with the reducible fraction (47-50%), except amendments with high $CaCO_3$ contents, and the organic fraction (26-74%); its residual fraction was variable from 4 to 56%.

The results from the sequential extraction procedure indicated that HF would be recommended for total digestion together with a strong oxidant acid in order to completely dissolve the organic and residual fractions of the amendments of interest.

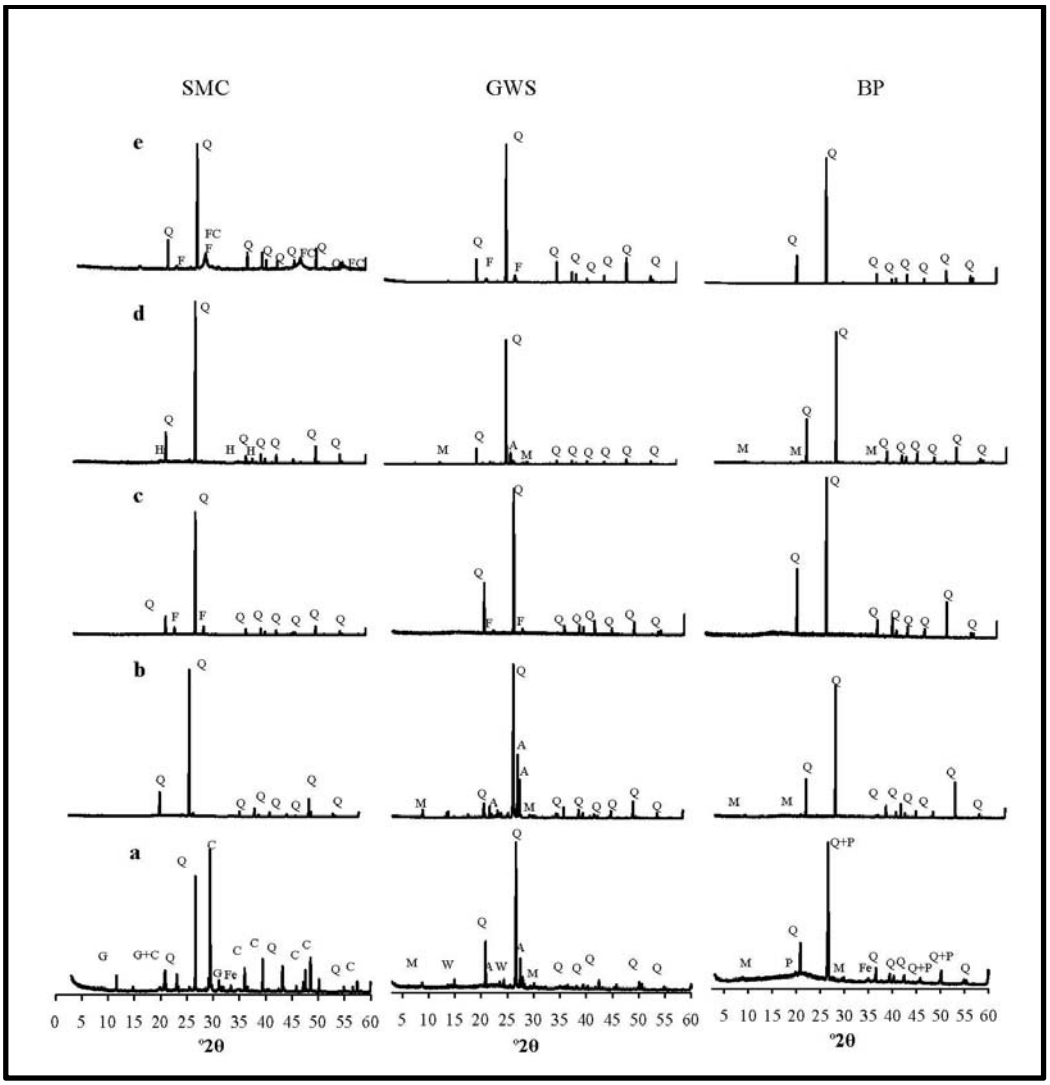

Fig. 3.1: X-ray diffractograms of SMC, GWS and BP (a) and their residues after four different acid mixture microwave-assisted digestion methods (b: AR; c: ARF; d: N; e: NF). A: anorthite; Al: aluminum hydroxides; C: calcite; CF: CaF_2; D: dolomite; F: $AlCaF_5$; Fe: iron oxides; G: gypsum; H: hallosite; I: illite; K: kaolinite; M: mica; P: $AlPO_4$; Q: quartz; W: whewelite.

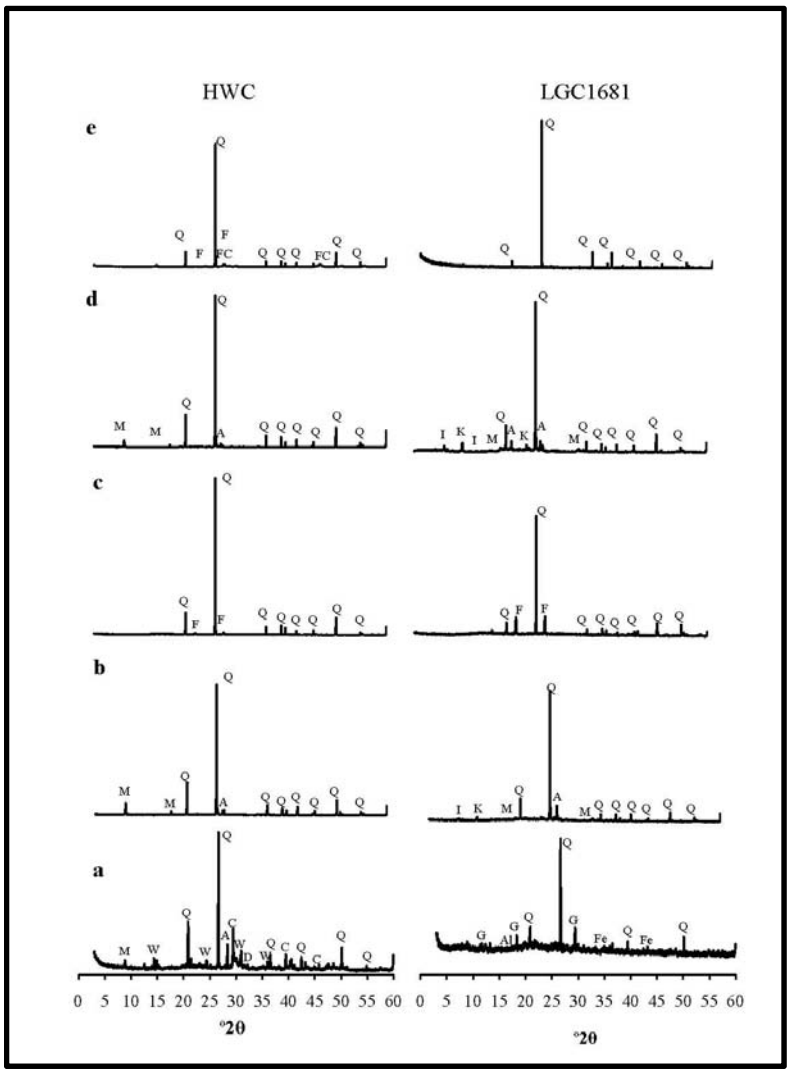

Fig. 3.1 (Continued)

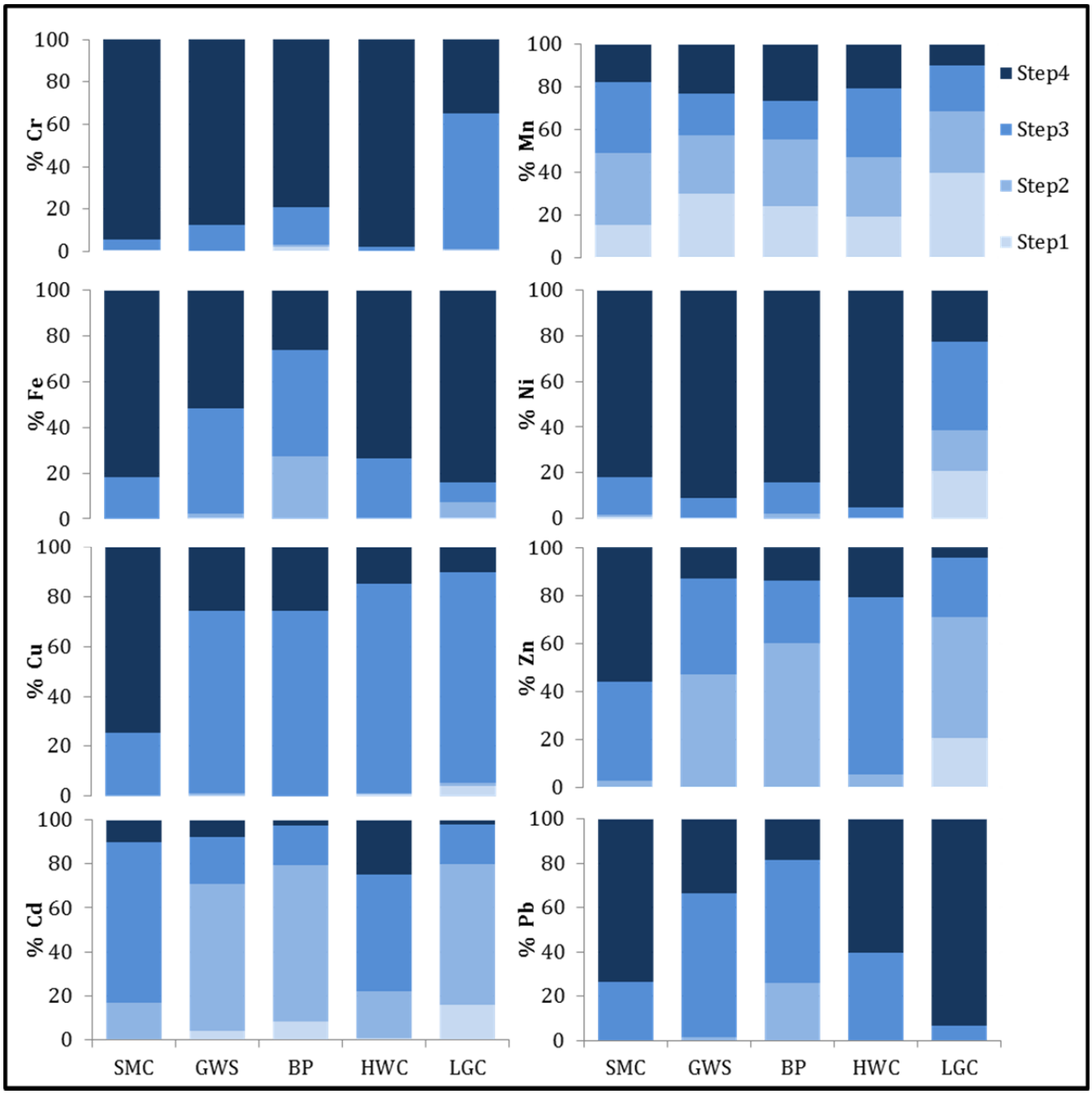

Fig. 3.2: Distribution of fractions for BCR sequential extraction of Cr, Mn, Fe, Ni, Cu, Zn, Cd and Pb of organic amendments. Step 1 is exchangeable, water and acid soluble fraction, Step 2 is reducible fraction, Step 3 is oxidizable fraction and Step 4 is residual fraction. Results are mean percentages for n = 3.

3.1.1. Comparison of open system digestions

Table 3.3 shows the results of aqua regia extraction (ISO 11466) and total digestion (HF-HNO_3-$HClO_4$) of organic amendments. The precision of the methods was assessed by the percentage relative standard deviation (%RSD). For both methods, %RSD was good, generally <10% for all the metals and amendments. Pseudo-total digestion according to ISO 11466 obtained equal or higher concentrations of Cr, Mn, Ni, Cu, Zn, Pb and Cd (except for SMC) than total digestion. Only Fe concentrations were higher in the extracts from tri-acid total digestion for all amendments, suggesting the release of Fe included in the aluminosilicate phase during this treatment. These results indicated that open total digestion produced metal losses specially Ni, Cu and Zn; similar results were obtained by other authors (Hseu et al., 2002; Nemati et al., 2010), suggesting the use of closed digestion methods.

3.1.1. Accuracy of open system digestions

The accuracy of the aqua regia extraction (pseudo-total digestion) and tri-acid total digestion was assessed with the analysis of the certified reference material LGC6181 which certified values corresponding to the extractable metal contents (Table 3.4). The recovery of each metal was calculated based on the mean value of CRM LGC6181 [(measured concentration (mg kg^{-1})/ mean certified value (mg kg^{-1})) x 100]. The accuracy was better for the aqua regia extraction (recovery ranged from 97 to 107%) compared to total digestion (recovery ranged from 39% to 101%). For both methods precision was very satisfactory, with %RSD <5%. According to the ERM (2010) aqua regia extraction using protocol ISO 11466 produces results that agree perfectly with certified values. However, HF-HNO_3-$HClO_4$ total digestion results only agreed for Cr and Mn. Due to these results, only the aqua regia extraction using ISO 11466 is considered adequate for multi-elemental analysis for this kind of organic amendments.

For further comparisons, concentrations of trace elements of amendments obtained with aqua regia extraction using ISO11466 are considered as 100% recovery.

Table 3.3: Multi-elemental analysis of four organic amendments using aqua regia extraction (ISO 11466) and HF-HNO_3-$HClO_4$ total digestion. Results are expressed in mg kg^{-1}. Letters indicate significant differences between digestion methods for each material and metal. RSD is given in brackets; *n* = 3.

	SMC		GWS		BP		HWC	
	Aqua Regia	HF-HNO_3-$HClO_4$	Aqua Regia	HF-HNO_3-$HClO_4$	Aqua Regia	HF-HNO_3-$HClO_4$	Aqua Regia	HF-HNO_3-$HClO_4$
Cr	83.0 (5)	86.6 (1)	355 (5)	383 (2)	99.8^a (1)	87.0^b (0.1)	322 (0.1)	324 (3)
Mn	318^a (6)	268 ^{b}(1)	237 (2)	225 (3)	22.2^a (2)	19.0^b (7)	372^a (1)	319^b (4)
Fe	8848^b (6)	11496^a (12)	8014^b (4)	9737^a (3)	2493^b (2)	3507^a (1)	9098^b (1)	11795^a (4)
Ni	36.5^a (6)	28.4^b (2)	130^a (4)	104^b (3)	29.5 (1)	28.3 (2)	122^a (2)	83.0^b (4)
Cu	30.1^a (7)	20.5^b (1)	94.4^a (4)	79.0^b (10)	3.96 (6)	3.55 (12)	153^a (2)	100^b (3)
Zn	139^a (6)	79.0^b (1)	120^a (5)	80.7^b (3)	45.5^a (23)	9.26^b (4)	248^a (2)	123^b (4)
Cd	0.144^b (5)	0.162^a (1)	0.273 (2)	0.263 (4)	0.147 (1)	0.140 (1)	0.453^a (7)	0.397^b (3)
Pb	10.1^a (3)	5.42^b (6)	20.2 (4)	21.4 (4)	6.13 (4)	6.11 (14)	21.0 (2)	20.5 (4)

Table 3.4: Multi-elemental analysis of Certified Reference Material LGC6181 using aqua regia extraction (ISO 11466) and HF-HNO_3-$HClO_4$ total digestion. Results are expressed as recovery percentage (Rec) of certified values and Relative Standard Deviation (RSD). Letters indicate significant differences between digestion methods for each metal; n = 3.

	LGC6181	Aqua regia		HF-HNO_3-$HClO_4$	
	Certified (mg kg^{-1})	Rec (%)	RSD(%)	Rec (%)	RSD(%)
Cr	78 ± 8	105[a]	0.4	96[b]	1
Mn	454 ± 23	102	2	101	3
Fe	40300 ± 2300	100[a]	1	88[b]	3
Ni	45 ± 3	107[a]	2	81[b]	1
Cu	354 ± 18	100[a]	2	71[b]	2
Zn	1100 ± 50	102[a]	2	92[b]	4
Cd	5.8 ± 0.3	103[a]	2	78[b]	2
Pb	105 ± 8	97[a]	3	39[b]	10

3.3.6. Quality control of microwave-assisted closed vessel digestions

The quality control of the microwave-assisted acid digestions was performed by comparison with the LGC6181 (Fig. 3.3). Reproducibility of four acid mixtures was very good with %RSD in most cases lower than 5%. However, recovery for the microwave-assisted AR procedure was lower than aqua regia extraction (ISO 11466) for all the metals with respect to certified values. Additionally, microwave-assisted N digestion led to similar results of those for LGC6181 with the exception of Zn. Nitric acid microwave digestion has been considered an alternative for aqua regia extraction for samples with high organic matter content (Sastre et al., 2002).

Total digestion procedures using HF (ARF and NF) showed higher concentrations than AR and N for all trace elements and major recoveries up to 100% for Cr, Mn, Fe and Pb, between 111 and 123% (Fig. 3.3). These results could be attributed to more complete digestion of silicate components present in the sewage sludge LGC6181. These higher recoveries meant that ISO 11466 (extractable aqua regia metals) did not completely digest the test material, so the acid mixtures ARF and NF were more effective in sewage sludge matrix digestion and therefore in metal extraction strongly associated with mineral matter.

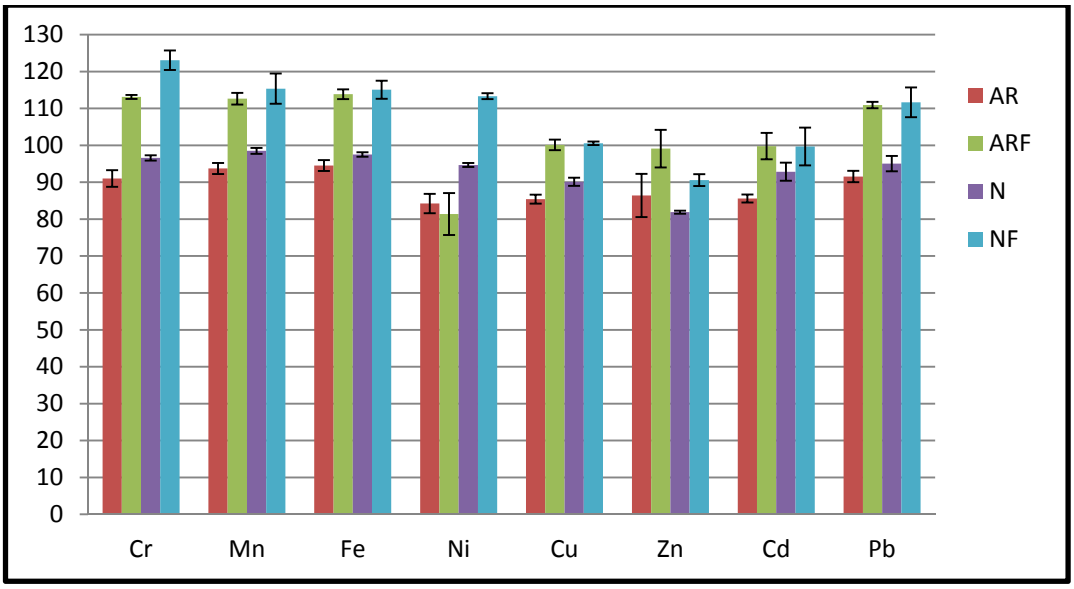

Fig. 3.3: Trace elements microwave-assisted acid digestion recoveries (%) of four acid mixtures (AR, ARF, N, NF) obtained from LGC1681. Bars indicate standard deviation; *n* = 3.

Limits of quantitation (LOQ) of microwave-assisted acid digestions (Table 3.5) were significantly lower than those obtained in the literature for determining and monitoring trace elements of organic amendments (Melaku et al., 2005). Especially remarkable were the low LOQ values for Cd (0.002 – 0.006 mg kg^{-1}). The use of HF in general increased LOQ, only Cd in ARF mixture and Ni and Zn in NF mixture got LOQ lower. For pseudo-total digestions, AR mixture produced LOQ major than N except Cu and Zn. For total digestion ARF mixture produced major LOQ except Mn. As general pattern, the mixture of acids produced an increase of LOQ values.

Table 3.5: Limits of quantitation (LOQ, mg kg^{-1}) of acid mixtures after ICP-MS analysis.

	AR	ARF	N	NF
Al	2	5	1	3
Si	179	188	128	147
Cr	0.2	0.2	0.03	0.03
Mn	0.03	0.06	0.01	0.08
Fe	9	9	3	7
Ni	0.2	0.3	0.1	0.07
Cu	0.08	0.1	0.08	0.07
Zn	0.5	0.8	0.8	0.3
Cd	0.006	0.004	0.002	0.002
Pb	0.04	0.06	0.02	0.02

3.3.7. Effects of microwave-assisted acid mixture digestions on the inorganic matrix of amendments

Visual observation of the solid residues remaining following the four microwave-assisted acid digestions showed remarkable differences. HF acid mixture digestion on amendments with high $CaCO_3$ contents (SMC, HWC) created a white precipitate, which was more abundant in SMC. Samples characterized by the absence of $CaCO_3$ content (BP, GWS, LGC6181) digested with HF acid mixtures showed a minor quantity of solid residue. In these amendments, the acid mixture NF looked to be most effective because very little residue remained after microwave digestion.

X-ray diffractograms of organic amendments and their residues after the four microwave-assisted acid mixtures digestions are shown in Fig. 3.1. In general, similar patterns were observed in the mineralogical composition of the samples after the digestion procedures. Interestingly, all residues after all microwave-assisted acid digestions showed residual quartz. In addition, calcite, dolomite, gypsum, whewellite, Al hydroxides and Fe oxides were attacked by the four acid mixtures assayed and not detected in the residues. Acid mixtures, ARF and NF, completely digested silicate compounds, such as mica, ilite, kaolinite and anorthite.

XRD patterns of the residues, except those obtained in BP, after ARF and NF microwave digestion (Fig. 3.1c and Fig. 3.1e, respectively) showed three peaks at

3.15, 3.93 and 1.82 Å for $CaAlF_5$, with different intensities for each material. This result suggested the formation of calcium fluoroaluminate during both ARF and NF digestions. These reflections were more intense for SMC and LGC6181, and a significant decrease in recoveries of Al (56 and 40% respectively) and Ca (18 and 45%) employing ARF vs. NF was also observed. These results suggested an important $CaAlF_5$ formation stage probably due to the high Ca and Al content of these materials (Fig. 3.4). However, $AlCaF_5$ was not detected for BP residues due to the low content of Ca and Al. In support of these observations, Marin et al. (2008) reported Ca-F and Al-F complexes in calcareous soils after microwave-assisted HF digestion

Additional broad peaks occurred at 1.93, 3.15 and 1.65 Å in the samples with high $CaCO_3$ content (SMC and HWC) only after NF microwave digestion. These reflections corresponded to CaF_2 and their intensities were higher in SMC than HWC, in agreement with the higher SMC Ca content (Fig. 3.4). The lack of CaF_2 in the solid residue after ARF digestion could suggest inhibition of formation by HCl. This hypothesis was confirmed by chemical speciation models performed in Visual Minteq 3.0. This model revealed that $CaCl^+_{(aq)}$ was the predominant Ca species under ARF digestion conditions.

Decomposition of aluminosilicates in each microwave-assisted acid mixture digestion was determined through the aluminum and silicon concentrations (Fig. 3.4). As can be seen, microwave ARF and NF digestions liberated higher concentrations of Al and Si than AR and N. Similar results were obtained by Sandroni and Smith (2002) for Al in sewage sludge when employing HF. However, aluminum concentrations of SMC decreased with ARF and did not increase with NF, while Al concentrations of LGC6181 with ARF did not increase due to the formation of $CaAlF_5$. Furthermore, microwave AR digestion liberated higher Si and Al concentrations than microwave N digestion, because hot aqua regia is capable of dissolving tri-octahedral clays, primary and secondary salts and hydroxy oxides of amorphous aluminosilicates (Raisanen et al., 1992). Silicon concentrations obtained with HNO_3 microwave extraction digestions were smaller than the quantitation limit except for black peat.

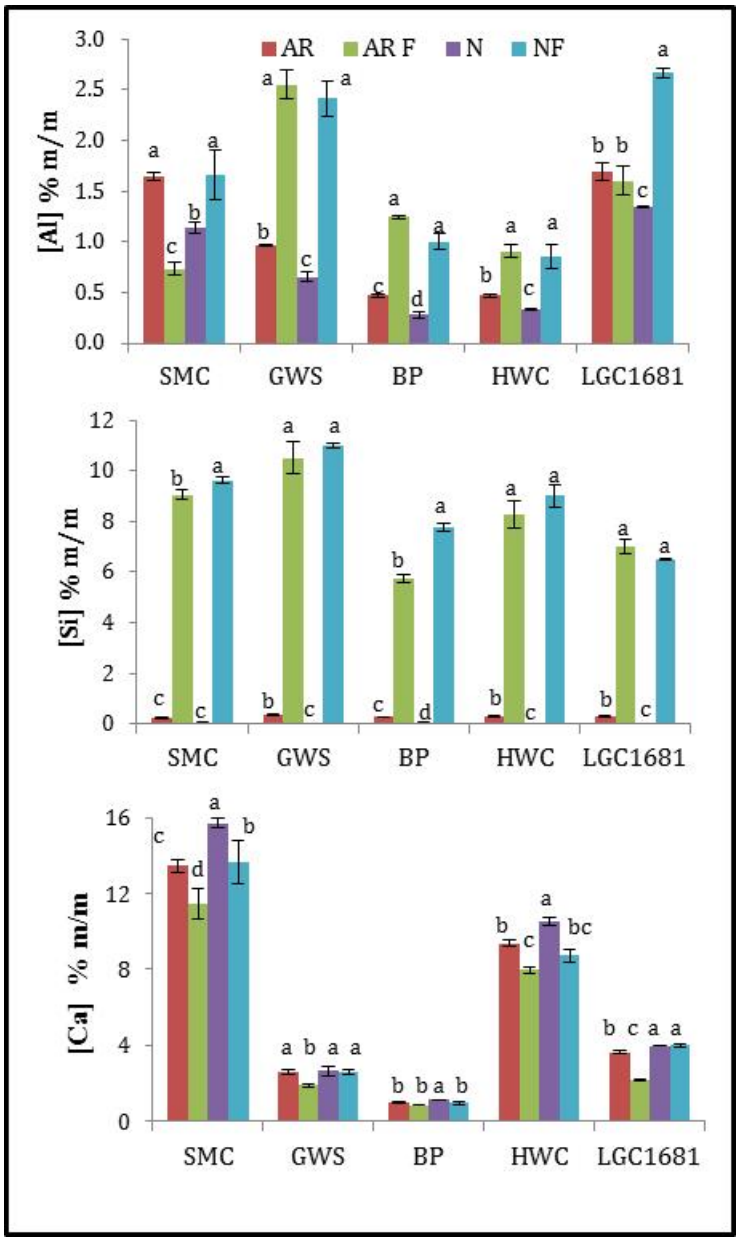

Fig. 3.4: Concentrations of Al, Si and Ca from four acid mixture microwave-assisted digestions (AR, ARF, N, NF). Bars indicate standard derivation (n=3), and different letters indicate significant differences at $p < 0.05$.

3.3.8. Comparison of microwave-assisted digestion of organic amendments

Microwave acid mixture digestion procedures were compared to each other and with the ISO11466 method that was previously checked in section *3.5* Results are shown in Fig. 3.5.

Microwave-assisted AR and N digestions liberated similar concentrations of trace elements to ISO11466, except for Cr and Ni, which were extracted mainly in the residual fraction during the BCR procedure. For these metals, microwave-assisted AR digestion released significantly larger amounts than microwave-assisted N digestion. Florian et al. (1998) reported similar behavior for microwave nitric acid and HCl:HNO_3 (3:1) digestions with respect to Ni and Cr.

Acid mixtures ARF and NF (with HF) more efficiently attacked aluminosilicates of samples, as is shown by the lack of aluminosilicate compounds in the XRD of residues after microwave HF digestions (Fig. 3.1) and higher Si recoveries (Fig. 3.4). Therefore, microwave-assisted HF digestions were more efficient and obtained higher trace elements concentrations than AR, N and ISO11466.

The increase in recoveries obtained when HF was used had a positive correlation with trace elements extracted in the fourth step of BCR (Table 3.6). This correlation indicated that metals extracted with HF were absorbed into aluminosilicate compounds, while AR and N were not able to extract them. Consequently, HF must be included for total acid digestion of organic amendments with high inorganic fractions. However, underestimation of certain element concentrations has been reported due to their trapping in Ca-F and Al-F complexes in calcareous soils after microwave-assisted HF digestion (Marin et al., 2008). For comparison, our results showed a significant decrease in Fe concentrations when CaF_2 was formed after NF microwave digestion of amendments (SMC and HWC) rich in carbonates. In addition, Zn showed lower recoveries in GWS and LGC6181 with NF than ARF. However, ARF microwave digestions had lower Ni recoveries for all materials tested. These results suggested a co-precipitation phenomenon for CaF_2 and $CaAlF_5$ detected by XRD analysis.

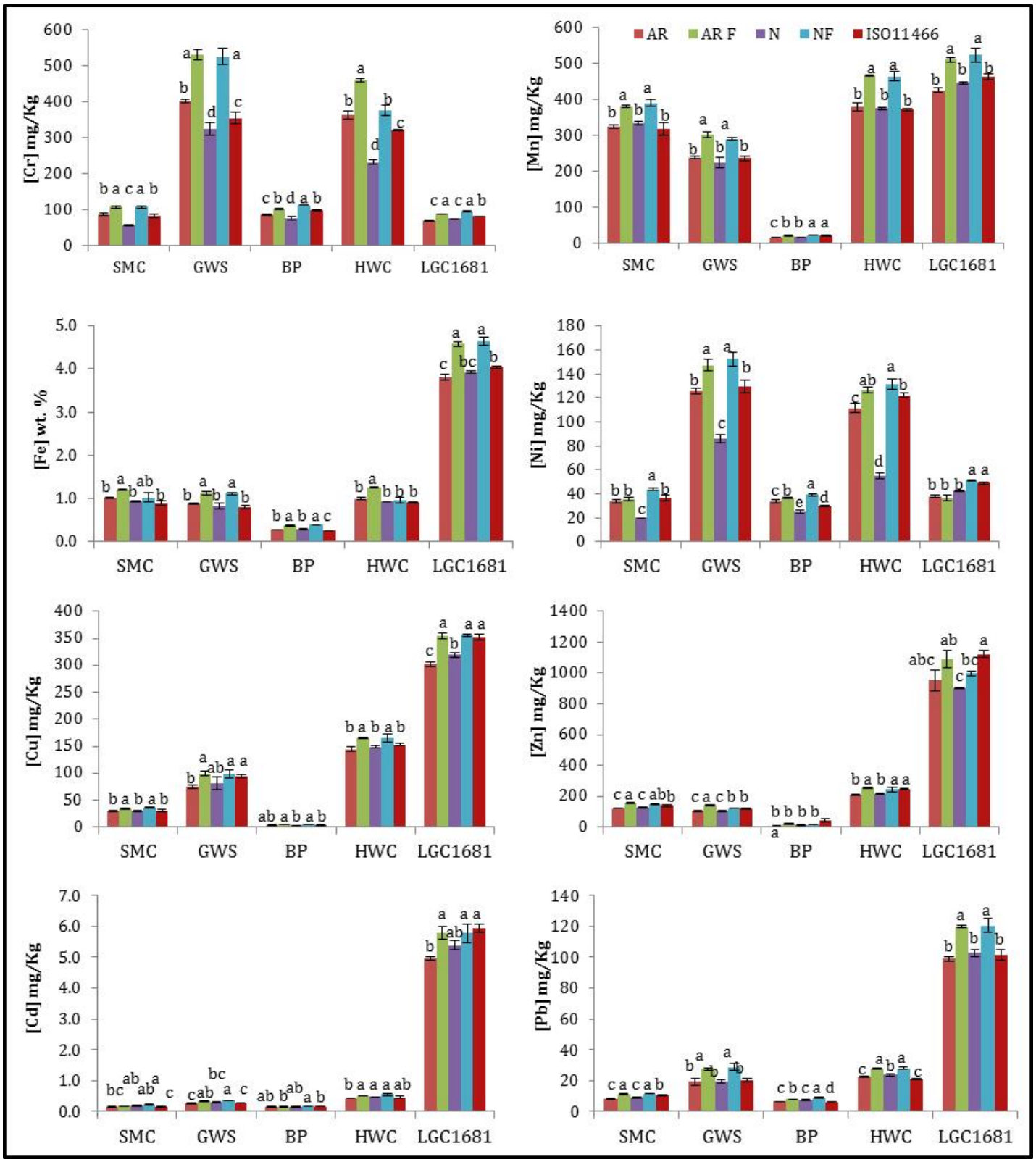

Fig. 3.5: Comparison of trace elements concentrations of four acid mixture microwave-assisted digestions (AR, ARF, N, NF) and ISO 11466. Bars indicate standard deviation (n=3), and different letters indicate significant differences at $p<0.05$.

Table 3.6: Linear correlation coefficients (r) between trace elements content of the residual fraction of BCR sequential extraction and metal concentration increase when HF was employed (n = 15).

	Cr	Mn	Fe	Ni	Cu	Zn	Cd	Pb
ARF-AR[a]	0.935***	0.827***	0.954***	0.917***	0.816***	0.529*	0.629*	0.916***
NF-N[b]	0.911***	0.850***	0.844***	0.974***	0.749***	0.565*	0.638*	0.798***

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

[a] Increase of metal concentration using acid mixtures ARF vs AR.

[b] Increase of metal concentration using acid mixtures NF vs N.

Fig. 3.6 summarizes the results obtained from the different microwave digestion procedures. This flowchart is proposed as a procedure decision tree for organic amendment digestion for trace elements analysis.

In the literature, there are few reference materials other than sewage sludge to be consulted for the validation of digestion methods for compost or organic amendments. The different behavior of organic amendments shown in this paper indicates that Certified Reference Materials other than sewage sludge are necessary for future method development.

3.4. Conclusion

Microwave-assisted HF acid mixtures were more effective in attacking the aluminosilicate fraction and increased trace elements and iron recoveries. Additionally, this study demonstrated the formation of precipitates in the residues of organic amendments rich in calcium minerals after microwave-assisted digestion with HF mixtures (i.e., $CaAlF_5$ formation after microwave ARF and NF digestion, and CaF_2 formation only after microwave NF digestion).

As was noted in the text, Al, Fe, Zn and Ni could be underestimated by microwave-assisted HF acid mixture digestion in amendments with high Ca content as a consequence of their trapping in calcium fluoroaluminates or calcium fluoride precipitates.

Based on the analytical results and the required time of extraction, microwave-assisted digestion with a mixture of 11 mL of HNO_3 (69%) and 1 mL of HF (48%) is recommended for complete digestion of organic amendments, while a mixture of 9 mL of HCl (37%) and 3 mL of HNO_3 (69%) is proposed for pseudo-total digestion.

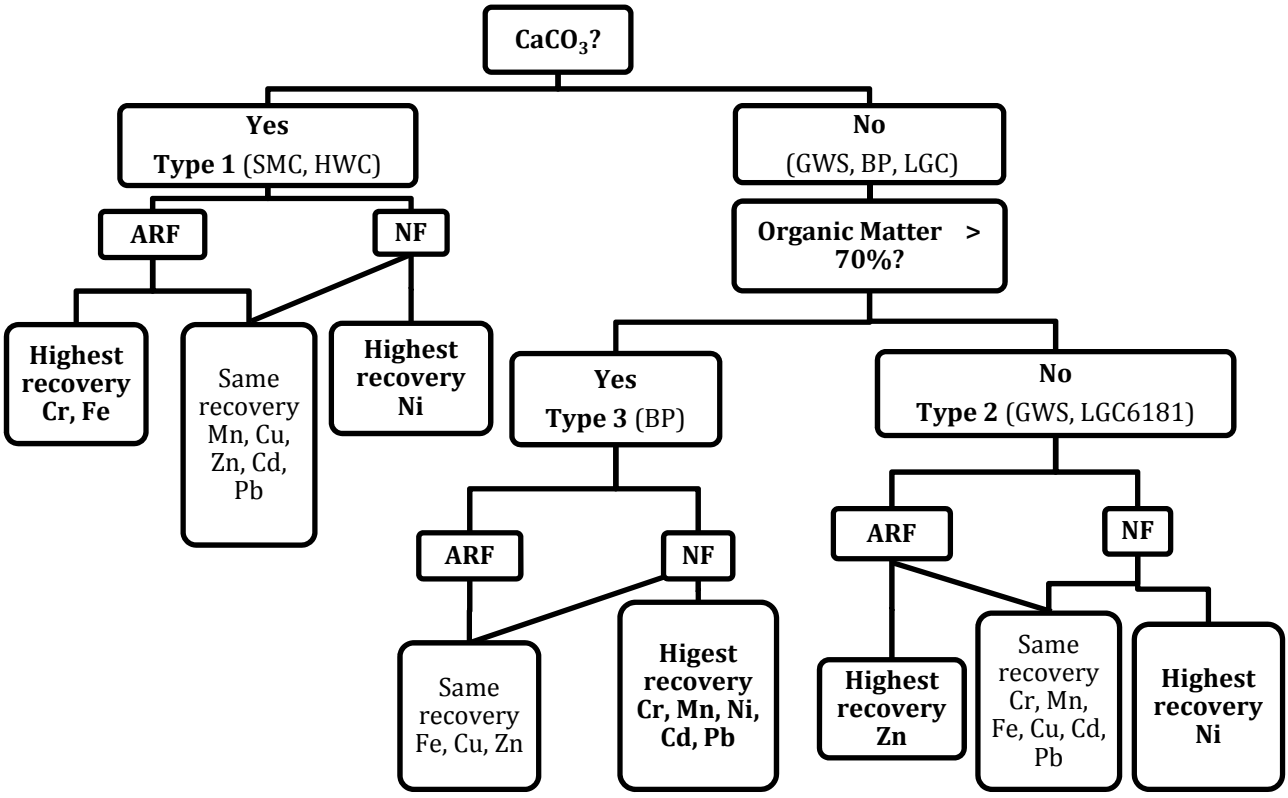

Fig. 3.6: Decision flowchart for the selection of a total digestion method for organic amendments according to chemical characteristics.

Capítulo 4:

Methodology for Polycyclic Aromatic Hydrocarbons extraction from either fresh or dry spent mushroom compost and quantification by high performance liquid chromatography – photodiode array detection.

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Abstract

Polycyclic Aromatic Hydrocarbons (PAH) are a family of compounds classified as Persistent Organic Pollutants, hazardous for environmental and human health. White rot fungi are organisms that are able to remediate PAH from polluted soils. Spent Mushroom Compost (SMC) is employed for soil bioremediation and environmental research. In this paper, four solvents (acetone:CH_2Cl_2 1:1 mixture; acetone:hexane 1:1 mixture; methanol, and acetone), which are among those already used for PAH solvents, were chosen to be combined with two extraction procedures (ultrasonic and orbital shaking). All extracted PAH were quantified by HPLC-PDA. Certified soil CRM141, containing 16 PAH included in the US EPA priority list, was used for above mentioned methodology validation. Orbital shaking procedure was selected because all detected and quantified PAH were within CRM141 prediction interval, and there was less variability for all checked solvents than in the ultrasonic procedure.

Once the orbital shaking methodology was selected, fluorene, phenanthrene, anthracene and pyrene standards were added to fresh (60% moisture content) and dry SMC. The orbital shaking procedure was carried out over 24 hours to avoid PAH degradation, and 1 month later to provide PAH interaction with solid matrix in this period of time. PAH orbital shaking extraction over dry SMC showed large recovery percentages for all tested solvents. PAH extracted from fresh SMC showed biodegradation after 24 hours. Acetone:CH_2Cl_2 (1:1), methanol and acetone solvents employed over fresh SMC obtained less recovery percentage than over dry SMC. Acetone:hexane (1:1) obtained recovery percentages higher than 80% and did not show any decrease in recovery over fresh SMC. Results indicate that is not necessary to include a drying step previous to PAH extraction if acetone:hexane (1:1) solvent mixture is employed.

The recommended methodology for PAH extraction includes orbital shaking of fresh compost with acetone:hexane (1:1) solvent mixture and quantification by HPLC-PDA.

4.1. Introduction

Polycyclic aromatic hydrocarbons (PAH) are a family of aromatic hydrocarbons with two or more condensed benzene rings formed during incomplete combustions. PAH are persistent organic pollutants with toxic, mutagenic and carcinogenic properties (IARC, 2010) and consequently, their presence in soil is hazardous for the environment and human health. These compounds are deposited on soils due to their low vapor pressure and water solubility. Today, soil bioremediation techniques, such as white rot fungi application are being researched. These organisms are able to biodegrade PAH due to unspecific enzymatic activities like laccase, peroxidase, manganese peroxidase and lignin peroxidase (Gramss et al., 1999). One of the ways in which the fungus is supplied to the soil is through spent mushroom compost (SMC) (Reid et al., 2002), so it is necessary to validate analytical procedures for detection and quantification of PAH extracted from SMC.

PAH extraction from their matrix is a critical step of the analytical sequence. PAH extraction by Soxhlet is the method most commonly used for soils, sediments and sewage sludge, but due to high solvent volumes and time consumption other methods have been developed such as ultrasonic (Santos et al., 2007; Sun, 1998), shaking (Song et al., 2002), microwave-assisted extraction (MAE) (Camel, 2000; Villar et al., 2004). Pressurized-liquid extraction (PLE) (Zuloaga et al., 2000) and supercritical fluid extraction (SFE) (Miège et al., 2003) can be used with good results although substantial outlay on equipment is required. Ultrasonic and shaking extraction methods are good alternative techniques which require moderate amounts of both time and solvent volumes (Song et al., 2002).

Sample moisture is a controversial point of PAH extraction. Some authors have reported either PAH losses or PAH extraction improvement depending on the sample moisture content, technique and solvent (Shu and Lai, 2001; Song et al., 2002).

The aims of this work were a) to validate an extraction analytical procedure for quantification of PAH from soil and b) to select a solvent for PAH extraction from fresh and dry spent mushroom compost (SMC).

4.2. Materials and methods

4.2.1. Chemicals and reagents

Acetonitrile, acetone and hexane (alkane mixture) HPLC grade, were obtained from Panreac (Barcelona, Spain). Dichloromethane Chromasolv® HPLC was obtained from Sigma-Aldrich (France). Ultrapure water was obtained from a Milli-Q water system (Millipore Corporation, France). Standard solutions mixture of 16 US EPA PAH (Naphthalene (Naph), Acenaphthylene, (Acy), Acenaphthene (Ace), Fluorene (Flu), Phenanthrene (Phe), Anthracene (Ant), Fluoranthene (Fla), Pyrene (Py), Benzo[a]anthracene (BaA), Chrysene (Ch), Benzo[b]fluoranthene (BbF), Benzo[k]fluoranthene (BkF), Benzo[a]pyrene (BaP), Dibenzo[a,h]anthracene (DBahA), Benzo[g,h,i]perylene (BghiP) and Indeno[1,2,3-c,d]pirene (IcdP)), at a range of concentration 100-2000 µg mL^{-1} in methanol: methylene chloride (1:1) were provided by Supelco (Bellefonte, USA). Standard solutions for single Flu at 5000 µg mL^{-1} in methanol, Phe at 5000 µg mL^{-1} in methanol, Ant 1000 µg mL^{-1} in acetone and Py at 1000 µg mL^{-1} in methanol were also provided by Supelco (Bellefonte, USA). Appropriate dilutions of the standards with acetonitrile or acetone were made to HLPC standards or PAH spiked in SMC, respectively.

4.2.2. Apparatus

An ultrasonic cleaning bath, Selecta® (Barcelona, Spain), with a 50-60 KHz operating frequency was used for soil PAH extraction by the ultrasonic method. An orbital shaker, Selecta (Barcelona, Spain), was used for PAH extraction from both soil and SMC by mechanical shaker. The HPLC system was a Waters 2695 Separation Module coupled with a Waters 996 Photodiode Array Detector (PDA).

4.2.3. Soil and spent mushroom compost samples

Certified reference material (loamy-clay soil CRM141) fortified with 16 US EPA PAH was used for analytical procedure validation. Limits of detection (LD) and quantification (LQ) of soil samples were determined on an unpolluted agricultural soil (texture sandy-loam, pH 7.3 and 1.4% of organic matter) as 3 SD and 10 SD of signal noise of extracts, respectively.

Mushroom compost was produced from wheat straw (55%), poultry litter (30%) and grape marc (15%). After composting for 21 days, a soil with high calcium carbonate content was added up to 30% of final mixture. After being used to grow *Agaricus bisporus* for 100 days, the compost was labelled as Spent Mushroom Compost (SMC). Dry SMC was obtained after drying at room temperature and grinding an aliquot. Moisture content of fresh SMC was 60% (w/w). One ml of acetone Flu, Phe, Ant and Py standard solutions at 125 μg mL^{-1} were added on 5 grams of both fresh and dry SMC to achieve 25 μg g^{-1}. Spiked dry SMC was aged in the dark at room temperature for 30 days. Fresh SMC was extracted 24 hours after PAH solution was applied, to permit acetone evaporation and in order to minimize PAH biodegradation.

4.2.4. The PAH extraction procedures in soil CRM141

Two ultrasonic and orbital shaking extraction methods were tested with four solvents, acetone:dichloromethane (1:1), acetone:hexane (1:1), methanol, and acetone. Three replicates per tested solvent were performed.

Ultrasonic extraction procedure: 0.5 g of sample plus 10 ml of each tested solvent were immersed in an ultrasonic bath for 30 minutes with occasional manual shaking in order to avoid sample caking.

Orbital shaking extraction procedure: 5 g of sample plus 25 mL of each tested solvent were shaken for 2 hours at 200 rpm. Solution was left for 30 min before solution decanting.

After the extraction step, solutions were filtered through nylon syringe filters with a 0.45μm pore size (Whatman International, Maidstone, UK). One mL of each resultant solution was taken to dryness by N_2 flow. Residue was redissolved in 1

mL of acetonitrile solvent. PAH detection and quantification from the resultant extract were performed by HPLC-PDA.

4.2.5. Effect of evaporation of extracts

Solvent change of extracts was necessary because all solvents were not miscible with mobile phase of HPLC or appropriate for the cartridge column used. The aim of this test was to assess the effect of drying acetone:hexane (1:1) extracts with N_2 flow on PAH analysis. One mL of diluted standard solution of each of the 16 PAH (10 µg mL^{-1} of Acy, 5 µg mL^{-1} of Naph and Ace, 1 µg mL^{-1} of Flu, Fla, BbF, DBahA and BghiP, 0.5 µg mL^{-1} of Phe, Ant, Py, BaA, Chr, BkF, BaP and IcdP) in acetone:hexane (1:1) was evaporated under N_2 flow to dryness (approximately 10 minutes). Residue was redissolved in 1 mL of acetonitrile. PAH detection and quantification from resultant extract were performed by HPLC-PDA. Results were compared with the same 16 PAH diluted standard solution in acetonitrile without the dryness step. Three replicates for each one were performed.

4.2.6. The PAH extraction from dry and fresh spent mushroom compost

According to the results obtained from soil PAH extraction procedures, orbital shaking extraction was chosen as the extraction method for PAH extraction from both dry and fresh SMC. The above-described four solvents were tested as well to assess the moisture content effect on the PAH extraction effectiveness. Dry and fresh SMC without any added PAH was processed in parallel to be considered as blank solutions so both LD and LQ were determined from them.

4.2.7. Liquid chromatography conditions

The analytical cartridge column was a Supelcosil™ LC-PAH 250x3 mm, 5µm. The cartridge column was protected by a Supelguard™ LC-18 20x3mm guard column. The mobile phase was an acetonitrile-water gradient at a flow-rate of 0.5 mL min^{-1}. The gradient elution program was 0-5 min: 60% acetonitrile and 40% water, then a linear gradient elution from 60% acetonitrile at 5 min to 100% acetonitrile at 15 min followed by isocratic elution for 20 min (Santos et al., 2007).

The column run temperature was fixed at 28ºC. Sample injection volume was 20µL. The chromatograms were monitored at 254 nm and processed by Empower® software (Waters, Milford, MA, USA). Each PAH was identified by both time retention and UV spectrum by comparing with standards.

4.2.8. Statistical analysis

Statistical analyses were performed with SPSS statistical software (version 19.0; SPSS Inc., Chicago, IL). Data were statistically evaluated by one-way analysis of variance (ANOVA). Levene's variance homogeneity test was performed before ANOVA, and Duncan or Games-Howell post-hoc tests at $p \leq 0.05$ were used, as parametric and non-parametric appropriate tests, respectively. Means comparison was used to find differences when recovery rates from extraction methods for each solvent were compared. Non-parametric Kruskal-Wallis test was applied to compare the number of PAH validated against both solvent and extraction method.

4.3. Results and discussion

4.3.1. Validation of PAH extraction methods

Recovery rates for all combinations of two extraction procedures, ultrasonic and orbital shaking, and four solvents, acetone:dichloromethane (1:1), acetone:hexane (1:1), methanol and acetone, each tested on CRM 141certified material for the 16 US EPA PAH are shown in Table 4.1. Naph and Acy might be not detected since they were below detection limit (DL) for any solvent and extraction method. Means of 14 PAH recovery rates and percentages of relative standard deviation (RSD) of each solvent, were compared between orbital shaking and ultrasonic extraction methods. For acetone:dichloromethane, acetone:hexane and acetone, no significant differences were found between ultrasonic and orbital shaking extraction when recovery rates were analyzed, but for methanol, orbital shaking showed higher recovery than ultrasonic extraction. RSD of ultrasonic extraction were significant higher than RSD of orbital shaking for the acetone:dichloromethane solvent mixture, methanol, and acetone solvents.

According to the IRMM (2010) protocol for comparison of the result of a measurement with a certified value, orbital shaking extraction with acetone:hexane solvent mixture was the only method in which the 14 PAH results agreed with the certified values. Many of the individual PAH results that did not agree with the certified value were overestimations, only Ant and BaP showed systematic underestimation. However, no significant differences were found among the numbers of PAH validated for ultrasonic and orbital shaking extraction for any solvent. The same behaviour was observed when the four solvents were compared using ultrasonic extraction. However, when orbital shaking extraction was entered as a factor, significant differences were found at p = 0.056 between acetone:hexane (14 validated PAH) and methanol (8 validated PAH) (Table 4.1).

4.1.1. The PAH recoveries of standard solutions evaporated to dryness

PAH recovery percentages and %RSD from 16 PAH standard solutions in acetone:hexane (1:1) are shown in Table 4.2. Recovery rates were calculated as percentages of the values obtained without applying the drying step. Large recovery rates were found for PAH with more than two aromatic rings. Among the PAH with two aromatic rings, satisfactory recovery rates for Acy (76%), Ace (77%) and Flu (88%) were found (Miège et al., 2003) and significant losses were found when the dryness step was applied only for the most volatile PAH, Naph.

Regarding the RSD values in general, low RSD were obtained for all tested PAH. As expected, Naph presented the largest RSD (25%), surely correlated with great losses. No more than 7% RSD was found for the other 15 PAH. This behavior is in accord with characteristic PAH vapor pressure values which decrease as the number of aromatic rings increase (WGPAH, 2001). According to these results, if N_2 flow is passed for 10 minutes to take the solution to dryness, 15 of 16 PAH may be properly detected and quantified by HPLC-PDA.

Table 4.1: Soil CRM 141 certified values and average recoveries (%) of acetone:dichloromethane, acetone:hexane, methanol and acetone with ultrasonic and orbital shaking extraction. RSD is in brackets. Different letters indicate significant differences at $p \leq 0.05$. n=3.

	CRM 141	Acetone:dichloromethane		Acetone:hexane		Methanol		Acetone	
	(µg Kg^{-1})	Ultrasonic	Shaking	Ultrasonic	Shaking	Ultrasonic	Shaking	Ultrasonic	Shaking
Naph	188±40.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Acy	176±45.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ace	693±174	106^{v} (21)	91^{v} (27)	297 (22)	51^{v} (84)	70^{v} (87)	157 (17)	117^{v} (20)	37 (140)
Flu	338±111	96^{v} (10)	99^{v} (11)	86 ^{v}(25)	68^{v} (53)	71^{v} (31)	136 (2)	88^{v} (26)	69 ^{v} (33)
Phe	719±221	116^{v} (9)	120^{v} (3)	107^{v} (11)	108^{v} (9)	109^{v} (4)	134 (3)	117^{v} (14)	108^{v} (7)
Ant	393±130	63 (27)	100^{v} (2)	69^{v} (11)	91^{v} (9)	66 (4)	105^{v} (1)	72^{v} (17)	75^{v} (11)
Fla	176±40.3	168 (24)	109^{v} (12)	78^{v} (9)	103^{v} (9)	101^{v} (22)	123 (3)	117^{v} (20)	83^{v} (8)
Py	331±62.0	112^{v} (14)	110^{v} (12)	93^{v} (7)	101^{v} (14)	82 (6)	107^{v} (29)	96^{v} (25)	93^{v} (5)
BaA	409±83.0	103^{v} (10)	116^{v} (3)	106^{v} (4)	118^{v} (16)	107^{v} (8)	116^{v} (3)	110^{v} (13)	111^{v} (3)
Chr	316±52.0	133 (11)	129 (4)	136 (7)	128^{v} (14)	123 (4)	129 (0)	134 (16)	122 (2)
BbF	364±48.6	89^{v} (4)	117 (2)	88^{v} (10)	117^{v} (17)	79 (18)	113^{v} (5)	89^{v} (16)	112^{v} (4)
BkF	253±43.9	94^{v} (22)	112^{v} (5)	103^{v} (5)	113^{v} (16)	97^{v} (9)	106^{v} (1)	110^{v} (12)	108^{v} (5)
BaP	198±25.8	33 (87)	102^{v} (11)	71 (4)	102^{v} (14)	51 (15)	85 (9)	55 (16)	82 (10)
DBhaA	451±70.4	97^{v} (8)	113^{v} (7)	111^{v} (6)	116^{v} (17)	81^{v} (17)	94^{v} (5)	110^{v} (11)	111^{v} (6)
BghiP	618±109	82^{v} (37)	109^{v} (6)	96^{v} (6)	110^{v} (19)	79 (14)	88^{v} (1)	101^{v} (10)	108^{v} (2)
IcdP	394±52.0	100^{v} (13)	115 (4)	105^{v} (5)	115^{v} (16)	80 (15)	91^{v} (5)	107^{v} (10)	112^{v} (3)
Mean Rec.		99^{ns}	110^{ns}	110^{ns}	103^{ns}	85^{b}	113^{a}	102^{ns}	95^{ns}
Mean RSD		16^{a}	8^{b}	9^{ns}	22^{ns}	18^{a}	4^{b}	16^{a}	8^{b}

n.d. indicates no detection of the compound in HPLC analysis
^{v} indicates recovery validated according IRMM (2010) criteria
^{ns} no significant differences

Table 4.2: Recovery rates and relative standard deviations (RSD) of 16 PAH standard solutions in acetone:hexane (1:1) evaporated to dryness with N_2 flow. *n*=3.

Compound	Aromatic rings	Recovery (%)	RSD (%)
Naph	2	20	25
Acy	2	76	2
Ace	2	77	7
Flu	2	88	4
Phe	3	96	3
Ant	3	98	3
Fla	3	102	3
Py	4	102	4
BaA	4	103	2
Chr	4	103	2
BbF	4	103	3
BkF	4	103	3
BaP	5	102	3
DBhaA	5	103	2
IcdP	5	103	3
BghiP	6	103	2

4.1.2. Moisture effects of PAH extraction from SMC

Due to the large values of PAH recoveries and/or minor RSD found for orbital shaking extraction, four solvents with orbital shaking were used to assay PAH extraction from both dry and fresh SMC. Recovery rates for each solvent are shown in Table 4.3. Recovery rates of Flu, Phe, Ant and Py from fresh SMC were significantly lower than those from dry SMC when using acetone:dichloromethane, methanol and acetone solvents. Methanol, as the most polar solvent, presented a mean 48% recovery decrease from fresh SMC. However, significantly higher recoveries of Flu and Phe were found for the acetone:hexane solvent mixture. No significant differences were found for Py. In the case of Ant, it showed a significant recovery decrease of 5%. Nevertheless, acetone:hexane solvent mixture recovery for Ant in fresh SMC (82%) was considered adequate (Santos et al., 2007). These

results indicate that for moist samples, polar solvents should be discarded since poor PAH recovery rates were achieved and the mixture with non polar solvent is necessary for PAH extraction yield improvement.

Table 4.3: Flu, Phe, Ant and Py average recoveries using acetone:dichloromethane, acetone:hexane, methanol and acetone with orbital shaking extraction of fresh and dry spent mushroom compost (SMC). Letters indicate significant differences for each solvent between fresh and dry SMC at $p \leq 0.05$. RSD is shown in brackets. n=4.

	Acetone:dichloromethane		Acetone:hexane		Methanol		Acetone	
	Fresh	Dry	Fresh	Dry	Fresh	Dry	Fresh	Dry
Flu	59 ^{b} (7)	73 ^{a} (11)	81 ^{a} (2)	63 ^{b} (3)	24 ^{b} (19)	73 ^{a} (4)	59 ^{b} (7)	72 ^{a} (3)
Phe	82^{ns} (7)	87^{ns} (7)	106 ^{a} (2)	91 ^{b} (3)	46 ^{b} (13)	91 ^{a} (4)	78 ^{b} (5)	87 ^{a} (1)
Ant	65 ^{b} (4)	83 ^{a} (7)	82 ^{b} (2)	87 ^{a} (3)	35 ^{b} (13)	87 ^{a} (3)	56 ^{b} (4)	83 ^{a} (1)
Py	84 ^{b} (6)	95 ^{a} (7)	108^{ns} (2)	104^{ns} (3)	50 ^{b} (11)	95 ^{a} (4)	79 ^{b} (4)	96 ^{a} (1)

^{ns} no significant differences.

Chromatograms from fresh SMC spiked with Flu, Phe, Ant and Py presented two peaks that did not appear in dry SMC spiked with the 4 PAH (Fig 4.1) or in fresh unspiked SMC. Possible PAH biodegradation could occur during the 24 hours between spiking and extracting the PAH. Comparing PAH recoveries obtained in dry SMC and losses of Flu in the dryness step of the extraction with nitrogen flow, the degradation at 24 hours was low.

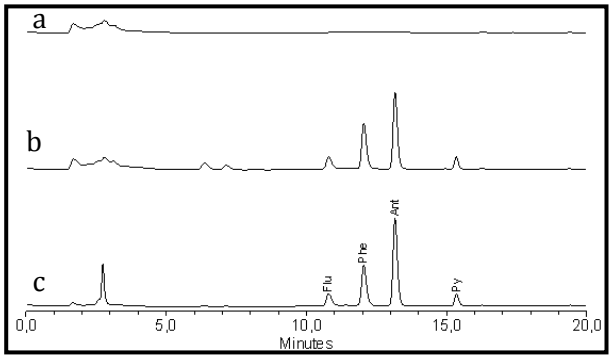

Fig. 4.1: HPLC-PDA chromatograms at 254 nm of acetone:hexane (1:1) orbital shaking extraction of spent mushroom compost (SMC). (a)fresh SMC without PAH spiking, (b) fresh spiked SMC and(c) dry spiked SMC. Samples were spiked with 25 mg Kg^{-1} of Flu, Phe, Ant and Py.

4.1.3. Limits of detection and quantification

LD and LQ were determined from extraction of soil, dry and fresh SMC using orbital shaking extraction with acetone:hexane (1:1) (Table 4.4). LD and LQ of soil and dry SMC were similar and presented values between 1 and 35 μg Kg^{-1} dw (Table 4). The highest LD and LQ were found in fresh SMC except for Chr. According to these results, only in mildly polluted SMC is it recommended to include the drying step.

Table 4.4: Limits of detection (μg Kg^{-1}dw) and quantification (μg Kg^{-1} dw) of PAHs in soil, fresh SMC and dry SMC extracted with acetone:hexane (1:1) using orbital shaking. *n*=3.

	Soil		Fresh SMC		Dry SMC	
	LD	LQ	LD	LQ	LD	LQ
Naph	1	3	9	29	3	9
Acy	6	20	76	252	11	35
Ace	3	10	18	62	9	31
Flu	<1	1	2	7	1	3
Phe	1	3	1	3	1	2
Ant	<1	1	<1	1	<1	1
Fla	<1	1	3	10	<1	1
Py	1	3	3	10	<1	1
BaA	<1	1	1	5	1	2
Chr	3	11	3	9	4	14
BbF	1	2	16	54	3	10
BkF	<1	1	2	7	1	3
BaP	<1	1	10	33	2	5
DBhaA	1	2	4	14	<1	1
BghiP	<1	1	11	37	1	2
IcdP	<1	1	2	6	<1	1

4.1. Conclusion

PAH extraction with acetone:hexane (1:1) solvent mixture and orbital shaking, and quantification by HPLC-PDA has been validated for 14 of the 16 PAH included in the CRM141 reference soil. In addition, PAH extraction from fresh spent mushroom compost with acetone:hexane/orbital shaking showed adequate recovery rates . Therefore, acetone:hexane (1:1)/ orbital shaking method and quantification by HPLC-PDA has been shown to be an adequate methodology for PAH analysis from soil, dry and fresh spent mushroom compost.

Capítulo 5:

Localización y selección de emplazamientos contaminados

5.1. Introducción

Se estima que en Europa hay 2,5 millones de emplazamiento potencialmente contaminados siendo los contaminantes más abundantes los metales pesados (JRC EC 2014). La identificación de los emplazamientos contaminados es la primera fase de la remediación de los suelos. En el RD 9/2005 (BOE, 2005) se establecen la relación de actividades potencialmente contaminantes del suelo y los criterios y estándares para la declaración de suelos contaminados. Dentro de estas actividades hay una serie de ellas que son susceptibles de producir contaminación tanto por compuestos orgánicos como inorgánicos. En el Registro Estatal de Emisiones y Fuentes Contaminantes (MAGRAMA) puede consultarse la emisión a la atmósfera, agua y suelo de sustancias contaminantes por complejo o actividad industrial, zona geográfica o contaminante. Este registro permite estudiar un histórico de las emisiones, tanto para la identificación de los contaminantes como de sus cantidades emitidas antes de realizar el muestreo de suelo y determinar con cierta seguridad, las zonas sospechosas de encontrarse contaminadas o afectadas por sustancias contaminantes.

La localización de muestras de suelo contaminadas por actividades humanas a lo largo del tiempo para su posterior uso en ensayos de remediación de suelo es de gran importancia. Los suelos contaminados en laboratorio de forma artificial (spiked soils) no presentan estabilización de la contaminación (aging) e invariablemente ésta se encuentra más biodisponible. Smith et al. (2011) determinó que la forma en que los PAH son añadidos al suelo tiene influencia en la susceptibilidad de éstos a ser degradados. Respecto a los metales pesados, de Santiago-Martín et al. (2014) estudió la disminución en la biodisponibilidad de varios metales a lo largo de 12 meses desde la contaminación del suelo. Por lo tanto los resultados obtenidos en suelos contaminados de forma “natural” reflejan mejor los efectos de los tratamientos de remediación a investigar.

Por otro lado, el uso de suelos multicontaminados por metales pesados e hidrocarburos aromáticos policíclicos (PAH) resulta muy interesante para la implementación de técnicas de descontaminación biológicas porque la

presencia de metales tiende a dificultar la degradación de PAH por hongos o bacterias (Baldrian et al., 2000; Ma et al., 2014; Thavamani et al., 2011).

El objetivo principal del presente capítulo consistió en la localización de emplazamientos contaminados, tanto por metales como PAH o la mezcla de ambos para su posterior uso en ensayos de evaluación de SAS como remediador de suelos contaminados.

5.2. Materiales y métodos

5.2.1. Selección de emplazamientos y muestreo de suelo

Con la finalidad de hallar emplazamientos multicontaminados (PAH + metales pesados), se seleccionaron una serie de actividades potencialmente contaminantes con un criterio de generación o dispersión en el medio tanto de metales pesados como de PAH atendiendo al RD 9/2005, datos del Registro Estatal de Emisiones y Fuentes Contaminantes (MAGRAMA) y publicaciones científicas (Eggen, 1999; Nadal et al., 2011, 2004; Peddicord and LaKind, 2000; Sahu et al., 2009; Sorvari et al., 2006).

Las actividades que se consideraron fueron las siguientes:

- Generación de electricidad por centrales termoeléctricas de carbón
- Industria petroquímica
- Tratamiento de madera mediante creosota
- Prácticas de tiro con armas de fuego
- Industria metalúrgica

Las actividades industriales seleccionadas aparecen en el Registro Estatal de Emisiones y Fuentes Contaminantes como emisoras de varios metales (Cu, Zn, Cd, Pb) y compuestos orgánicos (PAH entre otros) a la atmósfera, suelo o agua.

Como resultado de este criterio fueron muestreados diferentes emplazamientos en cuatro comunidades autónomas, Castilla y León, Navarra, Madrid y Castilla – La Mancha (Fig. 5.1, Tabla 5.1).

Fig. 5.1: Localización de los emplazamientos muestreados.

En Castilla y León se muestrearon suelos del entorno de centrales térmicas de carbón localizadas en la cuenca minera del rio Sil (León), en los municipios de Anllares del Sil (A), Cubillos del Sil (C) y La Robla (R)

En Navarra se muestrearon suelos procedentes de una planta de tratamiento de madera mediante creosota localizada en el municipio de Castejón (CJ).

En Madrid se muestrearon suelos de las cercanías de una industria metalúrgica localizada en Getafe (G) y en las proximidades del campo de tiro (CT) adyacente a la Universidad Autónoma de Madrid.

En la comunidad autónoma de Castilla – La Mancha se realizaron muestreos de suelo en la localidad de Puertollano (P) en las proximidades de las centrales térmicas de carbón (P1-5) y la industria petroquímica (P6,7) emplazada en dicho municipio.

Tabla 5.1: Coordenadas geográficas de los emplazamientos muestreados.

		Latitud (N)	Longitud (O)
Anllares del Sil	1	42º 50´ 5´´	6º 31´ 48´´
	2	42º 50´ 8´´	6º 32´ 24´´
	3	42º 50´ 14´´	6º 32´17´´
Cubillos del Sil	1	42º 36´ 55´´	6º 34´ 12´´
	2	42º 36´36´´	6º 33´ 30´´
	3	42º 36´30´´	6º 34´ 12´´
La Robla	1	42º 47´ 24´´	6º 37´ 54´´
	2	42º 47´ 40´´	5º 38´ 16´´
	3	42º 47´ 19´´	5º 38´ 48´´
Puertollano	1	38º 38´ 53´´	3º 57´ 57´´
	2	38º 38´ 54´´	3º 59´ 20´´
	3	38º 39´ 11´´	4º 7´ 5´´
	4	38º 39´ 11´´	4º 7´ 68´´
	5	38º 39´ 10´´	4º 7´ 26´´
	6	38º 39´ 50´´	4º 4´ 17´´
	7	38º 39´ 37´´	4º 3´ 33´´
Castejón	1	42º 10´ 29´´	1º 41´ 29´´
	2	42º 10´ 32´´	1º 41´ 50´´
	3	42º 10´ 31´´	1º 41´ 36´´
Campo tiro	1	40º 32´ 43´´	3º 40´ 56´´
Metalúrgica	1	40º 18´ 38´´	3º 42´ 31´´
	2	40º 18´ 36´´	3º 42´ 31´´
	3	40º 18´ 33´´	3º 42´ 32´´
	4	40º 18´ 29´´	3º 42´ 16´´

5.2.2. Análisis de elementos traza

Las muestras de suelo fueron secadas al aire y tamizadas a 2 mm, posteriormente se molieron en mortero. La extracción de elementos traza se realizó con agua regia en horno microondas en la mismas condiciones que las descritas en el bloque 1 capítulo 3 (García-Delgado et al., 2012b). Los elementos traza analizados fueron Mn, Cu, Zn, Cd y Pb. La determinación de estos elementos en los extractos obtenidos se realizó mediante espectrometría de absorción atómica (Perkin – Elmer AA800) o ICP-MS (Perkin-Elmer Sciex Elan 6000).

5.2.3. Análisis de PAH

Las muestras de suelo fueron secadas al aire, tamizadas a 2 mm y molidas. La extracción de PAH fue llevada a cabo por la metodología detallada en el bloque 1 capítulo 4 (García-Delgado et al., 2013a), así como la determinación de PAH mediante HPLC.

5.3. Resultados y discusión

Atendiendo a la actividad potencialmente contaminante que se desarrolla en las inmediaciones de las zonas muestreadas se observa que las muestras de suelo tomadas en las cercanías de centrales termoeléctricas de carbón (A, C, R y P) no muestran contenidos elevados de metales (Tabla 5.2). Únicamente las muestras R3 y P2 se encontraron concentraciones elevadas de Mn a pesar de que según el Registro Estatal de Emisiones y Fuentes Contaminantes, la generación de energía eléctrica mediante combustión de carbón emite a la atmósfera cantidades más que apreciables de metales entre otros compuestos como PAH.

La presencia de PAH es estas muestras de suelo tampoco fue elevada (Tabla 5.3), incluso en la muestra A3 no se detectó ninguno de los 16PAH analizados. Los PAH de 2 anillos fueron detectados en muy pocas muestras mientras que los de medio y alto peso molecular estuvieron presentes en la práctica totalidad. Este hecho evidencia la diferente dinámica de los PAH en la atmósfera en función de su peso molecular y presión de vapor. Los PAH más pequeños están presentes en la atmósfera en fase gaseosa mientras que según aumenta su peso molecular se encuentran de forma mayoritaria sorbidos en las partículas en suspensión (Mastral and Callén, 2000). Como resultado los PAH más pesados y que también presentan una menor presión de vapor son más proclives a depositarse en el suelo desde la atmósfera.

Las muestras de suelo procedentes de plantas termoeléctricas que presentaron mayor concentración total de PAH fueron C3 y R1 con valores de 14,0 y 9,23 mg kg^{-1} respectivamente. Estas muestras de suelo sobrepasan los niveles genéricos de referencia de BaA, BbF, BaP e IcdP, para la protección de la salud humana para usos de suelo diferentes a industrial o urbano (BOE, 2005) lo que indica cierto

impacto de la actividad industrial en ambos suelos. Sin embargo las concentraciones de PAH en estas muestras no se consideraron suficientes para el uso de estos suelos en ensayos de biorremediación con SAS.

Tabla 5.2: Concentraciones (mg kg^{-1}) de elementos traza en los emplazamientos muestreados (*n=3*).

		Mn	Cu	Zn	Cd	Pb
Anllares del Sil	1	440	20.1	50.3	n.d.	n.d.
	2	260	22.8	45.8	n.d.	1.27
	3	209	19.1	37.7	n.d.	n.d.
Cubillos del Sil	1	538	14.1	43.1	n.d.	n.d.
	2	462	22.6	69.5	0.34	n.d.
	3	440	64.9	78.4	n.d.	n.d.
La Robla	1	835	8.22	65.3	n.d.	8.08
	2	522	27.6	71.7	0.36	5.36
	3	2924	3.22	37.3	0.39	n.d.
Puertollano	1	451	18.7	21.0	0.12	n.d.
	2	2258	10.9	31.0	1.42	n.d.
	3	147	17.3	96.2	1.04	20.8
	4	421	22.3	140	0.94	58.8
	5	408	20.9	180	1.28	53.5
	6	409	7.02	45.8	0.42	20.2
	7	883	755	1229	4.25	941
Castejón	1	243	79.6	103	0.79	92.7
	2	350	30.1	84.8	1.36	40.3
	3	291	88.6	76.6	1.25	27.0
Campo tiro	1	226	19.6	66.0	0.13	1429
Metalúrgica	1	1937	64.2	924	2.00	210
	2	3317	100	1288	1.60	244
	3	400	35.0	1286	0.80	43.0
	4	425	18.2	1330	0.60	15.4

Las muestras P6 y P7 fueron tomadas en las inmediaciones de una planta petroquímica, sin embargo también se encontraban influenciadas por las plantas térmicas de carbón de la zona. La concentración de metales (Tabla 5.2) y PAH (Tabla 5.3) en la muestra P6 fue baja. En cambio la muestra P7 contenía elevadas concentraciones de metales y Σ16PAH. Individualizando la concentración de cada uno de los PAH se observa que casi el 70% corresponde a Naph, compuesto que es fácilmente degradable por microorganismos del suelo comparado con PAH de mayor peso molecular y relativamente volátil. Por otro lado, la muestra tomada no podía considerarse un suelo propiamente, ya que se trataba de un vertido de algún

tipo de residuo industrial (Fig. 5.2). Estos hizo que la muestra P7 a pesar su alto contenido en metales y concentración total de PAH, se tuviera que descartar para su uso en los futuros ensayos de degradación de PAH con muestras de suelo real.

Fig. 5.2: Detalle del perfil del emplazamiento P7 (Puertollano, Ciudad Real)

Las muestras de suelo procedentes de la planta de creosotado de madera muestran altas concentraciones de PAH (Tabla 5.4) y según el RD 9/2005 estas concentraciones se encuentran por encima de los niveles genéricos de referencia para la protección de la salud humana para uso de suelo industrial. El resultado fue el esperado teniendo en cuenta que la creosota está compuesta aproximadamente por un 85% de PAH y diversos trabajos reportan su acumulación en suelos de plantas de creosotado (Covino et al., 2010; Gallego et al., 2008; Tejeda-Agredano et al., 2013). Las muestras CJ 1 y 2 fueron tomadas en la zona de secado de la madera a la salida del autoclavado con creosota, mientras que la CJ3 corresponde al suelo de una antigua zona de almacenado de madera tratada. Por lo tanto la contaminación de muestra de suelo CJ3 se encuentra más estabilizada que en las muestras CJ 1 y 2. En cuanto a la concentración pseudo-total de metales, no se detectaron concentraciones altas. Esto es debido a que a pesar de que en el pasado la creosota contenía importantes cantidades de metales (Cd, Pb, Cu) y As, la legislación actual es más restrictiva y actualmente se encuentran prohibidos. Por otra parte se nos comunicó durante la toma de muestra que unos años antes la planta había descontaminado las zonas más problemáticas. Estas muestras de suelo fueron consideradas para los posteriores ensayos de degradación de PAH en

muestras de suelo real y ausencia de concentraciones elevadas de metales (Capítulo 7).

Las muestras de suelo tomadas en las cercanías de una metalúrgica mostraron concentraciones de metales ampliamente superiores a los valores VR90 para suelos de la Comunidad de Madrid (BOCM, 2007). Esto evidencia el impacto que está teniendo la actividad industrial en los suelos cercanos. Según la legislación actual ninguno de los metales analizados sobrepasa los valores establecidos para el uso industrial del suelo. Sin embargo desde un punto de vista medioambiental la concentración de Zn puede considerarse como alta. Las concentraciones de PAH encontradas en estos suelos (Tabla 5.4) fueron bajas o incluso indetectables por lo que estos suelos no pudieron ser usados para ensayos de degradación de PAH.

La muestra de suelo tomada en las cercanías del campo de tiro adyacente a la Universidad Autónoma de Madrid mostró una alta concentración de Pb (1429 mg kg^{-1}) que sobrepasa ampliamente los 75 mg kg^{-1} establecidos como nivel genérico de referencia en la legislación aplicable a este suelo para su actual uso, ni urbano ni industrial (BOCM, 2007). La alta concentración de Pb muy probablemente se deba a la munición, tanto a los perdigones como al fulminante, empleada en el campo de tiro y su posterior dispersión en el medio. De hecho durante el tamizado del suelo se observaron perdigones. Cabe destacar que durante la molienda del suelo para su posterior digestión en microondas se eliminaron estos perdigones. Por lo tanto la concentración de Pb correspondió al Pb del suelo y no a la digestión de perdigones. En bibliografía se encuentran otros trabajos que también han encontrado concentraciones altas de Pb en muestras procedentes de campos de tiro (Hashimoto et al., 2008), incluso en Finlandia aproximadamente el 10% de los suelos potencialmente contaminados corresponde a campos de tiro (Sorvari et al., 2006). El resto de metales no presentó concentraciones importantes.

Los análisis de PAH mostraron elevadas concentraciones de estos compuestos (Tabla 5.3) llegando a sobrepasar ampliamente los niveles genéricos de referencia de la normativa española (BOE, 2005). Peddicord and LaKind (2000) reportaron la presencia de estos compuestos en suelos de capo de tiro, sin

embargo las concentraciones de PAH de la muestra de suelo analizada sobrepasaba ampliamente las concentraciones reportadas por el trabajo citado. Profundizando en los resultados se observa que el 11% de la concentración total corresponde a PAH de 3 anillos, 40% a PAH de 4 anillos y 49% a PAH de 5 y 6 anillos. Esta distribución hace de este suelo un buen candidato para su estudio en ensayos de degradación de PAH ya que la mayoría de la concentración de PAH se debe a HMW-PAH, compuestos altamente recalcitrantes y tóxicos. Entre ellos destaca el BaP, compuesto declarado como carcinógeno por la IARC (2010), y que se encuentra en alta concentración (93,1 mg kg^{-1}) en este suelo. Por todo esto, la muestra de suelo procedente del campo de tiro se seleccionó para la evaluación del SAS en biorremediación de suelos contaminados por metales (Pb) y PAH (Capítulo 8).

5.4. Conclusiones

Los emplazamientos cercanos a plantas de generación eléctrica mediante combustión de carbón mostraron una acumulación baja de metales y PAH. Los suelos procedentes de las inmediaciones de la industria metalúrgica mostraron bajas concentraciones de PAH pero una apreciable acumulación de metales, especialmente Zn. El emplazamiento situado en la planta de creosotado de madera mostró una elevada concentración de PAH pero ausencia de contaminación por metales. El suelo procedente de las inmediaciones del campo de tiro reportó una alta acumulación de Pb y PAH. Por todo esto, para la evaluación de la efectividad de SAS en biorremediación de suelos reales contaminados, se seleccionaron los suelos afectados por el creosotado de la madera como modelo de suelo contaminado por PAH y el suelo cercano al campo de tiro como modelo de suelo multicontaminado por Pb y PAH.

Tabla 5.3: Concentraciones (mg kg^{-1}) de hidrocarburos aromáticos policíclicos en los alrededores de centrales térmicas de carbón (*n=3*).

		Naph	Acy	Ace	Flu	Phe	Ant	Fla	Py	BaA	Ch	BbF	BkF	BaP	DBhaA	BghiP	IcdP	Σ16PAH
Anllares del Sil	1	1.36	n.d.	n.d.	n.d.	0.02	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.38
	2	0.57	n.d.	n.d.	n.d.	0.81	n.d.	n.d.	n.d.	n.d.	0.14	n.d.	n.d.	n.d.	n.d.	0.17	0.03	1.73
	3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Cubillos del Sil	1	n.d.	n.d.	n.d.	n.d.	0.04	0.01	0.03	0.00	0.01	0.02	n.d.	0.00	0.03	0.00	0.01	n.d.	0.15
	2	n.d.	n.d.	n.d.	n.d.	0.10	0.01	n.d.	n.d.	0.00	0.03	0.08	n.d.	0.02	0.01	0.01	0.01	0.26
	3	n.d.	n.d.	n.d.	n.d.	1.22	0.16	3.38	2.36	1.11	1.15	0.95	0.57	1.21	0.01	1.10	0.83	14.0
La Robla	1	n.d.	n.d.	n.d.	n.d.	0.98	0.11	1.34	1.24	0.73	0.89	1.05	0.43	1.03	0.04	0.77	0.61	9.23
	2	n.d.	n.d.	n.d.	n.d.	0.21	0.02	0.15	0.07	0.03	0.08	0.17	0.03	0.07	0.00	0.04	0.04	0.91
	3	n.d.	n.d.	n.d.	n.d.	0.14	n.d.	0.27	0.08	0.02	0.10	n.d.	0.06	0.18	n.d.	n.d.	0.05	0.91
Puertollano	1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.02	0.03	0.05
	2	n.d.	n.d.	n.d.	n.d.	0.03	0.01	n.d.	n.d.	0.00	0.00	n.d.	n.d.	0.01	n.d.	0.01	n.d.	0.06
	3	n.d.	n.d.	n.d.	n.d.	1.19	0.09	n.d.	0.21	0.16	0.40	n.d.	n.d.	0.14	n.d.	n.d.	n.d.	2.19
	4	n.d.	n.d.	n.d.	n.d.	0.68	0.04	n.d.	0.04	0.11	0.21	n.d.	n.d.	0.17	n.d.	0.09	0.04	1.38
	5	n.d.	n.d.	n.d.	n.d.	0.70	0.02	n.d.	0.04	0.05	0.10	n.d.	n.d.	0.09	n.d.	0.09	0.02	1.12
	6	n.d.	n.d.	n.d.	n.d.	0.06	0.01	n.d.	0.01	0.01	0.01	n.d.	n.d.	0.01	n.d.	0.01	n.d.	0.11
	7	34.5	3.09	n.d.	n.d.	4.34	0.03	4.75	3.38	n.d.	0.00	n.d.	n.d.	0.01	n.d.	0.03	0.01	50.1

n.d.: no detectado

Tabla 5.4: Concentraciones (mg kg^{-1}) de hidrocarburos aromáticos policíclicos en planta de creosotado, alrededores de campo de tiro e industria metalúrgica (*n=3*).

		Naph	Acy	Ace	Flu	Phe	Ant	Fla	Py	BaA	Ch	BbF	BkF	BaP	DBhaA	BghiP	IcdP	Σ16PAH
Castejón	1	127	17.9	506	439	286	56.7	91.1	62.9	22.6	38.1	34.5	14.8	17.9	0.28	11.4	10.7	1737
	2	n.d.	n.d.	n.d.	21.5	339	30.8	342	298	84.5	56.0	18.2	6.28	7.56	1.28	3.19	3.39	1212
	3	n.d.	n.d.	3.59	6.71	16.1	53.6	19.3	12.5	5.26	7.77	5.96	3.13	2.86	0.07	2.06	1.85	141
Campo tiro	1	n.d.	n.d.	49.9	0.82	28.9	7.40	71.0	95.8	56.8	76.4	112	32.3	93.1	8.47	74.1	49.5	757
Metalúrgica	1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.04	0.33	0.04	n.d.	0.06	0.03	0.04	n.d.	0.04	n.d.	0.60
	2	n.d.	n.d.	n.d.	n.d.	0.22	0.04	0.42	0.29	0.13	0.23	0.10	0.07	0.12	n.d.	0.09	0.07	1.81
	3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.01	n.d.	0.04	n.d.	0.01	n.d.	0.06	0.03
	4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d.: no detectado

Bloque II: Evaluación del Sustrato post-cultivo de *A. bisporus* como remediador de suelos contaminados

En el segundo bloque de capítulos se encuentran recogidos los resultados obtenidos durante los ensayos sobre la capacidad de degradación de PAH e inmovilización de Cd y Pb por el sustrato post-cultivo de champiñón (*A. bisporus*) y ensayos de biorremediación de suelos reales contaminados con PAH y multicontaminado con PAH y Pb.

Como paso inicial para la evaluación de la capacidad remediadora del sustrato post-cultivo de champiñón (*A. bisporus*) se muestran los resultados de ensayos de adsorción de Cd y Pb, así como degradación de PAH en presencia o ausencia de Cd o Pb y determinación de los efectos de estos metales en la actividad de enzimas ligninolíticas.

En un segundo paso de la evaluación del SAS, se muestran los resultados de un ensayo de remediación de suelo real contaminado con PAH en el que se utilizaron diferentes formulaciones de SAS con el objetivo de evaluar sus capacidades como bioestimulador de la microbiología del suelo, utilidad como inóculo de *A. bisporus* y toda la microbiología inherente a este sustrato o únicamente como inóculo de *A. bisporus*. Todos los tratamientos se compararon con un control sin SAS y se evaluaron parámetros relacionados con la colonización del suelo por hongos, modificaciones en la microbiología del suelo, capacidad degradadora de PAH, biodisponibilidad de PAH y finalmente detoxificación del suelo.

En tercer lugar se llevó a cabo una experiencia similar a la anterior pero utilizando un suelo multicontaminado con plomo y PAH. En este caso se prestó especial atención al efecto del Pb sobre los microorganismos del suelo y SAS, su efecto en la degradación de PAH y a los cambios en la movilidad del Pb producidos por los diferentes modos de aplicación de SAS.

En este bloque se exponen los resultados incluidos en los siguientes trabajos:

- "Cadmium and lead bioavailability and their effects on polycyclic aromatic hydrocarbons biodegradation by spent mushroom substrate." Environmental Science and Pollution Research (2013) 20:8690-8699. DOI: 10.1007/s11356-013-1829-0
- "Implications of polluted soil biostimulation and bioaugmentation with spent mushroom substrate (*Agaricus bisporus*) on the microbial community and polycyclic aromatic hydrocarbons biodegradation." Science of the Total Environment (2015) 508: 28-28 DOI: 10.1016/j.scitotenv.2014.11.046
- "Bioremediation of multi-polluted soil by spent mushroom substrate (Agaricus bisporus): polycyclic aromatic hydrocarbons degradation and lead availability." Journal of Hazardous Materials (Under revision).

Capítulo 6:

Cadmium and lead bioavailability and their effects on polycyclic aromatic hydrocarbons biodegradation by spent mushroom substrate

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Abstract

Bioremediation of mixed metal-organic soil pollution constitutes a difficult task in different ecosystems all around the world. The aims of this work are to determine the capacity of two spent mushroom substrates (*Agaricus bisporus* and *Pleurotus ostreatus*) to immobilize Cd and Pb, to assess the effect of these metals on laccase activity and to determine the potential of spent *A. bisporus* substrate to biodegrade four polycyclic aromatic hydrocarbons (PAH): fluorene, phenanthrene, anthracene and pyrene, when those toxic heavy metals Cd and Pb are present. According to adsorption isotherms, spent *P. ostreatus* and *A. bisporus* substrates showed a high Pb and Cd adsorption capacity. Pb and Cd interactions with crude laccase enzyme extracts from spent *P. ostreatus* and *A. bisporus* substrates showed Cd and Pb enzyme inhibition; however laccase activity of *A. bisporus* presented lower inhibition. Spent *A. bisporus* substrate polluted with PAH and Cd or Pb was able to biodegrade PAH, although both metals decrease the biodegradation rate. Spent *A. bisporus* substrate contained a microbiological consortium able to oxidize PAH with high ionization potential. Cd and Pb were immobilized during the bioremediation process by spent *A. bisporus* substrate. Consequently, spent *A. bisporus* substrate was adequate as a multi-polluted soil bioremediator.

6.1. Introduction

Many industrial polluted soils simultaneously hold inorganic and organic pollutants, such as heavy metals and polycyclic aromatic hydrocarbons (PAH) (Beesley et al. 2010; Thavamani et al. 2011). However, many soil remediation research studies are focused on organic or inorganic pollutants individually, but not as a mixture. The mixture of different kinds of pollutants complicates the soil remediation process, even more so if bioremediation is the selected remediation technique (Megharaj et al. 2011). This environmentally friendly technique includes the use of plants, bacteria, algae or fungi as organisms and compost, as well as manure or straw as organic matter sources to immobilize heavy metals or enhance the activity of PAH-degrading organisms (Hwang et al. 2007; Haritash and Kaushik 2009; Tandy et al. 2009).

Over the past few years, a growing interest has been shown in assessing the bioremediation potential of fungi. One of the more studied fungi for organic pollutant biodegradation is white rot fungi. It is a group with the capacity to degrade lignin by extracellular enzymes. Ligninolytic enzymes are mainly lignin peroxidase (LiP; EC 1.11.1.14), manganese-dependent peroxidase (MnP; EC 1.11.1.13), versatile peroxidase (VP; EC 1.11.1.16) and laccase (Lac; EC 1.10.3.2) (Wong 2009). These enzymes are nonspecific and oxidize a wide variety of organic compounds. The PAH oxidation mechanism by fungal ligninolytic enzymes is similar to the degradation of nonphenolic lignin (Peng et al. 2008). The white rot fungi most studied for PAH degradation were *Pleurotus ostreatus* and *Phanerochaete chrysosporium* (Haritash and Kaushik 2009), instead of other successfully used fungi, such as *Irpex lacteus* and *Lentinus tigrinus* (Covino et al. 2010). However, the presence of heavy metals affects fungi soil penetration, consequently affecting PAH biodegradation (Baldrian et al. 2000) and playing an important role in the regulation of extracellular enzyme activities. In this sense, Cu increases Lac induction and its stabilization, Cd increases Lac activity and Pb and Hg decrease Lac activity in *P. ostreatus* (Baldrian and Gabriel 2002).

Agaricus bisporus is able to biodegrade organic xenobiotics present in soil, such as phenolic compounds (Trejo-Hernández et al. 2001) and PAH (Li et al. 2010; Reid et al. 2002). Spent *A. bisporus* substrate (SAS) is a source of mycelia and ligninolytic

enzymes, especially Lac (Trejo-Hernández et al. 2001). A large amount of SAS is generated; just in Europe, more than 3.5 x 10^6 t are produced every year. The accumulation of SAS causes important environmental pollution in mushroom production areas due to salts and soluble organic carbon leaching during SAS storage (Guo et al. 2001). Some authors have researched how to reduce the amount of SAS produced (Royse 2010), or how to reuse it (Pardo-Giménez and Pardo-González 2008) so as to minimize the adverse environmental impact of SAS.

SAS used as *A. bisporus* inoculum carrier can be useful for soil bioremediation, producing simultaneous effects, such as the immobilization of heavy metals which alter fungi development, an increase in the activities of ligninolytic enzymes and consequently, the enhancement of PAH biodegradation.

The aims of this study were: 1) to assess the Cd and Pb immobilization properties of spent *A. bisporus* substrate and spent *P. ostreatus* substrate, 2) to evaluate the effects of Cd and Pb on laccase activity of *A. bisporus* and *P. ostreatus* from crude extracts of spent *A. bisporus* substrate and spent *P. ostreatus* substrate, compared with a purified laccase enzyme from *T. versicolor*, 3) to determine the potential of spent *A. bisporus* substrate to biodegrade PAH in the presence of Cd or Pb, and its effects on Cd and Pb bioavailability.

6.2. Materials and methods

6.2.1. Spent mushroom substrates and characterization

The selection of spent substrates was made bearing in mind the most significant edible fungi cropped around the world (*P. ostreatus* and *A. bisporus*) and, therefore, the substrate wastes that present the highest environmental implications. *A. bisporus* substrate was produced from wheat straw (55%), poultry litter (30%) and grape marc (15%). After composting for 21 days, gypsum was added. During the crop, a mixture of sphagnum blond peat and soil with high calcium carbonate content as casing was added to up to 30% (m/m) of the final mixture (Pardo-Giménez and Pardo-González, 2008). After *A. bisporus* harvest, the compost was labeled as spent *A. bisporus* substrate (SAS) (pH=6.04; %C=27;

%N=1.7). The *P. ostreatus* substrate component is 100% wheat straw (Michael et al. 2011). After the *P. ostreatus* harvest, spent substrate was labeled as spent *P. ostreatus* substrate (SPS) (pH=6.95; %C= 42; %N=0.7). The SAS and SPS samples were provided by commercial farms (Cuenca, Spain) which traditionally use these substrates.

In order to describe the most important functional groups contained in the organic matter of SAS and SPS, they were subjected to cross-polarization and magic angle spinning nuclear magnetic resonance (CP-MAS ^{13}C NMR) in a Bruker AV-400-WB apparatus (Billerica, MA, USA) operating at 100.61 MHz (rotor spin rate 10 kHz; contact time 3.5 ms; acquisition time 4 s; 18,000 scans). Samples were packed in 4 mm zirconia rotors with Kel-F caps. The pulse sequence was applied with a 1 h ramp. Chemical-shift (δ) regions were divided into the main resonance regions according to Tapia et al. (2010) and the characterization of organic matter shown on Table 6.1.

Table 6.1: Relative areas (percentage of total area) of the chemical shift (δ) regions in 13C cross-polarization and magic angle spinning nuclear magnetic resonance (CP-MAS 13C NMR) spectra of spent *P. ostreatus* (SPS) and *A. bisporus* (SAS) substrates.

chemical shift (ppm)	Type of C bond	SPS	SAS
		% total area	
0 < δ ≤ 45	Alkyl (aliphatic)	4.4	21.4
45 < δ ≤ 60	N-alkyl	3.9	7.3
60 < δ ≤ 93	O-alkyl	67.8	39.2
93 < δ ≤ 110	Di-O-alkyl	14.6	10.0
110 < δ ≤ 140	Aromatic	1.8	4.2
140 < δ ≤ 160	Phenolic	2.7	2.8
160 < δ ≤ 190	Carboxyl	3.9	14.6
190 < δ ≤ 220	Amide-ketonic	0.9	0.5

The highest spectra region corresponded to the O-alkyl group for both, SPS and SAS, attributable above all to cellulose and hemicellulose. However, SPS showed more O-alkyl carbon than SAS, probably because SPS was only wheat straw and SAS contains other sources of carbon such as poultry litter in its initial composition. Attending to the aliphatic/O-alkyl ratio that is an indicator of the evolution of organic matter (Tapia et al. 2010), SAS displayed organic matter more mature than SPS (ratio values 0.55 and 0.06, respectively). This result is consistent,

considering that SAS had suffered a more intense composting process than SPS before the mushroom harvest. SAS showed a higher alkyl and carboxyl region than SPS. These higher amounts of alkyl and carboxyl groups may be related to the composting process, which probably produced fatty acids and humic acids with aliphatic structure. The high presence of carboxyl acidic groups can increase the cation exchange capacity of the material and would induce the formation of heavy metal complexes.

6.2.2. Heavy metals adsorption isotherms in SAS and SPS

Cd and Pb adsorption isotherms were constructed according to Chen et al. (2005). SAS and SPS were air-dried, milled and sieved at 0.2 mm. 0.0100 g of sample were suspended in 10 ml of $Cd(NO_3)_2$ or $Pb(NO_3)_2$ solution at 0, 10, 25, 50, 75, 100, 150, 300 mg l^{-1} adjusted to pH 6.5. After 24 h of interaction at 25ºC, suspension was centrifuged at 3000 rpm for 15 min and the remaining supernatant was filtered through an ashless Whatman 42 filter. Interactions were made per triplicate. The solutions obtained were analyzed by a Perkin Elmer AA800 atomic absorption spectrometer. Data obtained were adjusted to the Langmuir adsorption model according to the equation: $q_e = \frac{qmax*aL*Ce}{1+(aL*Ce)}$ where q_e is the heavy metal amount adsorbed (mg g^{-1}), q_{max} is the maximum amount of heavy metal adsorbed (mg g^{-1}), a_L is the adsorption constant (l mg^{-1}) and C_e is the heavy metal concentration after interaction (mg l^{-1}).

6.2.3. Extraction and quantification of ligninolytic enzyme activity

Enzymatic extraction was carried out per triplicate with a modified method by Trejo-Hernández et al. (2001): 50 g of SAS or SPS were mixed with 1 L of 0.1 M Tris-HCl buffer (pH 7.5) and 1 h shaken at 4ºC, then centrifuged at 5000 rpm and the supernatant was used directly as the enzyme source and was labeled as enzymatic crude extract.

Lac activity was evaluated spectrophotometrically by following the oxidation of 0.2 mM 2,2´-azino-bis-(3- ethylbenzothiazoline-6-sulphonic acid) (ABTS) in 100 mM sodium acetate buffer (pH 4.5) at 420 nm (ε= 36000 M^{-1} cm^{-1}).

MnP activity was assayed by the oxidation of 1 mM $MnSO_4$ in 50 mM sodium malonate buffer (pH 4.5), in the presence of 0.1 mM H_2O_2. Manganic ions, Mn^{3+}, form a complex with malonate, which absorbs at 270 nm (ε= 11590 M^{-1} cm^{-1}) (Wariishi et al. 1992). One unit of enzyme activity (IU) is defined as the amount of enzyme which produced 1µmol of product per minute under assay conditions.

6.2.4. Heavy metals – laccase enzyme interaction

Increasing concentrations of Cd and Pb were assayed to assess the effect on Lac activity from low to very high bioavailable Cd and Pb. $Cd(NO_3)_2$ or $Pb(NO_3)_2$ were added to SAS and SPS crude enzymatic extracts, and purified laccase of *Trametes versicolor* provided from Sigma-Aldrich, as Lac model, dissolved in 0.1M Tris-HCl buffer (pH 7.5). Final metal concentrations were 0 (control) 5, 50 and 500 µM. Interactions were incubated per triplicate at 25 ^oC in the dark with orbital shaking (160 rpm). Lac activity was analyzed at 1 h, 24 h and 7 days later, according to Baldrian and Gabriel (2002).

6.2.5. Biodegradation of PAH by SAS in the presence of Cd and Pb

The assay was conducted per quadruplicate, in a growth chamber in the dark at 20 ^oC and 80% relative air humidity. Twenty grams of fresh SAS (56% moisture content) and fresh SAS sterilized twice in autoclave at 120 ^oC for 45 min as an abiotic control were incubated in 100 ml Erlenmeyer flask for 28 days. In order to assess the PAH biodegradation rate of SAS with and without Cd or Pb, the following treatments were assayed: fresh SAS without PAH, sterilized SAS with PAH, fresh SAS with PAH, fresh SAS with PAH plus 500 µmol kg^{-1} Cd and fresh SAS with PAH plus 500 µmol kg^{-1} Pb. PAH were fluorene 98% (Flu), phenanthrene 98% (Phe), anthracene 97% (Ant) and pyrene 98% (Py) provided for Sigma-Aldrich. PAH were added as acetone HPLC grade (Panreac) solution (1 ml) to achieve a final concentration in SAS of 100 mg kg^{-1}. Acetone was added to treatment without PAH, to equal the initial solvent content in SAS. The samples were placed 24h in a chemical hood to acetone evaporation after acetone solution addition. Cd and Pb were added as $Cd(NO_3)_2.4H_2O$ and $Pb(NO_3)_2$ (Panreac) aqueous solution (2ml). For

treatments without heavy metals, $Ca(NO_3)_2.4H_2O$ (Panreac) was added to equal the nitrate content of SAS. Cd and Pb concentrations were selected according to results obtained in the adsorption assay and Lac-heavy metals interactions in order to get an appreciable heavy metals bioavailability and previously published works (Baldrian and Gabriel, 2002). PAH concentration was based on previously published works (Marquez-Rocha et al. 2000).

To assess the quick and long term effect of SAS in heavy metals bioavailability, ligninolytic activity and PAH biodegradation, both soluble and bioavailable Cd and Pb content, Lac and MnP activities and total PAH were determined, respectively, on days 7, 21 and 28.

Cd and Pb availability was determined per quadruplicate by extracting with water as soluble fraction or 0.05 M EDTA-Na_2 pH 7 as bioavailable fraction in soils (Zhang et al. 2002). One gram of dry and milled SAS was extracted for 1 h with 10 ml of Milli-Q water (Millipore Corporation) or 0.05 M EDTA respectively. Heavy metals analyses were carried out by atomic absorption spectrometry (Perkin-Elmer AA800).

Lac and MnP were extracted and activities determined per quadruplicate as described in the "Extraction and quantification of enzymatic activity" section.

6.2.6. PAH analysis and metabolites identification

PAH extraction and determination were carried out per quadruplicate according to García-Delgado et al. (2013a). Five grams of fresh SAS sample were extracted with 25 ml of acetone:hexane (1:1) in an orbital shaker for 120 min, and then the solution was left for 30 min for solution decanting. After the extraction step, the solutions were filtered through a nylon syringe filter with 0.45µm pore size (Whatman International). One ml of each resulting solution was taken to dryness by N_2 flow. The residue was redissolved in 1 ml of acetonitrile. PAH were analyzed by a HPLC Waters 2695 Separation Module coupled with a Waters 996 photodiode array detector (PDA). The analytical cartridge column was a Supelcosil™ LC-PAH 250x3 mm, 5µm. The cartridge column was protected by a Supelguard™ LC-18 20x3 mm guard column. The mobile phase was acetonitrile-

water gradient at a flow-rate of 0.5 ml min^{-1}. The gradient elution program was 0-5 min: 60% acetonitrile and 40% water, then a linear gradient elution from 60% acetonitrile at 5 min to 100% acetonitrile at 15 min followed by isocratic elution for 20 min. The column run temperature was set at 28ºC. Each PAH was identified both by time retention and by the UV spectrum by comparing with standards, and was determined at 254 nm. PAH metabolites were identified according to Aranda et al. (2010).

6.2.7. Statistical analysis

All statistical tests were carried out using the SPSS (Statistical Product and Service Solutions) 15.0 software package. One-way analysis of variance (ANOVA) after previously performing a Levene variance homogeneity test were carried out. To compare the differences between treatments, the Duncan or Games-Howell post-hoc test (according to variance homogeneity) at $p < 0.05$ for interaction metal-laccase and $p < 0.1$ for PAH biodegradation assay was used.

6.3. Results and discussion

6.3.1. Spent *P.ostreatus* and *A. bisporus* substrates Cd and Pb adsorption isotherms

Results of Cd and Pb adsorption on SAS and SPS were modeled according to the Langmuir equation (Fig. 6.1). SAS Langmuir curve fits for Cd and Pb were good with 0.973 and 0.960 R^2 values, respectively (Fig. 6.1a). Maximum amounts of adsorbed heavy metals (q_{max}) were 33.9 and 19.7 mg g^{-1} for Cd and Pb, respectively.

SPS showed Langmuir curve fits for Cd and Pb with 0.947 and 0.982 R^2 values, respectively (Fig. 6.1b). Maximum amounts of adsorbed heavy metals (q_{max}) were 16.6 mg g^{-1} for Cd, and 51.5 mg g^{-1} for Pb.

SAS presented higher Cd affinity than Pb; however, SPS showed higher Pb adsorption capacity. The different behavior towards Pb adsorption could be

partially explained according to the CP-MAS ^{13}C NMR O-alkyl content of substrates (Table 6.1), which was higher for SPS than for SAS. Cation Pb^{2+} formed a complex with cellulose and precipitation of the hydrolysis product of the Pb^{2+} complex (Zhou et al. 2005). Additionally, the pH value of SPS (6.95) was higher than SAS (6.04) and it would help in heavy metal precipitation such as hydroxides.

The higher SAS Cd adsorption could be justified by two factors: firstly, the SAS composition that was more complex (organic and inorganic components) than SPS (only wheat straw), and that underwent the composting process that produced more stabilization of organic matter and higher carboxylic acids amount according to CP-MAS ^{13}C NMR analysis. The second reason is that SAS contained inorganic compounds such as clays, Fe and Mn oxides, $CaCO_3$ and $CaSO_4$ (Tapia et al. 2010, García-Delgado et al. 2012b) which may be responsible for heavy metal adsorption or precipitation phenomena.

Throughout the bioremediation process, cellulose, hemicellulose and lignin components of SPS and SAS will be gradually degraded and then adsorption properties of heavy metals will be altered. However, SAS is a more stable material than SPS and holds more stabilized organic matter and inorganic components that probably will produce smaller changes throughout time. As such, all of these reasons suggest that heavy metal adsorption properties would be maintained longer on SAS than on SPS.

In view of the high Cd and Pb adsorption capacity of SAS and SPS, these agricultural wastes could be reused as organic amendments of polluted soils to immobilize Cd and/or Pb. Both, SAS and SPS could be used in polluted soils where heavy metals extraction in not possible and, therefore, immobilization will be the best option to minimize their bioavailability and hence, the environmental damage or heavy metals entering the food chain.

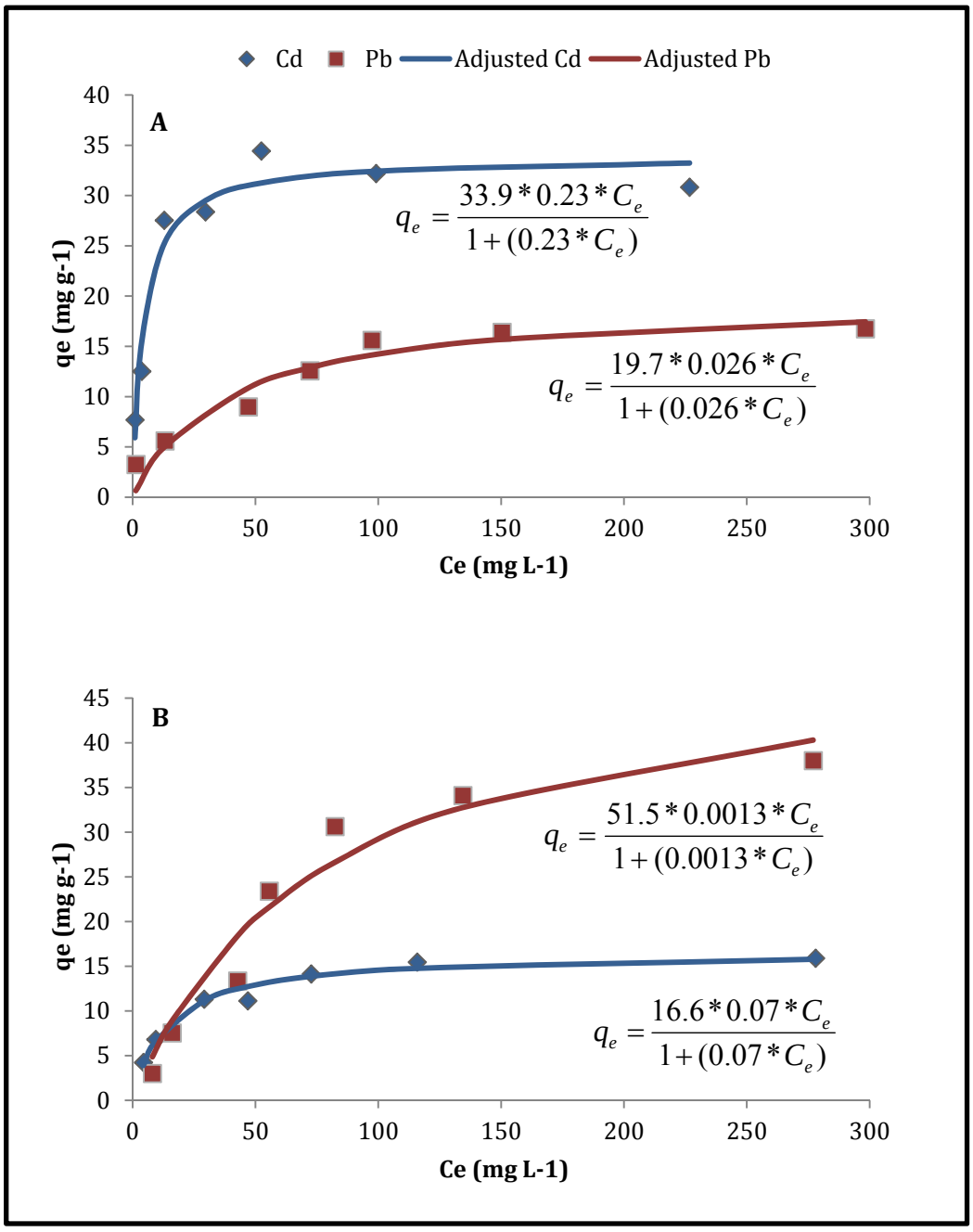

Fig. 6.1: Adsorption of Langmuir isotherms (25 °C) of Cd^{2+} and Pb^{2+} by spent *A. bisporus* substrate (A) and spent *P. ostreatus* substrate (B) where q_e is the heavy metal amount adsorbed (mg g^{-1}) and C_e is the heavy metal concentration after interaction (mg l^{-1}). *n* =3.

6.3.2. Interaction of heavy metals with laccase enzyme obtained from *T. versicolor*, SAS (*A. bisporus*) and SPS (*P. ostreatus*)

Interactions of Lac-heavy metals were made to determine the inhibition or increase of Lac activity in presence of Cd or Pb from different sources of this ligninolytic enzyme. Purified Lac from *T. versicolor* was used as model of purified enzyme. Table 6.2 presents the results of percentage of Lac activity after 1 h, 24 h and 7 days of incubation with Cd or Pb. The substrate SAS shows higher Lac activity (8670±460 U kg^{-1}) than SPS (3540±86 U kg^{-1}). Initial Lac activity quantified in extracts for *T. versicolor, SAS (A. bisporus)* and SPS (*P. ostreatus)* were 0.25±0.02, 0.43±0.02 and 0.177±0.004 IU ml^{-1}, respectively. *P. ostreatus* Lac activity showed the highest decline (49-77% of initial activity) at 24 h. For *T. versicolor,* the reduction was about 0-54% and *A. bisporus* Lac activity reduction was between 8 and 31%. However, on the 7th day, *A. bisporus* Lac activity was not detected and *P. ostreatus* Lac activity showed a high reduction (> 90%) except in treatments with heavy metal at 500 µM. *T. versicolor* Lac was the most resistant at 7 days and showed a reduction of 42-69% in comparison with initial activity.

Table 6.2: Percentage of Laccase activity (mean ± standard deviation) of purified enzyme of *T. versicolor* and crude extracts from spent *A. bisporus* (SAS) and *P. ostreatus* (SPS) substrates after incubation with Cd and Pb in Tris-HCl 0.1M pH 7.5 buffer (*n=3*).

		T. versicolor			SAS			SPS		
Treatment		1h	24h	7 days	1h	24h	7 days	1h	24h	7 days
Control		100±8	102±4	46±14	100±5	82±1	1 ±1	100±2	33 ± 1	3 ± 3
Cd	5 µM	91 ± 3	91 ± 5	58 ± 5	107±3	92±8	n.d.	95 ± 2	25 ± 1	3 ± 4
	50 µM	80±12	86 ± 3	56 ± 6	96±5	90 ±2	n.d.	91 ± 4	23 ± 2	21 ± 6
	500µM	75 ± 8	79 ± 5	52 ± 6	90 ± 4	79 ±5	5 ± 2	81 ± 5	51 ± 1	25 ± 4
Pb	5 µM	91±10	86 ± 4	46 ± 5	85 ± 3	77 ±4	n.d.	101±4	29 ± 6	2 ± 2
	50 µM	93 ± 5	78 ± 7	48±14	81 ± 5	69 ±3	n.d.	96±16	27 ± 5	6 ± 7
	500 µM	74 ± 8	46 ± 8	31 ± 5	74 ± 3	75 ±3	2 ± 1	83 ± 5	45 ± 2	21±21

n.d.: not detected

The different behavior towards time and heavy metals found between purified Lac from *T. versicolor* and crude Lac of SAS and SPS could be explained by two reasons. Firstly, because enzyme purification could increase the life of enzymes during the incubation time due to the lack of microbial content and therefore lower microbial degradation than crude extracts of SAS and SPS; and secondly, according to Baldrian (2006), there is considerable heterogeneity in the composition and properties of Lac from different fungi. In this sense, *T. versicolor* and *A. bisporus* Lac were negatively affected by Cd and its increasing concentrations produced a decrease in Lac activity. However, *P. ostreatus* only showed this tendency at 1 h sampling because at 24 h and 7 days, increasing Cd concentrations increase Lac activity. Baldrian and Gabriel (2002) found similar results for interactions of *P. ostreatus* Lac extracts at Cd 50 μM, or at higher concentrations. Lead strongly inhibited Lac activity and showed a clear trend to reduce activity, according to Pb concentration increases for all fungi tested, with the exception of Lac from *P. ostreatus* that showed a positive effect with Pb (500 μM) at 24 h. However, the Lac activity remaining at 24h of SAS extracts was higher at any concentrations of Cd and Pb.

Higher initial Lac activity of SAS (*A. bisporus*) and its lower inhibition towards Cd and Pb than SPS (*P. ostreatus*) at 24 h may indicate that SAS could be more effective as a Lac source for PAH biodegradation in the presence of Cd or Pb. In addition, Li et al. (2010) reported that crude extracts from SAS were excellent for PAH detoxification in liquid medium.

6.3.3. PAH biodegradation by SAS substrate in the presence of Cd and Pb

According to previous results of heavy metals adsorption capacity and Lac – heavy metals interactions, a biodegradation assay, in solid phase, of PAH in presence of Cd and Pb with SAS was carried out. The aim of the assay consisted in the assessment of the SAS potential to immobilize Cd and Pb and to biodegrade PAH simultaneously.

6.3.3.1. Cd and Pb inmobilization during PAH biodegradation

Fig. 6.2 shows percentages of water (Fig. 6.2a) and 0.05 M EDTA (pH=7.0) (Fig. 6.2b) soluble by both Cd and Pb during the PAH biodegradation process. Water soluble contents were lower than 6 and 1% of Cd and Pb added (500 μmol kg^{-1}) respectively. No differences were found in water soluble Pb during the three sampling times. However, Cd decreased its water soluble content by about 50% from the 7th to the 21st day (Fig. 6.2a). Metals extracted with EDTA (bioavailable fraction) were lower than 30% of Cd and Pb added. On day 7, Cd-EDTA and water soluble percentages were higher than Pb; however, on the 21th and 28th days, their bioavailable fractions were similar. Therefore, Cd immobilization, according to water soluble content, was slower than Pb. No differences were found for bioavailable Cd during the three sampling times, but the bioavailable Pb fraction increased gradually from 19 to 29% throughout the 28 days. These results may be explained according to the expected evolution of cellulose and carboxylic acid content of SAS, because Pb have a high tendency to be adsorbed onto cellulose materials (Wong 2009). During the biodegradation process, cellulose was degraded; therefore, the Pb could be desorbed. On the other hand, lignine, hemicelluloses and other organic compounds after degradation increased the amount of carboxylic groups in their structure that were able to fix Cd and therefore decreased its solubility. Not only compost chemistry affected Cd and Pb adsorption; fungi are able to immobilize heavy metals mainly attributable to defense mechanisms that produce extracellular compounds such as oxalic acid, melanins and phenolic molecules associated with the cell wall (Baldrian 2003; Jarosz-Wilkolazka et al. 2006).

The low heavy metal bioavailability regarding the total content due to adsorption or precipitation phenomena indicates that the mere measurement of total heavy metal content in a solid medium would not be adequate for making a realistic interpretation, and heavy metal bioavailability must be considered.

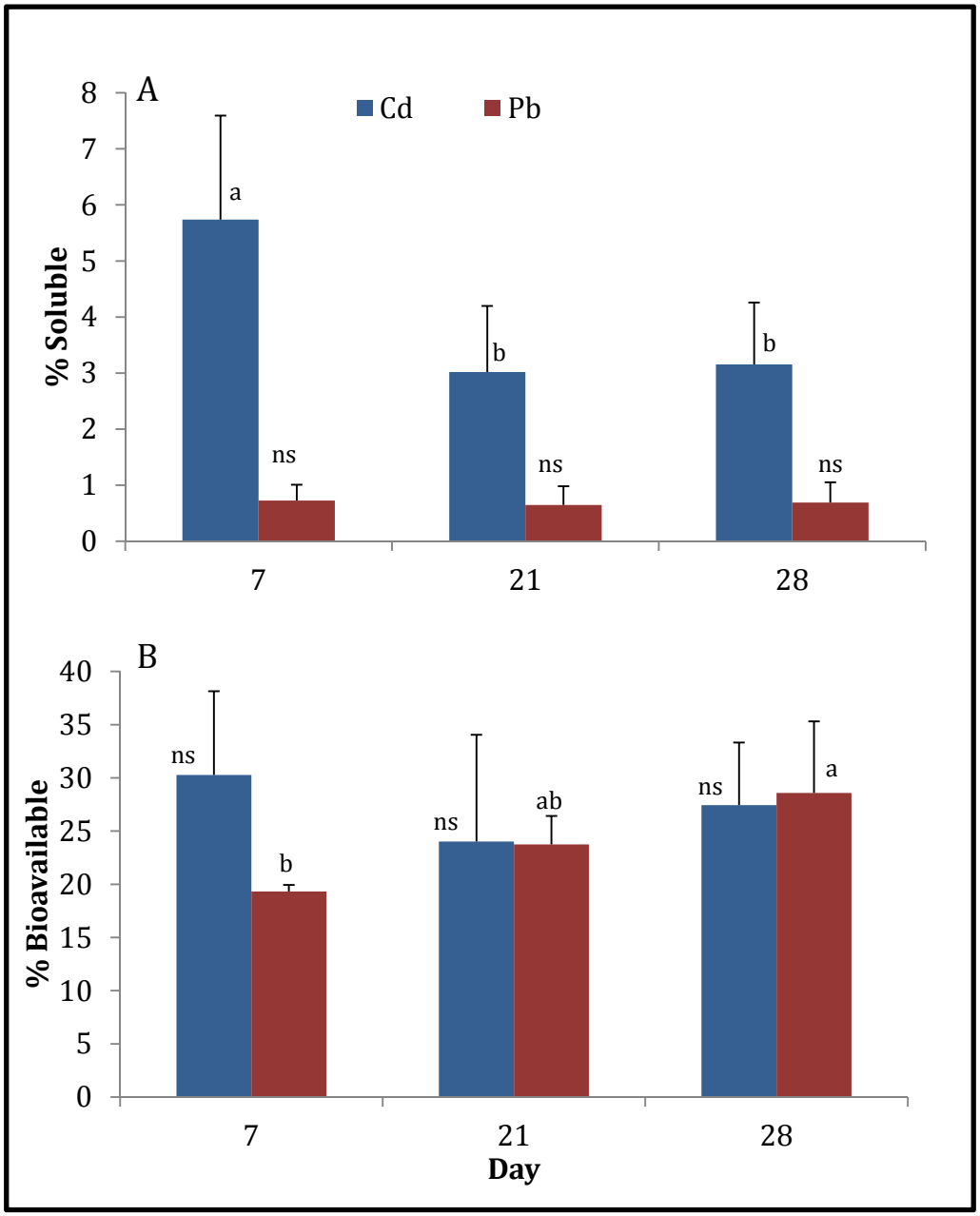

Fig. 6.2: Percentage of soluble (water extractable) (A) and bioavailable (0.05M EDTA-Na2 pH 7 extractable) (B) Cd and Pb of spent *A. bisporus* substrate spiked with 500 µmol kg-1 of Cd or Pb. Significant differences were provided at $p < 0.1$. Bars indicate standard deviation. *n =4*. ns indicates no significant differences.

6.3.3.2. Cd and Pb effects over laccase and Mn-Peroxidase activity of SAS during PAH biodegradation

Initial values for SAS enzymatic activity showed Lac as a major enzyme, about 23 times higher than MnP (Fig 6.3 a, b). Neither LiP nor VP were detected using veratril alcohol and ABTS in the presence of H_2O_2 methods (Tanaka et al. 1999), respectively. Lac and MnP patterns were similar; both enzymes decrease their activities throughout the assay period. Lac activity reduction went from 7860 to 950 IU kg^{-1} and MnP activity dropped from 350 to 1.5 IU kg^{-1}. Baldrian et al. (2000) obtained a similar decrease for Lac activity throughout the 30 days for a soil bioremediation assay using *P. ostreatus*. Cadmium did not decrease Lac or MnP activity. The bioavailability of Cd during the assay was lower than 150 µmol kg^{-1}, and as was shown in the Lac-Cd interaction in liquid medium, low Cd concentration did not inhibit Lac activity. Lead produced significant differences during the assay. On the 7th day, MnP activity of PAH+Pb treatment was lower than treatments without Pb. No significant differences were obtained in Lac activity on the 7th day, but on the 21th day, PAH+Pb proved to be the treatment with highest Lac activity. These results contrast with those obtained in the Lac-Pb interaction in liquid medium, where Pb inhibited Lac activity at all concentrations tested (Table 6.2). The different behavior can be explained because, although Pb inhibited Lac activity, Pb could promote the enzyme production by *A. bisporus*. Baldrian et al. (2005) reported a similar pattern for *P. ostreatus* during lignocellulose degradation in the presence of Pb. At the end of the assay (28 days), the treatments that use xenobiotics, PAH with and without Cd or Pb, showed higher Lac activity than control (without both PAH and metals).

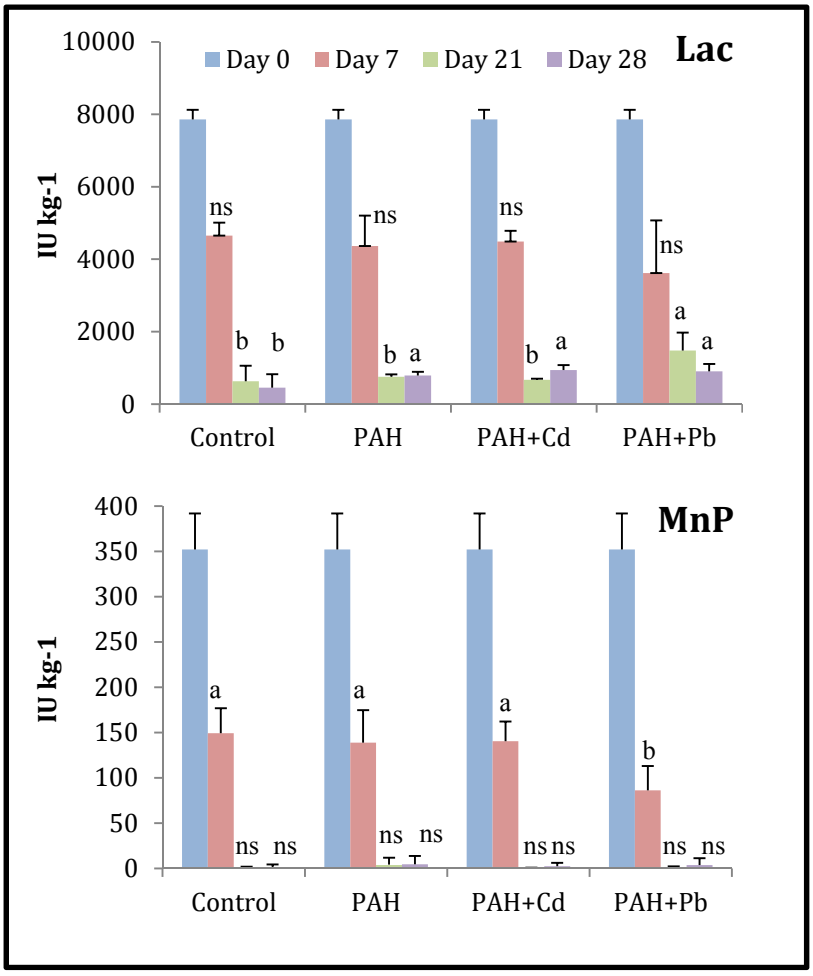

Fig. 6.3: Laccase and Mn-Peroxidase activities (IU g^{-1}) during the PAH biodegradation process by spent *A. bisporus* substrate with and without Cd or Pb 500 µmol kg^{-1}. Control treatment was spent *A. bisporus* substrate without PAH, Cd nor Pb. Significant differences were provided at $p < 0.1$. Different letters indicate significant differences between treatments in the same sampling time. Bars indicate standard deviation. *n =4*. ns indicates no significant differences.

6.3.3.3. Biodegradation of PAH by SAS in the presence of Cd and Pb

Flu, Phe, Ant and Py biodegradation with SAS in the absence of, and with 500 µmol kg^{-1} of Cd or Pb was comparatively determined (values of the remaining PAH are shown in Fig. 6.4) (Appendix Fig. A1). No biodegradation nor PAH abiotic losses in sterilized SAS throughout the 28 days of the assay were detected. SAS polluted with PAH, PAH+Cd and PAH+Pb was able to biodegrade the 4 PAHs tested (Flu, Phe, Ant and Py). Biodegradation was more intense on the first 7 days. This higher biodegradation at the beginning of the assay agrees with the highest Lac

and MnP activities (Fig. 6.3) and with a probable higher PAH bioavailability due to PAH aged decrease their bioavailability (Smith et al. 2011). On day 7, biodegradation of Flu was lower in the presence of Pb (51%) than Cd (59%) or no heavy metal treatment (61%). Biodegradation of Phe in the presence of Cd was 15% higher than in the Pb treatment, and no differences were shown between Cd and no heavy metal treatment. No significant differences were found between treatments for Ant and Py, although their biodegradation in the presence of Cd showed a tendency to be increased. On the 21st day, Phe, Ant and Py biodegradation in the presence of Pb was lower than in the treatment without heavy metals, but on the other hand, the addition of Cd to SAS did not change PAH biodegradation. Flu biodegradation did not show differences in this sampling period.

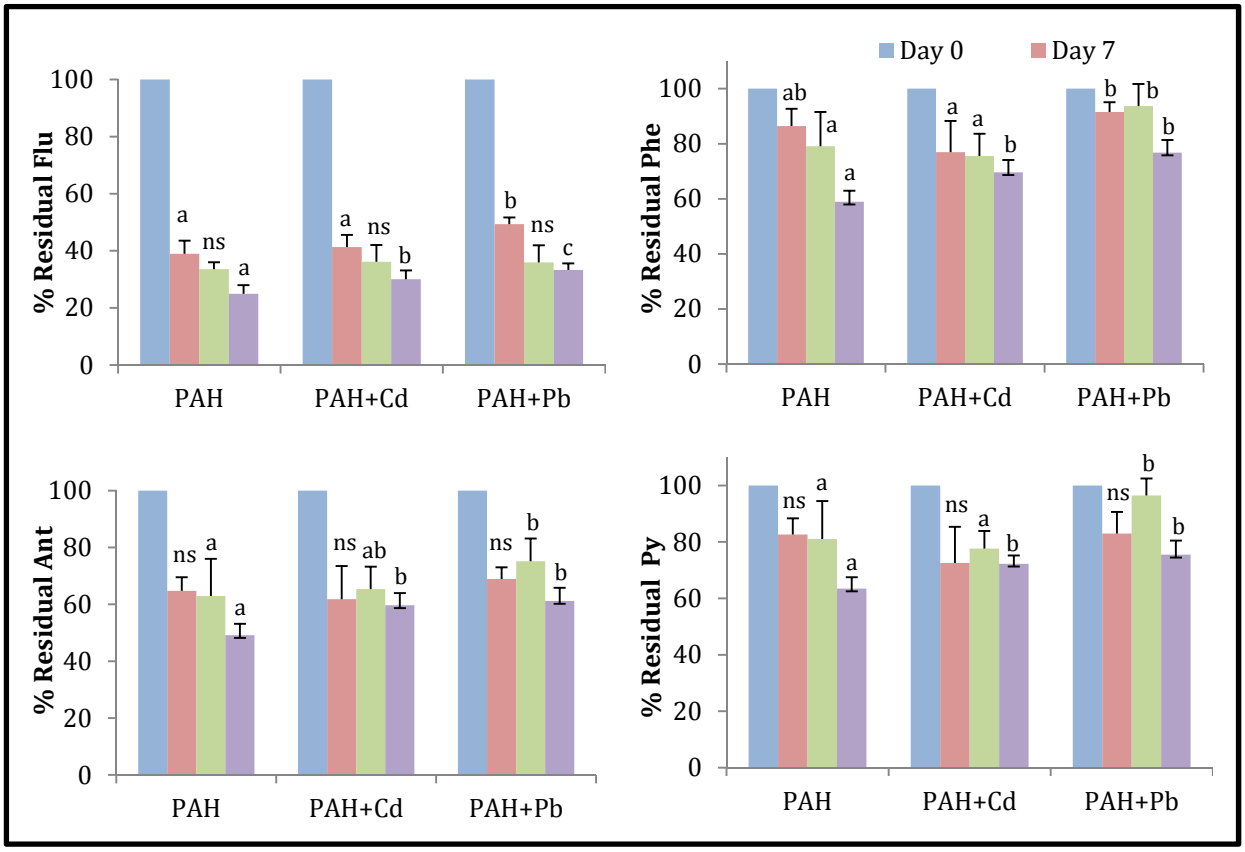

Fig. 6.4: Percentage of residual PAH (initial Flu, Phe, Ant, Py 100 mg kg^{-1}) during the 28 days of biodegradation process by spent *A. bisporus* substrate with and without Cd or Pb 500 µmol kg^{-1}. Significant differences were provided at $p < 0.1$. Different letters indicate significant differences among treatments in the same sampling time. Bars indicate standard deviation. n =4. ns indicates no significant differences.

At the final sampling time (28 days), treatment without heavy metals showed the highest biodegradation rates for Flu (75%), Phe (41%), Ant (51%) and Py (37%). Flu biodegradation was lower in the presence of Pb than with Cd. No differences were found between Cd and Pb treatments for Phe, Ant and Py biodegradation, in contrast with the other sampling times where Pb inhibited PAH biodegradation. Therefore, the inhibition effect of Cd was only presented at the final sampling time. According to Baldrian et al. (2003) the mechanism for Cd^{2+} uptake by white rot fungi is linked to the transportation of Ca^{2+}; due to the high Ca^{2+} contents in SAS (García-Delgado et al. 2012b), a competition between both cations can occur and the negative effects of Cd on *A. bisporus* were delayed. In the case of Flu, lower biodegradation occurred in the presence of Pb than with Cd. These results mean that Cd and Pb tend to decrease biodegradation potential of *A. bisporus* and other microorganisms present in SAS. A similar pattern was reported in the case of lignocellulose biodegradation in the presence of heavy metals by *P. ostreatus* (Baldrian et al. 2005). Nevertheless, taking into account that the presence of heavy metals in soil inhibited the mycelium penetration, and therefore PAH biodegradation (Baldrian et al. 2000), and that SAS adsorbs heavy metals as is demonstrated in this work, the use of SAS as a fungi carrier to multi-polluted soil could decrease the heavy metal bioavailability and increase mycelium development.

As a general pattern, the number of aromatic rings of each PAH is an indicator of biodegradation difficulty for microorganisms (Haritash and Kaushik 2009). According to this, Flu with 2 aromatic rings was the most biodegradable PAH (67-75%). Both Phe and Ant are formed by 3 aromatic rings, although Ant was more biodegradable than Phe, which presents similar behavior to Py, with 4 aromatic rings. Leonardi et al. (2008) reported similar Phe biodegradation behavior for *P. ostreatus* and *I. lacteus* in soil, due to Phe higher ionization potential (IP=8.03 eV) than Ant (IP=7.43 eV) and its higher resistance to oxidation. Mayolo-Deloisa et al. (2011) reported that Lac from SAS of *A. bisporus* was not able to biodegrade Phe (IP=8.03 eV) and Flu (IP = 7.89 eV) in liquid medium due to their high IP. Farnet et al. (2008) reported that Lac partially purified from *Marasmius quercophilus* strain19 were not able to biodegrade PAH with IP > 7.55 eV (e.g. Naphtalene or Phe). Majcherczyk et al. (1998) obtained similar results for Flu and

Phe (IP > 7.55) using Lac extract from *T. versicolor,* and suggest that PAH with IP below 7.45 eV revealed very good oxidation by Lac without a mediator redox compound. Instead, Li et al. (2010) got Flu biodegradation rates between 25 and 79%, and very low Phe biodegradation using crude extract from SAS and others spent mushroom substrates. The mechanisms of PAH biodegradation by Lac and the production of the oxidative enzymes are not directly correlated with the PAH metabolism (Haritsah and Kaushik 2009). This can be confirmed in our results with the PAH+Pb treatment, which showed higher Lac activity on the 21st day and equal Lac activity as the other treatments on the 28th day; however, their biodegradation percentages were the same as, or less than PAH+Cd and PAH without heavy metal treatment.

In this work, *A. bisporus* was not the only PAH degrader agent. The PAH biodegradation process was carried out in SAS where a consortium of *A. bisporus* and other microorganisms was present. Some of them were probably able to biodegrade PAH and their metabolites and increase their ability to oxidize PAH with high IP. PAH metabolites detected by HPLC-PDA were fluoren-9-one; 9,10-anthracenedione and pyrene-hydroxides for Flu, Ant and Py, respectively. These compounds have been described as final biodegradation metabolites of white rot fungi (Aranda et al. 2010).

Consequently, SAS behaves adequately to be reused and revaluated to bioremediate heavy metals and PAH simultaneously. The main environmental effects of SAS input in soil will be the adsorption of heavy metals, which causes their immobilization, and PAH degradation by *A. bisporus* and other microorganisms present in the material. In addition, the well-known advantages of adding organic matter to soil, such as the improvement of soil texture, water retention capacity, nutrients content and microbial activity, have to be taken into account in soil remediation. On the other hand, the reuse of SAS will prevent or decrease the environmental impact in mushroom production areas, mainly soil and groundwater pollution, caused by leaching of salts and soluble organic carbon during SAS storage.

6.4. Conclusions

SAS showed a greater tendency to immobilize Cd than SPS, probably because SAS has a higher degree of humification. Nevertheless, SPS presented higher capacity to adsorb Pb than SAS because of the higher cellulose-hemicellulose content. Different Lac activity behavior in the presence of Cd and Pb was found for *T. versicolor*, *A. bisporus* and *P. ostreatus*, although all Lac were inhibited by Pb. Lac extracted from SAS was more resistant to Cd and Pb than Lac extracted from SPS. SAS was able to biodegrade PAH in the presence of Cd and Pb. However, both Cd and Pb decreased PAH biodegradation rate. This study shows the potential of SAS to biodegrade PAH and to immobilize Cd and Pb simultaneously.

Capítulo 7:

Implications of Polluted Soil Biostimulation and Bioaugmentation with Spent Mushroom Substrate (Agaricus bisporus) on the Microbial Community and Polycyclic Aromatic Hydrocarbons Biodegradation.

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Abstract

Different applications of spent *Agaricus bisporus* substrate (SAS), a widespread agro-industrial waste, were investigated with respect to the remediation of a historically polluted soil with Polycyclic Aromatic Hydrocarbons (PAH). In one treatment, the waste was sterilized (SSAS) prior to its application in order to assess its ability to biostimulate, as an organic amendment, the resident soil microbiota and ensuing contaminant degradation. For the other treatments, two bioaugmentation approaches were investigated; the first involved the use of the waste itself and thus implied the application of *A. bisporus* and the inherent microbiota of the waste. In the second treatment, SAS was sterilized and inoculated again with the fungus to assess its ability to act as a fungal carrier. All these treatments were compared with natural attenuation in terms of their impact on soil heterotrophic and PAH-degrading bacteria, fungal growth, biodiversity of soil microbiota and ability to affect PAH bioavailability and ensuing degradation and detoxification. Results clearly showed that historically PAH contaminated soil was not amenable to natural attenuation. Conversely, the addition of sterilized spent *A. bisporus* substrate to the soil stimulated resident soil bacteria with ensuing high removals of 3-ring PAH. Both augmentation treatments were more effective in removing highly condensed PAH, some of which known to possess a significant carcinogenic activity. Regardless of the mode of application, the present results strongly support the adequacy of SAS for environmental remediation purposes and open the way to an attractive recycling option of this waste.

7.1. Introduction

Polycyclic aromatic hydrocarbons (PAH) are ubiquitous organic contaminants which comprise two or more condensed benzene rings with toxic, mutagenic and carcinogenic properties (IARC, 2010). As a consequence of their physico-chemical properties, these compounds are accumulated in soil *via* their sorption onto organic matter (Krauss et al., 2000; Liang et al., 2006) and/or clay fractions (Hundal et al., 2001). The selection of a particular remediation technique for PAH-polluted soils is not an easy choice. There are many alternatives including *in situ* or *ex situ* techniques which rely on either physico-chemical or biological approaches (Gan et al., 2009; Pelaez et al., 2013). Among them, bioremediation is increasingly deemed to be the most environmentally friendly technique to clean-up polluted soils. Bioremediation of PAH-contaminated soils is based either on the biostimulation of the indigenous microbiota (Sayara et al., 2010) or on the addition of exogenous microorganisms, the latter approach being referred to as bioaugmentation (Haritash and Kaushik, 2009; Lladó et al., 2012). The biostimulation of resident microbial communities of PAH-impacted soil can be achieved in a variety of ways including the addition of organic matter, which has been found to be successful (Covino et al., 2010b; Sayara et al., 2010).

Bioaugmentation with lignin-degrading fungi (LDF) to perform the clean-up of PAH-contaminated soils has received increasing attention in recent years due to its reported efficacy (Covino et al., 2010b; Federici et al., 2012a; Li et al., 2012). LDF are known to produce extracellular lignin-modifying enzymes (LME) with low substrate specificity which enable them to degrade a wide range of organic pollutants, including PAH (Covino et al., 2010c; Majcherczyk et al., 1998). The main LME enzymes include multi-copper oxidases, such as laccase, and heme-peroxidases (Mn-peroxidase (MnP), versatile peroxidase and lignin peroxidase).

Soil colonization and the ensuing contaminant degradation by LDF in soil requires the addition of lignocellulosic materials either as amendments, or inoculum carriers (Covino et al., 2010a; Lestan and Lamar, 1996). The use of these additives has been found to have a favorable impact on the resident microbiota, including specialized populations (Federici et al., 2012a). PAH degradation *via* bioaugmentation with LDF has been shown to involve either synergistic or

antagonist interactions between the fungi added and the autochthonous microbiota. In one study a cooperative effect on the degradation of highly condensed PAH was reported between resident bacteria and *Bjerkandera* sp. (Kotterman et al., 1998) or *Trametes versicolor* (Borràs et al., 2010). However, in another study *Pleurotus ostreatus* enhanced PAH degradation in non-sterile, artificially spiked soils but also inhibited the growth of the indigenous bacteria and changed the composition of the bacterial community (Andersson et al., 2000). Appropriate inocula formulations of LDF, relying on lignocellulosic materials as the carriers have been shown to improve the competitive ability of the fungi added to the resident microbiota (Covino et al., 2010a, 2010b; Federici et al., 2012a). The use of pent mushroom substrate of some LDF as a source of viable inocula for soil clean up applications has been proposed (Li et al., 2012). However spent *Agaricus bisporus* substrate (SAS), without previous treatment, has never been used for bioremediation of PAH polluted soil. Spent *Agaricus bisporus* substrate (SAS), has been shown to enhance the ability of the fungi to endure the toxic effects of both cadmium and lead in a PAH-contaminated substrate (García-Delgado et al., 2013b). Marín-Benito et al. (2014; 2012a,b) reported the ability of pesticide degradation by SAS composted with spent *P. ostreatus* substrate (75:25) and its adsorption capacity for fungicides with low polarity that reduce their mobility in the environment. In addition, SAS has been reported to be an excellent source of LME, mainly laccase, that were able to biodegrade PAH in aqueous solutions (Mayolo-Deloisa et al., 2011). The annual production of this organic waste in Europe has been estimated at 3.5 x 10^6 t (Pardo-Giménez and Pardo-González, 2008) consequently its potential use in bioaugmentation applications would certainly help to reduce this figure.

Therefore, in view of the profitable and environmentally sound use of SAS, this study has thoroughly investigated its feasibility in PAH remediation applications. This necessarily implies a variety of manipulations of the waste prior to its application, to gain more insight into its ability to act as an organic amendment, fungal carrier or a supplier of exogenous complex microbiota.

To elucidate the isolated and/or combined effects exerted by SAS, several application options were compared using an historically PAH-contaminated soil. All were assessed for their ability to (i) affect the densities of heterotrophic and PAH-degrading bacteria (ii) enable fungal growth (iii) modify the biodiversity of the bacterial and fungal communities (iv) remove PAH (v) modify the bioavailability of PAH and finally to (vi) detoxify the soil. A non-amended contaminated soil microcosm was incubated in parallel and was used as a natural attenuation control.

7.2. Materials and Methods

7.2.1. Materials

The polluted soil was collected from a creosote wood treatment plant (42º 10´ 31´´N 1º 41´ 36´´ W Navarra, Spain). The total soil sample (40 kg) was obtained by mixing 20 sub-samples taken from an area close to the treated wood stock zone at a depth of 0-20 cm. The soil sample was homogenized, air-dried at room temperature and finally passed through a 2 mm-sieve. The main properties of the soil are shown in Table 7.1. According to the US textural classification, the soil was a clay loamy soil (sand 39%, silt 39% and clay 22%) with a water holding capacity of 37 %. Thirteen out of the 16 PAH US EPA were present in the polluted soil. The PAH concentrations are shown in Table 2.

SAS was collected from a composting plant of agricultural waste located at 39º 22’ 16’’N 1º 59’ 43’’ W (Cuenca, Spain). The main characteristics of the SAS are shown in Table 7.1. Total carbon, nitrogen, hydrogen and sulfur were determined by elemental analysis (LECO CHNS-932 analyzer, St. Joseph, MI).

Table 7.1: Characteristics of the PAH-polluted soil and spent *Agaricus bisporus* substrate (SAS) (*n*=3).

Parameter	Soil	SAS
pH	8.20 ± 0.03	6.7 ± 0.3
Electronic conductivity (dS m^{-1})	0.58 ± 0.02	7.1 ± 0.8
Organic matter (%)	1.2 ± 0.1	61.9 ± 1.7
Carbonates (%)	30 ± 1	n.d.†
C/N ratio	n.p.§	7.96 ± 0.13
% C	n.p.	32.4 ± 0.02
% N	n.p.	4.07 ± 0.07
% H	n.p.	1.95 ± 0.06
% S	n.p.	0.92 ± 0.12

†n.d.: not detected; § n.p.: not performed

7.2.2. Preparation of remediation microcosms

Irrespective of the type of microcosm, the experiments were carried out in 1l glass reactors using unsterilized contaminated soil (50 g dry mass). The microcosms were designed in order to simulate:

- Natural attenuation: the preparation of this microcosm simply involved the adjustment of the soil moisture content to 70 % of its water-holding capacity prior to the beginning of the incubation. This microcosm will be referred to as soil microcosm (SM)
- Biostimulation: the moisture content of the polluted soil was adjusted to 70 % as above and amended with sterilized SAS (121 °C, 30 min) at a 4:1 mass ratio to yield the SSAS microcosm. This approach was designed to assess the stimulatory effect of a sterilized organic waste on resident microbiota in the soil.
- Bioaugmentation I: the moisture content of the polluted soil was adjusted to 70 % as above and amended with SAS (4:1 mass ratio) to yield the SAS microcosm. This approach was designed to assess the combined effect of both *A. bisporus* and the indigenous SAS microbiota.
- Bioaugmentation II: to prepare this microcosm, sterilized SAS was inoculated with 3 agar plugs (1 cm diameter) from a 14 day old culture of *A.*

bisporus grown on malt extract agar and incubated for 10 d at 20 °C (Appendix Fig. A2). The colonized matrix was mixed with the contaminated soil (1:4 mass ratio) and the moisture content adjusted to 70 % as described above. This bioaugmentation approach, called the Abisp microcosm, was designed to eliminate the contribution of the SAS microbiota.

Each microcosm was carried out in triplicate and incubated at 20 °C for 0, 7, 21, 42 and 63 days under static conditions in the dark.

7.2.3. Extraction and analysis of Ergosterol and PAH

Total ergosterol was extracted and analyzed as described in the method by Covino *et al.* (2010b). Samples (0.5 g) were sonicated at 70 ˚C for 90 min with 3 ml methanolic solution KOH (10 %, w/v). Distilled water (1 ml) was added to each sample and the sample extracted three times with 2 ml of n-hexane. The solvent was evaporated under a nitrogen stream and the solid residue dissolved in methanol (1 ml). The samples were analyzed using high performance liquid chromatography (Waters 2695 Separation Module) coupled with a Waters 996 photodiode array detector equipped with Phenomenex Luna C18 column (250 mm × 4.60 mm; particle size 5 µm; pore size 100 A) equilibrated with methanol:water (95:5) at a flow rate of 1ml min^{-1}. The sample injection volume was 20 µl. The elution profile was monitored at 282 nm.

The extraction of PAH was performed by pressurized liquid extraction (PLE) (ASE350, Dionex). Soil samples (10 g) were loaded into the extraction cell (32 ml) and subsequently extracted with a dichloromethane–acetone mixture (DAM, 1:1, v/v). Static heating was applied to the vessel (100 ˚C, 5 min) and the extraction performed for 7 min at the same temperature under 1500 psi. The cell was then flushed with 7 ml DAM and finally the solvent purged from the cell with argon for 60 s. This extraction cycle was repeated twice for each sample. The resultant organic extract was dried under gentle N_2 flow at room temperature and finally dissolved in acetonitrile. HPLC analyses were performed using a system consisting of a 2695 Separations Module (Waters, Milford, MA) equipped with a Superguard™ LC-18 guard column (20 x 3 mm) prior to the separation

Supelcosil™ LC-PAH column (250 × 3.0 mm; particle size 5 µm) and a 2996 diode-array detector (Waters). Separation of the PAH was achieved using a gradient elution program, using (A) acetonitrile and (B) Milli-Q water. The elution program was: isocratic elution with 60 % (A) for 5 min, gradient to 100 % (A) for 15 min, isocratic elution at 100 % A for 20 min. The column temperature was fixed at 28 ^{o}C. The sample injection volume was 20 µl. The chromatograms were monitored at 254 nm. PAH were identified on the basis of both UV spectra and matching the retention times with commercially available standards (Sigma-Aldrich). The PAH detected and quantified were: fluorene (FLU); phenanthrene, (PHE), anthracene (ANT), fluoranthene (FLT), pyrene (PYR), benzo[a]anthracene (BaA), chrysene (CHR), benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF), benzo[a]pyrene (BaP), dibenzo[a,h]anthracene (DBahA), benzo[g,h,i]perylene (BghiP), indeno[c,d]pyrene (IcdP). Naphthalene, acenaphthylene, and acenaphthene were below detection limits probably due the volatilization of these compounds (García-Delgado et al., 2013a).

7.2.4. Estimation of PAH bioavailability

The bioavailable fraction of each PAH was determined in all microcosms by hydroxypropyl-β-cyclodextrin (HPCD) extraction according to the method described by Stokes et al. (2005). The extraction was carried out by mixing the soil (1.5 g) with a 25 ml solution of 50 mM HPCD in deionized water and incubating the mixture for 20 h on an orbital shaker (250 rpm). The mixture was then centrifuged (2500 rpm, 15 min) and the supernatant discarded. The resulting soil pellet was washed with deionized water (25 ml) and manually shaken for 10 s, centrifuged and the supernatant discarded again to remove residual HPCD. Finally, the soil pellet underwent exhaustive PLE extraction (as described above) to determine residual PAH concentration after HPCD extraction.

7.2.5. Biochemical determinations and toxicity test

Lignin-modifying enzymes (LME) were extracted from the remediation microcosms (3 g) at 5 ˚C for 1 h using the buffered solution described by D´Annibale et al. (2006). The aqueous suspension was centrifuged (6000 g, 30 min) and the supernatant assayed for LME activities.

Laccase activity was spectrophotometrically determined by following the oxidation of 0.2 mM 2,2´-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid in 100 mM sodium acetate buffer (pH 4.5) at 420 nm (ε= 36000 M^{-1} cm^{-1}). MnP activity was assayed by the oxidation of 1 mM $MnSO_4$ in 50 mM sodium malonate buffer (pH 4.5) in the presence of 0.1 mM H_2O_2. Manganic ions, Mn^{3+} form a complex with malonate which absorbs at 270 nm (ε= 11590 M^{-1} cm^{-1}) (Wariishi et al., 1992). One unit of enzyme activity (IU) is defined as the amount of enzyme which produced 1µmol of product per minute under the assay conditions.

Ecotoxicological assessment of remediation microcosms was carried out using two independent methods. The first was an acute toxicity test on the springtail *Folsomia candida* Willem based on the percentage mortality of adults, as previously reported by Leonardi et al., (2008). The second test was based on the determination of dehydrogenase activity which has been shown to be a sensitive ecological index in soils contaminated with hydrocarbons (Dawson et al., 2007). Remediation microcosms were assayed for dehydrogenase activity as follows: 1 ml 1.5 % 2,3,5-triphenyltetrazolium chloride dissolved in 0.1 M Tris-HCl buffer at pH 7.5 and added to 1 g fresh samples. The reaction mixture was incubated at 30 °C for 24 h in the dark. At the end of incubation, the triphenylformazan was extracted with 8 ml acetone, the extract was centrifuged (3500 rpm, 15 min) and the absorbance of the supernatant measured at 546 nm. (ε= 15.4 mM^{-1} cm^{-1}).

The percentage reduction of carcinogenic risk assessment (RCRA) of the PAH present in the soil was based on the Nisbet and LaGoy, (1992) toxic equivalency factors (TEF) of PAH for environmental exposure and was determined as follows:

$$RCRA(\%) = \frac{\sum_{i=1}^{13} [PAH_i]_{to} \bullet DR \bullet TEF_i}{\sum_{i=1}^{13} [PAH_i]_{to} \bullet TEF_i} \bullet 100$$

where $[PAH_i]_{to}$ is the initial concentration of PAH, DR and TEF the degradation rate and toxic equivalency factor (Table 7.2).

7.2.6. Enumeration of cultivable heterotrophic and PAH-degrading bacteria

Soil bacterial counts were performed using a miniaturized most probable number (MPN) method using 96-well microtiter plates, with eight replicate wells per dilution according to the method of Lladó et al. (2009). The total number of heterotrophs were counted in tryptone soy broth and the PAH-degrading bacteria were counted in a mineral medium containing a PAH mixture composed of PHE 0.5 g l^{-1} and FLU, ANT, PYR, at a final concentration of 0.05 g l^{-1} as the sole carbon sources. To avoid fungal contamination, cycloheximide at a final concentration of 100 mg l^{-1} was added to both growth media (i.e., tryptone soy broth and mineral medium). The MPN plates were incubated at room temperature for 30 days. Positive wells were detected by turbidity for heterotrophs, and the presence of a brownish/yellow coloration for PAH degrading bacteria. The MPN calculation was carried out using US EPA MPN Calculator v1.1 software.

7.2.7. DNA extraction

Whole genomic DNA from each soil was extracted using the Power Soil DNA extraction Kit (Mo Bio Laboratories Inc., Carlsbad, CA) according to the manufacturer's instructions. The bacterial V3 region of the 16S rRNA gene was amplified using the universal bacterial 341-f (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3') and 534-r (5′-ATT ACC GCG GCT GCT GG-3′) primers with a length of approximately 200 bp (Muyzer et al., 1993). Fungal 18S ribosomal DNA was amplified using the universal fungal primers FUN_NS1 (5′-GTAGTCATATGCTTGTCTC-3′) and GC fung (5′-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCATTCCCCGTTACCCGTTG-3) (Das et al., 2007). The reaction mixture consisted of 2 µL of template DNA (ca. 20 ng), Tris–HCl (20 mM, pH 8.3), KCl (100 mM), $MgCl_2$ (3 mM), Taq-polymerase (0.1 IU) (NzyTech, Lisbon, Portugal), primers (0.2 mM of each) and double deionized

water to bring the final volume up to 50 µL. For Bacterial amplification, the touchdown PCR program was performed in a *Primus* PCR thermo cycler (MWG biotech, Ebersberg, Germany) using the same procedure (Muyzer et al., 1993). For fungal amplification the PCR program previously reported by Das et al., (2007) was used. All amplicons (5 µL) were analyzed on agarose gel before being used for Denaturing Gradient Gel Electrophoresis (DGGE).

7.2.8. Denaturing gradient gel electrophoresis (DGGE) analysis

The INGENY phorU DGGE system (Ingeny, Goes, NL) was used for sequence-specific separation of PCR amplified fragments. For PCR amplicons obtained with the 341f-GC and 534r primers, electrophoresis was performed in a polyacrylamide gel (8 % (w/v) acrylamide/bis-acrylamide gel 37.5:1), containing 40 – 60 % urea-formamide denaturing gradient (100 % corresponds to 7 M urea and 40 % (w/v) formamide). For fungal amplicons the urea-formamide denaturating gradient ranged from 20 to 35 %. After DGGE electrophoresis the gels were stained with Gel star solution (Lonza, Ltd group, USA) at room temperature for 45 min and photographed using a UV-transillumination table with a GelDoc XR digital camera (Bio-Rad, Carlsbad, CA). The fingerprinting profile obtained from DGGE was investigated using the Quantity one software (Bio-Rad). To characterize the community composition obtained by DGGE analyses, the Shannon diversity index H' was calculated as follows:

$$H' = - \Sigma (P_i \times \ln P_i)$$

where P_i represents the ratio of the single intensity band to the sum of the bands intensity of each lane, Richness (S) is the number of bands revealed, and Evenness (E) was calculated as H'/ln S.

Table 7.2. Initial (t_o) and residual PAH concentrations after 63 d incubation (t_f) in the non amended soil microcosm (SM), amended with sterilized spent *Agaricus* substrate (SSAS), spent *Agaricus* substrate (SAS) and sterilized spent *Agaricus* substrate reinoculated with the fungus (Abisp). Data are mean±standard deviation of 3 replicated microcosms; the asterisk denotes significant differences between the initial and residual concentrations for each microcosm (ANOVA, p <0.05).

Contaminant	Toxic Equivalency Factor†	Contaminant concentration (mg kg^{-1}) in							
		SM		SSAS		SAS		Abisp	
		t_o	t_f	t_o	t_f	t_o	t_f	t_o	t_f
FLU	0.001	6.11±3.78	2.96±1.77	3.47±0.64	0.859±0.159*	3.86±1.13	1.27±0.29*	2.54±0.57	1.38±0.16*
PHE	0.001	16.8±5.9	6.30±3.80	13.3±2.1	1.39±0.43*	12.1±3.9	2.39±0.52*	10.2±1.6	4.62±0.43*
ANT	0.01	23.7±10.3	13.8±7.6	20.7±7.1	3.69±1.19*	20.1±3.1	8.95±3.9*	14.8±2.2	16.6±1.2
FLT	0.001	21.7±2.6	7.59±1.39*	16.7±0.5	8.05±4.08*	16.5±1.5	5.11±0.35*	1.60±1.9	8.73±4.08*
PYR	0.001	10.1±1.1	4.11±0.25*	8.02±0.04	4.84±2.68	8.46±0.96	2.60±0.15*	7.42±1.0	3.70±2.68*
BaA	0.1	4.35±0.77	2.61±0.82	3.21±0.04	2.72±1.16	3.68±0.35	2.16±0.31*	3.50±0.39	1.81±1.16*
CHR	0.01	11.8±5.0	9.55±4.61	8.01±1.22	8.99±3.84	8.61±0.75	5.97±1.1*	8.25±0.46	5.49±3.84*
BbF	0.1	5.94±0.59	5.45±0.18	4.81±0.09	5.40±1.04	5.35±0.23	4.42±0.21*	5.09±0.37	4.26±1.04*
BkF	0.1	2.42±0.22	2.09±0.08	1.94±0.02	2.03±0.42	2.19±0.10	1.73±0.13*	2.05±0.16	1.52±0.42*
BaP	1	2.91±0.27	2.91±0.05	2.13±0.03	3.02±.059	2.78±0.08	2.29±0.13*	2.59±0.22	1.26±0.59*
DBhaA	5	0.755±0.071	0.853±0.047	0.576±0.022	0.782±1.16	0.676±0.062	0.643±0.066	0.683±0.022	0.466±0.162*
BghiP	0.01	2.51±0.17	2.54±0.03	2.03±0.06	2.31±0.41	2.33±0.06	1.99±0.05*	2.23±0.11	1.79±0.41*
IcdP	0.1	2.18±0.18	2.15±0.04	1.75±0.02	2.03±0.29	1.98±0.04	1.68±0.02*	1.87±0.10	1.47±0.29*
Σ3rings		43.6±19.8	23.1±13.1	37.5±9.8	5.94±1.75*	36.1±8.0	12.6±4.5*	46.2±9.8	22.6±1.7
Σ4rings		48.0±8.3	23.9±7.0*	36.0±1.2	24.6±10.1	37.3±3.3	15.8±1.8*	35.1±1.2	19.7±10.1*
Σ5-6rings		16.7±1.5	16.0±0.1	13.2±0.2	15.6±2.9	15.3±0.6	12.8±0.55*	14.5±0.18	10.8±2.9*
ΣPAH		111±29	63.0±15.4	86.7±10.2	46.1±13.7*	88.6±4.7	41.2±6.6*	82.4±10.2	53.1±13.7*

† Nisbet and LaGoy (1992)

7.3. Results and discussion

7.3.1. Time- and Microcosm-Dependent Evolution of Fungal Biomass and Cultivable Bacteria

In the SM microcosm control the ergosterol content, a specific indicator of fungal biomass was invariably lower than the detection limits (Fig. 7.1A). Irrespective of the sampling time, no fungal growth was observed in the SM throughout the incubation period (Appendix Fig. A3).

There were no significant ($p \leq 0.05$) time dependent changes in ergosterol in the SSAS microcosm which indicates that despite the presence of the sterilized *Agaricus* substrate, fungal growth was not stimulated at all. This result was unexpected because several studies have shown that the application of sterilized lignocellulose waste exerts a positive effect on the resident fungi in contaminated soils (Federici et al., 2012a, 2011; Lladó et al., 2013). Conversely, in the SAS microcosm an approximate three-fold increase in ergosterol content was observed in the first week of incubation. This was followed by a decline in the values which remained constant in subsequent harvests (Fig. 7.1A).

No changes in the ergosterol content were observed throughout the incubation in the Abisp microcosm which suggests that the sterilized SAS was neither an adequate carrier for the subsequent growth of *A. bisporus* in the soil, nor a valuable trophic supplement for the resident fungi. This could be explained by the fact that most of the available organic components in the sterilized SAS had been consumed by the reinoculated *A. bisporus* prior to its addition to the soil, resulting in a microcosm with a low nutrient status. Therefore, the potential trophic contribution of the carrier to the competitive ability of the added inoculum was impaired (Covino et al., 2010b; Lestan and Lamar, 1996).

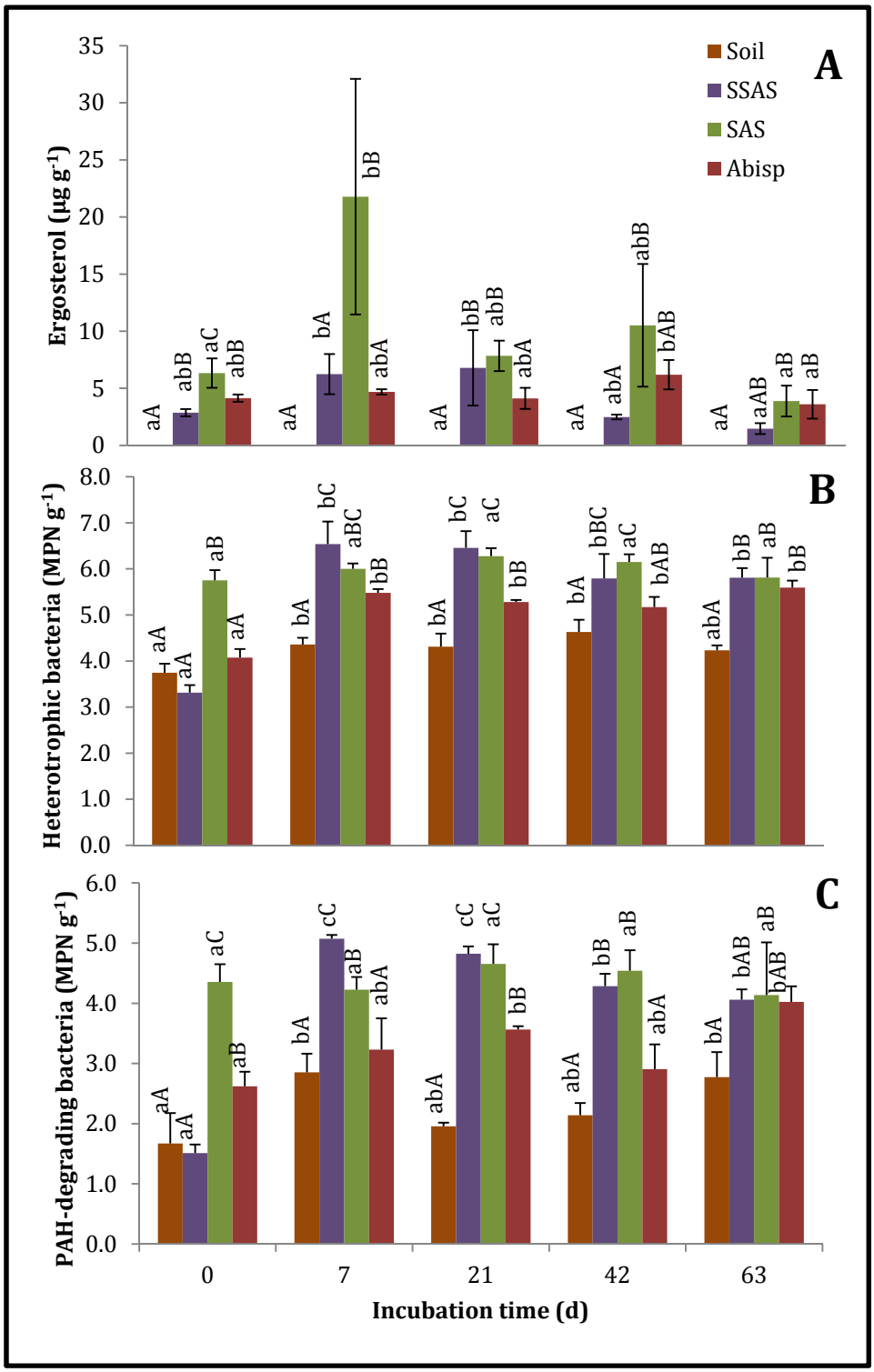

Fig. 7.1: Time course of ergosterol concentrations (A), total heterotrophic (B) and PAH degrading (C) bacteria in non amended soil microcosm (SM), amended with sterilized spent *Agaricus* substrate (SSAS), spent *Agaricus* substrate (SAS), and sterilized spent *Agaricus* substrate reinoculated with the fungus (Abisp). Data are the mean ± standard deviation of three replicated microcosms. Different uppercase and lowercase letters indicate significant differences between microcosms at the same incubation time and between incubation times within the same microcosm (Tukey post-hoc test; $p \leq 0.05$), respectively.

The density of the cultivable heterotrophic and PAH-degrading bacteria over time is shown in Fig. 7.1B and C respectively. Heterotrophic microbial counts in the SAS and SSAS microcosms increased by approximately two orders of magnitude and were significantly higher than those in the SM microcosm over the whole incubation period (Fig. 7.1B). The same result was also found in the bioaugmented Abisp microcosm where heterotrophic bacterial counts increased by more than one order of magnitude (Fig. 7.1B). In the amended microcosms, the lowest densities of heterotrophic bacteria throughout the incubation period were found in the Abisp microcosm. This may be explained by the low nutrient input associated with this inoculant. Its preparation had involved the reinoculation of *A. bisporus* into spent Agaricus substrate and subsequent incubation for 14 d prior to its application onto soil with ensuing consumption of organic nutrients by the growing fungus. This is also corroborated by the observed increase in heterotrophs in the other bioaugmented SAS microcosm where the addition of the inoculant ensured a higher nutrient input than in Abisp. Similar results were also found for the cultivable PAH-degrading bacteria. The highest densities were observed in the SSAS and SAS microcosms (Fig. 7.1C). However, it should be noted that the spent *Agaricus* substrate contained viable PAH-degrading bacteria which would have resulted in a higher initial density in the SAS microcosm than in the SM (2.3×10^4 vs. 0.5×10^2 MPN g^{-1}) (Fig. 7.1C). In the SSAS microcosm, the addition of the sterilized organic waste also exerted a high stimulation on the PAH-degrading bacteria which were increased by more than three orders of magnitude after one and three weeks of incubation, although after this their densities declined. As previously observed for heterotrophic bacterial counts in the amended remediation microcosms, the Abisp exhibited the lowest densities of PAH-degrading bacteria for most of the incubation period. Regardless of the different application options in addition to leading to a higher density of heterotrophs, when compared with the SM, it also enhanced specialized ones. As described above, the non-sterile spent *Agaricus* substrate in the SAS microcosm contained allochthonous PAH-degrading bacteria. This is not surprising because a molecular characterization of the bacterial community in this substrate has revealed the presence of several genera *Paenibacillus*, *Arthrobacter*, *Comamonas* and *Sphingobacterium*, (Ntougias et al., 2004; Watabe et al., 2004) which included

several species with reported PAH-degrading capacity (Haritash and Kaushik, 2009). However, the addition of the sterilized SAS, irrespective of whether it had been reinoculated with *A. bisporus* or not, also appeared to stimulate PAH-degrading bacteria. Previous studies have also found that the addition of sterilized organic wastes such as wheat straw and maize stalks activated the specialized resident bacterial populations in soils contaminated with PAH (Lladó et al., 2013), and polychlorobiphenyls (Federici et al., 2012a, 2012b). It has been suggested that the stimulatory effect exerted by organic waste is due to an enhancement of the oxygen transfer via an increase in soil porosity without necessarily involving either trophic factors or modifications in contaminant bioavailability (Federici et al., 2012b).

7.3.2. Microcosm-Dependent Impact on Bacterial and Fungal Community Structure

It is widely known that the cultivable microbiota in soil represents a minor fraction of the whole microbial community (Daniel, 2005). Therefore, an investigation into the structure of both the bacterial and fungal communities as a function of the remediation treatment necessitated a cultivation-independent approach which relied on DGGE. Table 7.3 shows the Shannon Weaver Index, which gives the richness and evenness values of bacterial and fungal communities in the microcosms at the start, and at the end of incubation. These values have been calculated by a numerical analysis of the DGGE profiles of PCR-amplified 16S and 18S rDNA fragments respectively. Both the richness and the diversity of the bacterial and fungal communities in the SM did not significantly change during incubation. This result is not surprising because both communities presumably had a well established adaptation to the conditions exerted by the historical contamination of the soil.

Table 7.3: Shannon Weaver Index (H'), richness (S) and evenness (E) values of bacterial and fungal communities at start (t_0) and at the end (t_{63}) of the incubation in the non amended soil microcosm (SM), amended with sterilized spent *Agaricus* substrate (SSAS), spent *Agaricus* substrate (SAS) and sterilized spent *Agaricus* substrate reinoculated with the fungus (Abisp).

	Bacteria			Fungi		
	H'	**S**	**E**	**H'**	**S**	**E**
SM t_0	2.27±0.08^{Aa}	11±0	0.95±0.03^{Aa}	2.61±0.02^{Ba}	17±1	0.91±0.00^{Ca}
SM t_{63}	2.23±0.03^{Aa}	10±0	0.97±0.01^{Ba}	2.59±0.04^{Ba}	19±1	0.96±0.12^{Aa}
SAS t_0	2.64±0.08^{Ba}	18±1	0.90±0.04^{Aa}	2.55±0.13^{ABa}	23±3	0.82±0.01^{Ba}
SAS t_{63}	2.95±0.08^{Ca}	27±1	0.89±0.01^{Aa}	2.74±0.03^{Ba}	27±0	0.83±0.01^{Aa}
SSAS t_0	2.42±0.03^{Aa}	12±1	0.97±0.02^{Aa}	2.25±0.10^{Aa}	15±1	0.83±0.01^{Ba}
SSAS t_{63}	2.86±0.04^{BCb}	21±1	0.95±0.01^{Ba}	2.66±0.07^{Bb}	25±2	0.83±0.00^{Aa}
Abisp t_0	2.41±0.02^{Aa}	12±1	0.96±0.00^{Aa}	2.09±0.01^{Aa}	15±0	0.77±0.00^{Aa}
Abisp t_{63}	2.63±0.11^{Bb}	16±2	0.94±0.01^{Ba}	2.41±0.02^{Ab}	20±0	0.81±0.03^{Aa}

*Data are the mean ± standard deviation of 3 independent microcosms. Multiple pair-wise comparisons were performed by the Fisher LSD test ($p \leq 0.05$): same uppercase and lowercase letters denote the absence of statistically significant differences between different microcosms at same time of treatment and between the same microcosm at different time, respectively.

As a result of the fungi and bacteria already present in the SAS microcosm which contained the unsterilized spent *Agaricus* substrate, higher initial richness values for bacterial and fungal biota were found than in those in the SM microcosm (18 *vs*. 11, respectively, and 23 *vs*. 17, respectively). In addition, the 63 day old SAS microcosms produced a significantly higher H' value for the bacterial community than that from the coeval SM (2.95±0.08 *vs*. 2.23±0.08, respectively), as well as a higher richness in the fungal biota (27 *vs*. 17, respectively) but without substantial differences in their respective evenness.

Conversely, the presence of the sterilized spent *Agaricus* substrate in the SSAS microcosm did not substantially modify the test parameters with respect to those found in the SM. In the 63 day old SSAS microcosm, significantly higher S and H' values for the bacterial community than those in the coeval SM were found, although no substantial differences were observed in their fungal biota. Regardless of the sampling time, similar results to those described for SSAS were found in the Abisp microcosm. Interestingly, the microcosms to which sterilized spent *Agaricus* substrate had been added, irrespective of whether they had been reinoculated with Agaricus or not, i.e. the SSAS and Abisp microcosms had the same impact on the

fungi. They both failed to promote fungal growth throughout incubation (Fig. 7.1A) and/or to substantially change the richness and diversity of the fungal community when compared to the SM microcosm (Table 7.3).

7.3.3. PAH Removal in the Remediation Microcosms

Table 7.2 shows the initial and residual PAH concentrations in the remediation microcosms after 63 days incubation. In the SM, no significant reduction in the total PAH concentration was observed (p=0.07). Significant differences between the initial and final concentrations were only found for FLT and PYR, with a percentage removal of 65 %, and 59 % respectively. In the biostimulation SSAS microcosm a statistically significant decrease (p<0.05) in 3-ring PAH content, FLU (75 %), PHE (90 %), ANT (82 %), and FLT (52 %), was observed and the overall PAH residual content significantly differed from that at the start. Therefore, the biostimulation treatment was effective at degrading low molecular weight PAHs which were the most abundant contaminants in the soil. In the bioaugmented microcosms, namely SAS and Abisp, significant differences between the initial and the residual concentrations were found for the majority of the individual contaminants as well as the overall PAH concentrations (Table 7.2). These findings indicate that the bioaugmentation treatments with *A. bisporus* were efficient at degrading both low and high molecular weight PAH.

A comparison of the degradation performances of SSAS, SAS and Abisp microcosms, revealed that SSAS and SAS, which exhibited the highest density of PAH-degrading bacteria, were very efficient at degrading low molecular weight PAH. However, Abisp was superior to SSAS and SAS in the removal of highly condensed PAH (Table 7.2). Therefore, for PAH which are potentially carcinogenic to humans or animals (IARC, 2010), the Abisp microcosm was more efficient than the SAS in degrading compounds such as BaP and DBahA (52 vs. 18 % respectively, and 32 vs. 6 % respectively). In this respect, a very limited number of bacteria able to grow in pure cultures on either 5- or 6-ring PAH have been identified (Haritash and Kaushik, 2009).

However, in the SAS microcosm, the augmented fungus appeared to exert a lower inhibitory action on the resident bacterial biota as deduced by comparing its

better degradation performance on low molecular weight PAH and its higher density of PAH-degrading bacteria than those found in the Abisp microcosm. One of the possible synergistic mechanisms which might occur between bacteria and fungi, relies on the ability of the latter to convert PAH into more polar degradation intermediates, such as PAH diones and hydroxylated derivatives (Covino et al., 2010b).

Linear regression analyses were performed to relate the percentage removal of each PAH in the microcosm with respect to the chemical characteristics of these contaminants (Table 7.4). Therefore, the degradation results were related to those parameters which have been suggested to significantly affect PAH degradation, such as molecular weight (MW), organic carbon sorption coefficient (log K_{oc}), hydrophobicity (log P), water solubility (WS) and ionization potential (IP), (Table 7.4). These analyses showed that the degradation results were positively and significantly ($p< 0.05$) correlated with WS in all the microcosms tested. Conversely, with the exception of the Abisp microcosm, PAH degradation results were negatively correlated ($p < 0.01$) with their respective MW, log K_{oc} and log P values (Table 7.4). These results clearly indicate that the bioavailability of PAH contaminants strongly affect their ability to be degraded in the SM, SSAS and SAS microcosms, where the bacteria make an important contribution to PAH depletion. Bacterial uptake of PAH and their subsequent metabolism have been shown to be governed by the mass transfer rates of contaminants from the solid to the liquid phase of soil, which are in turn affected by the aforementioned physico-chemical properties (Haritash and Kaushik, 2009). The distinctive behavior of the Abisp microcosm, namely the lack of correlation between PAH degradation and MW, log K_{oc} and log P, might indicate that different PAH degradation mechanisms are involved here. In particular, the direct involvement of PAH oxidation by LME which was high during the initial incubation phases (See subsection 3.5) might be as result of their ability to diffuse into the soil matrix and potentially oxidise PAH with low bioavailability (Haritash and Kaushik, 2009). This hypothesis may explain why the Abisp microcosm was able to partially degrade 5- and 6-ring PAH.

Table 7.4: Pearson product moment correlation coefficients between percent PAH removal in non amended soil microcosm (SM) or amended with either sterilized spent Agaricus substrate (SSAS) or spent Agaricus substrate (SAS) or sterilized spent Agaricus substrate reinoculated with the fungus (Abisp) and respective PAH physico-chemical properties including molecular weight (MW), organic carbon adsorption coefficient (log K_{oc}), hydrophobicity (log P), water solubility (WS) and ionization potential (IP).

Microcosm	MW	log K_{oc}§	log P§	WS§	IP†
SM	-0.720**	-0.705**	-0.688**	0.402*	0.339*
SSAS	-0.896**	-0.895**	-0.872**	0.582*	0.490**
SAS	-0.848**	-0.839**	-0.813**	0.546**	0.468**
Abisp	n.s.	n.s.	n.s.	0.327*	n.s.

§These parameters were calculated using the Advanced Chemistry Development v. 11.02 software package (ACD/Labs, Toronto, Canada) available from the on-line SciFinder chemical database (American Chemical Society, Columbus, OH); †From Covino et al., 2010a; n.s., no significant correlation; * significant correlation at $p < 0.05$: ** significant correlation at $p < 0.01$.

The IP has been shown to affect *in vitro* PAH oxidation by either laccase or fungal heme-peroxidases, whose activities were found in the bioaugmented microcosms (see subsection 3.4). The susceptibility of PAH to oxidation by fungal LME has been shown to increase as the IP decreases (Majcherczyk et al., 1998). In the present study the opposite trend was found when relating PAH depletions and respective IP values in all the microcosms. However, the Pearson Coefficients related to these regressions, which were significant with the exception of Abisp, were the lowest among the physico-chemical properties tested (Table 7.4). Similar findings were obtained with two historically PAH-contaminated soils augmented with either *Irpex lacteus* or *Lentinus tigrinus* (Covino et al., 2010a). This clearly suggests that even in augmented microcosms, LME-triggered PAH oxidation is not the sole mechanism involved in PAH degradation. This was made particularly evident in the bioaugmented microcosms by the high depletion extents of PHE and FLT, their high IP values (8.03 and 7.91 eV respectively) make them poorly susceptible to mono-electronic oxidation by LMEs (Majcherczyk et al., 1998; Mayolo-Deloisa et al., 2011). In addition to the action of the PAH-degrading bacteria, it should be taken into account that PAH degradation in fungi also relies

on the intracellular cytochrome P-450/epoxide hydrolase complex whose activity is not dependent on IP (Haritash and Kaushik, 2009).

7.3.4. Time- and Microcosm-Dependent Modifications in the PAH Bioavailable Fraction

The bioavailable fraction of each PAH was determined in all the microcosms at the start and after 63 days incubation using HPCD extraction (Stokes et al., 2005). At the start the different soil application options, i.e. SSAS, SAS and Abisp microcosms did not significantly affect the percentage bioavailable fraction of the large majority of contaminants in each microcosm (Table 7.5). The only exceptions were observed in some highly condensed PAH such as DBahA. The bioavailable fractions were lowest in the SAS and Abisp microcosms. BghiP showed the highest bioavailability in the SSAS and Abisp microcosms.

After 63 days incubation a microcosm-dependent modification of the bioavailable fraction of individual PAH was observed when compared to the beginning of the experiment. In particular, in the SM an increase in the bioavailable fraction was observed for high molecular weight PAH (i.e., DBahA, BghiP and IcdP). In the SSAS microcosm, the bioavailable fractions of BbF and DBahA increased while those of PHE and ANT decreased (Table 7.5). For the latter two compounds, it could be envisaged that their high degradation extents in this microcosm (90 and 82 %, respectively) left only the most recalcitrant fraction. In the bioaugmented microcosms (SAS and Abisp) a different scenario was found. In particular, in the SAS a decrease in the bioavailable fraction of PHE, FLT, PYR, CHR and BaP was observed. Conversely, in the Abisp microcosm, decreased bioavailabilities were found for FLU, PHE, BbF, BkF, BaP, BghiP and IcdP. The reason underlying the decrease in the bioavailable fraction of the majority of individual PAH was not solely due to their respective depletions since these parameters were not found to be correlated in all the microcosms. Therefore, the changes observed might have derived from a combination of PAH sorption onto organic matter and partial degradation. In this respect, the organic matter of spent *Agaricus* substrate analyzed by cross-polarization and magic angle spinning nuclear magnetic resonance, was found to contain a high percentage of aliphatic carbon (about 21

%) that could contribute significantly to PAH sorption *via* hydrophobic interactions (García-Delgado et al., 2013b).

Table 7.5: Percent bioavailable PAH fraction at start (t_0) and at the end (t_{63}) of the incubation in non amended soil microcosm (SM) or the same soil added with sterilized spent Agaricus substrate (SSAS) or spent Agaricus substrate (SAS) or sterilized spent Agaricus substrate reinoculated with the fungus (Abisp).

PAH	Percent bioavailable PAH fraction† in							
	SM		SSAS		SAS		Abisp	
	t_0	t_{63}	t_0	t_{63}	t_0	t_{63}	t_0	t_{63}
FLU	92	98^{A}	94	99^{A}	99	96^{A*}	97	86^{B*}
PHE	75	83^{A}	87	0^{D*}	89	57^{C*}	88	68^{B*}
ANT	86	93^{A}	82	29^{B*}	90	86^{A}	79	90^{A}
FLT	71	69^{A}	71	59^{AB}	74	39^{B*}	76	58^{AB}
PYR	17	0	7	4	14	0^{*}	3	0
BaA	0	2	8	4	0	0	5	3
CHR	43	61^{A}	64	68^{A}	58	30^{B*}	56	44^{AB}
BbF	5	19^{B}	7	33^{A*}	0	0^{C}	13	0^{C*}
BkF	23	30^{B}	46	44^{A}	10	1^{C}	14	1^{C*}
BaP	10	25^{B}	45	52^{A}	16	0^{C*}	20	0^{C*}
DBahA	28^{a}	54^{B*}	33^{a}	64^{A*}	0^{b}	34^{C}	2^{b}	16^{D}
BghiP	0^{b}	21^{A*}	24^{a}	20^{A}	0^{b}	0^{B}	12^{a}	0^{B*}
IcdP	0	14^{A*}	30	24^{A}	0	0^{B}	8	0^{B*}

† Data are the mean of three independent microcosms. Different lowercase letters indicate that differences between microcosms at start (t_0) were significant (Tukey post-hoc test, $p < 0.05$). Different uppercase letters indicate that differences between 63-d-old microcosms (t_{63}) sampling time were significant (Tukey post-hoc test, $p < 0.05$). The asterisk * denotes significant differences between sampling times within the same microcosm (ANOVA, $p < 0.05$).

7.3.5. Lignin-Modifying Enzyme Activity and Residual Toxicity in Remediation Microcosms

The remediation microcosms were assayed for their ligninolytic activity due to the involvement of these enzymes in the early oxidation steps of PAH (Li et al., 2010; Majcherczyk et al., 1998). Among them, laccase and to a much lower extent Mn-peroxidase activity (Fig. 7.2A and B, respectively) were only detected in the bioaugmented microcosms (SAS and Abisp). The highest laccase and MnP activity were found in the Abisp microcosms where the activity of the former enzyme markedly declined with incubation time (Fig. 7.2A). Noteworthy, in this microcosm the best depletions were observed for BaP and DBahA which despite being high molecular weight PAH, are characterized by low IP values (i.e., 7.12 and 7.38

respectively) and therefore highly susceptible to oxidation by both laccase and MnP (Bogan and Lamar, 1995). In this regard the results of the present study are in agreement with those of Li et al. (2010) who showed high removal rates of BaP and DBahA by crude laccase extracts from spent *Agaricus* substrate.

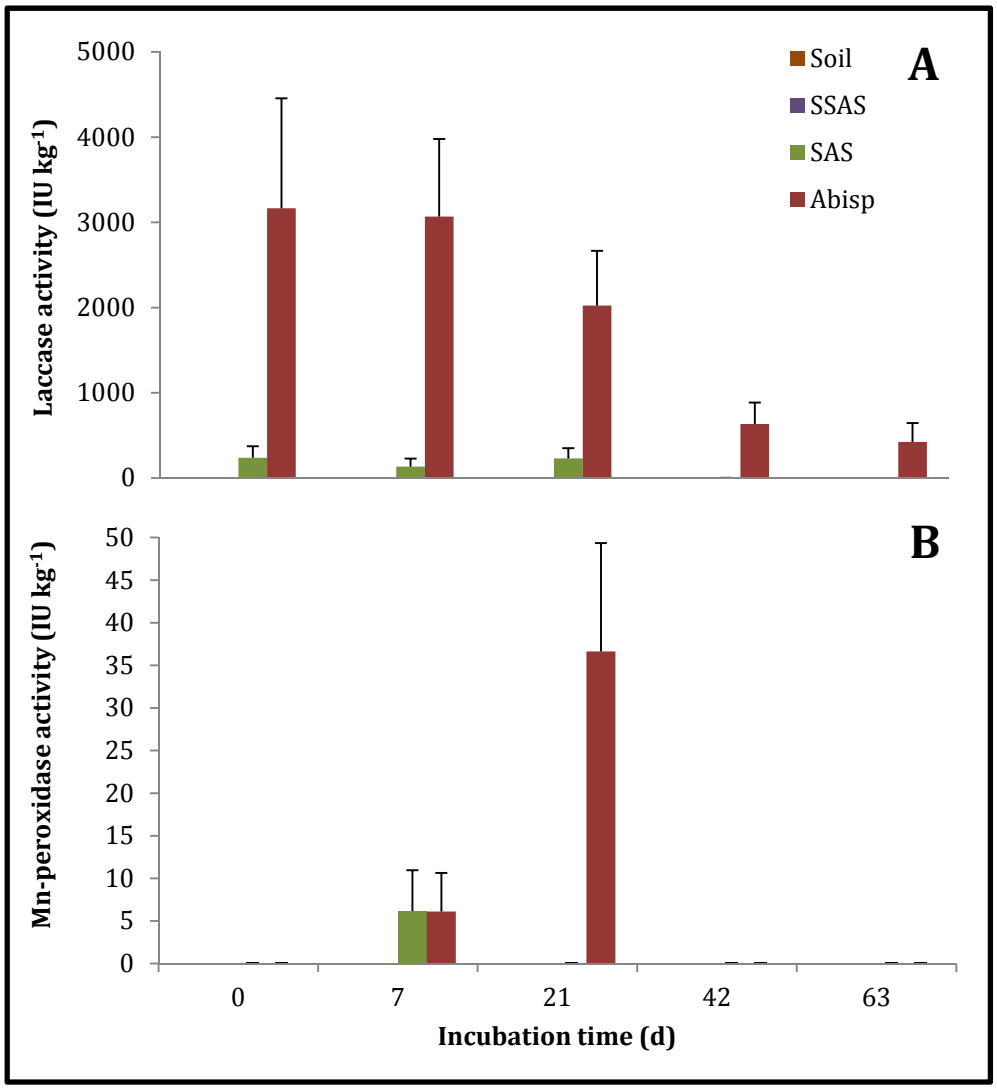

Fig. 7.2: Time courses of laccase (A) and Mn-peroxidase (B) activities in non amended soil microcosm (SM), amended with sterilized spent Agaricus substrate (SSAS), spent Agaricus substrate (SAS) and sterilized spent Agaricus substrate reinoculated with the fungus (Abisp). Data are the mean ± standard deviation of three replicated microcosms.

Dehydrogenase activity was used as a possible index of detoxification in the remediation microcosms as previously suggested for hydrocarbon-impacted soils (Dawson et al., 2007) in addition to the *F. candida* mortality test. In the present study, the adoption of this parameter did not provide an unequivocal clue to the detoxification by augmented microcosms. This was the result of a low dehydrogenase activity in the contaminated soil at the start (Table 7.6). The level of dehydrogenase activity was markedly and predictably boosted by the addition of viable fungal inocula in the SAS and Abisp microcosms (Table 7.6). The retention of high levels of activity in the 63 day old SAS and Abisp microcosms, albeit being lower in the SAS and equal in the Abisp, indicate that the soil microbiota retained a high functional activity despite a prolonged incubation time.

Table 7.6: Reduction of carcinogenic risk assessment (RCRA) and dehydrogenase activity and *F. candida* mortality at start (T_0) and after 63 d (T_f) incubation in non amended soil microcosm (SM) or the same soil added with sterilized spent *Agaricus* substrate (SSAS), spent Agaricus substrate (SAS) or sterilized spent *Agaricus* substrate reinoculated with the fungus (Abisp).

Microcosm	RCRA‡ (%)	Dehydrogenase activity† (IU kg^{-1})		*F. candida* mortality† (%)	
	T_f	T_0	T_f	T_0	T_f
SM	5.3±2.2AB	0.011±0.004Aa	0.041±0.003Ab	89.5±2.5Bb	60.6±3.2Ca
SSAS	4.5±0.6A	0.106±0.006Aa	0.223±0.075Ab	77.5±4.1Ab	58.7±2.1BCa
SAS	15.3±6.3B	0.598±0.140Cb	0.195±0.006Aa	89.6±3.8Bb	49.2±3.1Aa
Abisp	37.6±8.6C	0.336±0.060Ba	0.499±0.162Ba	85.5±4.1Bb	50.1±2.6Aa

† Data are the mean± standard deviation of 3 independent microcosms; same lowercase and uppercase letters denote the absence of statistically significant differences between column and row means, respectively, as assessed by the Tukey post-hoc test ($P< 0.05$).‡ Reduction of carcinogenic risk assessment expressed as percentage was based on toxic equivalency factors proposed by Nisbet and LaGoy (1992).

Table 7.6 also shows that the initial toxicity of the microcosms towards *F. candida* was high, leading to mortalities that ranged from 77.5 to 89 %. Although a partial detoxification was observed in all microcosms, the best results were observed with SAS and Abisp where mortality was reduced by 45.1 and 41.4 %, respectively. In these microcosms, a generalized decrease in the bioavailable fractions of individual contaminants were observed together with the highest

percentage removal of both 4-ring and highly condensed PAH. The lowest mortality reduction (26.8 %) was observed in SSAS where the lowest depletion of 4-ring PAH, and no depletion of highly condensed PAH were found. In this respect, the higher detoxification observed in Abisp than SSAS suggest that 3-ring PAH were less toxic to *F. candida* than 4-ring and 5,6-ring PAH. This might be explained by the fact that in the SSAS the former contaminant group was degraded at a significantly higher extent and, in the same microcosm, with the exception of PHE, their bioavailable fractions were comparatively lower than in Abisp.

A comparison of the 63 day old microcosms showed that the best reduction in the carcinogenic risk assessment (RCRA) was observed in the bioaugmented microcosms (SAS and Abisp). This is a consequence of the higher reduction in the amount of 4- and 5,6-ring PAH which have the highest carcinogenic properties (IARC 2010). Significant differences were also found when comparing the bioaugmented microcosms. The Abisp microcosm showed higher RCRA than the SAS because of its better efficacy in the removal of BaP and DBahA (Table 7.2) which exhibited the highest TEF values.

7.4. Conclusions

The addition of the sterilized spent *A. bisporus* substrate to the PAH-polluted soil was effective in stimulating the resident soil bacteria which resulted in higher levels of 3-ring PAH being removed. Bioaugmentation treatments with *A. bisporus*, in the SAS and Abisp microcosms, were more effective in removing 5, 6-ring PAH in particular BaP. The best detoxification results were obtained in the Absip microcosm where a high retention of microbiological functional activity, a significant decrease in *F. candida* mortality, and a reduction in carcinogenic risk assessment were observed. The wide spatio-temporal availability of this agro-waste combined with its proved efficacy in PAH biodegradation make its use technically feasible for environmental remediation purposes.

Capítulo 8:

Bioremediation of multi-polluted soil by spent mushroom (*Agaricus bisporus*) substrate: Polycyclic Aromatic Hydrocarbons degradation and lead availability.

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Abstract

This study investigates the effect of three spent *Agaricus bisporus* substrate (SAS) application methods on bioremediation of soil multi-polluted with Pb and PAH from close to a firing range with respect natural attenuation (SM). The remediation treatments involve i) use of sterilized SAS to biostimulate the inherent soil microbiota (SSAS) and two bioaugmentation possibilities ii) its use without previous treatment to inoculate *A. bisporus* and inherent microbiota (SAS) or iii) SAS sterilization and further *A. bisporus* re-inoculation (Abisp). The efficiency of each bioremediation microcosm was evaluated by: fungal activity, heterotrophic and PAH-degrading bacterial population, PAH removal, Pb mobility and soil eco-toxicity. Biostimulation of the native soil microbiology (SSAS) achieved similar levels of PAH biodegradation as SM and poor soil detoxification. Bioaugmented microcosms produced higher PAH removal and eco-toxicity reduction via different routes. SAS increased the PAH-degrading bacterial population, but lowered fungal activity. Abisp was a good inoculum carrier for *A. bisporus* exhibiting high levels of ligninolytic activity, the total and PAH-degrading bacteria population increased with incubation time. The three SAS applications produced slight Pb mobilization (<0.3%). SAS sterilization and further *A. bisporus* re-inoculation (Abisp) proved the best application method to remove PAH, mainly BaP, and detoxify the multi-polluted soil.

8.1 Introduction

Multi-polluted soils with both inorganic and organic pollutants are a growing global problem (Zhang et al., 2013). However, many studies on soil bioremediation focus their attention onto either organic or inorganic pollutants individually and they do not delve into the interaction between both types of pollutants and the organisms, or the materials that are involved in the technique applied. In fact, a paucity of information on the bioremediation of organic pollutants in co-contaminated environments exists (Olaniran et al., 2013).

Lead and polycyclic aromatic hydrocarbons (PAH) as inorganic and organic compounds are two of the most widespread pollutants throughout the world. Both, Pb and PAH, show toxic and even carcinogenic properties (IARC, 2010, 2006) and are listed as priority pollutants by the EU and US-EPA. They are found in multiple polluted soils such as those in areas surrounding industrial plants (Beesley et al., 2010; D'Annibale et al., 2007), high-ways (Koeleman et al., 1999), manufacturing gas plants (Thavamani et al., 2011) or firing ranges (Peddicord and LaKind, 2000) amongst others. Firing range soils typically contain high Pb concentrations because lead is the most common material used in ammunition. Sorvari et al. (Sorvari et al., 2006) reported that in Finland about 10% of the total number of potentially polluted places corresponded to firing ranges. These places have been widely studied with respect to Pb remediation. However, there is a lack of remediation experiments which address the problem of PAH biodegradation and Pb mobility, simultaneously.

The bioremediation of multi-polluted soils is not an easy task (Thavamani et al., 2011) because the technique applied can produce contradictory effects on the mobilization or immobilization of different pollutants (Beesley et al., 2010) and heavy metals often reduce the degradation of organic pollutants by microorganisms (Baldrian et al., 2000; Obuekwe and Semple, 2013; Olaniran et al., 2013; Shen et al., 2005). PAH biodegradation by lignin-degrading fungi (LDF) has shown great potential because LDF segregate extracellular ligninolytic enzymes that have low substrate specificity, and are able to diffuse into the soil matrix and potentially oxidize PAH beyond their respective bioavailable quantity in soil (Covino et al., 2010b). However, the presence of high concentrations of metals

interferes with the soil colonization (Baldrian et al., 2000) and ligninolytic enzymes activities (García-Delgado et al., 2013b). This technology necessitates the use of inoculum carriers that are organic materials such as wheat straw, maize stalks or mushroom substrates (Federici et al., 2011; García-Delgado et al., 2015a, 2015b; Li et al., 2012). The addition of organic materials modifies the pH, the organic matter content and other soil properties, and therefore affects metal availability (Pérez-Esteban et al., 2014). Nevertheless, metal inhibition of biodegradation is commonly related to the total metal concentration in a system and this may not be the most appropriate predictor of metal toxicity (Thavamani et al., 2011).

Previous work carried out by our research group (Carlos García-Delgado et al., 2013b; García-Delgado et al., 2015a; Tapia et al., 2010) and others (Herrero-Hernández et al., 2012; Li et al., 2010; Marín-Benito et al., 2014) has demonstrated the ability of the fungus *Agaricus bisporus* and spent *A. bisporus* substrate (SAS) and its inherent microbiota to biodegrade PAH and other organic pollutants, and that the SAS has an effect on heavy metal mobilization/immobilization. In addition, the reuse of SAS can contribute to a reduction in its adverse environmental impact in mushroom production areas.

The aim of this study is to assess the efficiency of three different application methods for spent *A. bisporus* substrate, i.e as an organic amendment, a supplier of exogenous complex microbiota or an inoculum carrier for *A. bisporus*, to bioremediate a multi-polluted soil with PAH and Pb, close to firing range with respect to natural attenuation. To determine the efficiency of each bioremediation treatment, the fungal activity, the populations of heterotrophic and PAH-degrading bacteria, PAH removal, Pb mobility and soil eco-toxicity were evaluated.

8.2 Materials and Methods

8.2.1. Materials

Multi-polluted soil with PAH and Pb was collected next to a shooting range located in Madrid (Spain) at a depth of 0-20 cm. The soil sample was homogenized, air-dried at room temperature and finally passed through a 2 mm-sieve. The *A. bisporus* substrate was originally produced from wheat straw and poultry litter. After composting for 21d gypsum was added. Following the development of the *A. bisporus* mycelium throughout the compost, a layer of black peat is placed on top as casing layer. The SAS was collected from a production plant located in Quintanar del Rey (Cuenca, Spain). The main soil and SAS characteristics are shown in Table 8.1.

Table 8.1: Characteristics of the multi-polluted soil and spent *Agaricus bisporus* substrate (SAS). (mean ± standard deviation, n=3).

Parameter	Soil	SAS
pH	8.13 ± 0.04	6.28 ± 0.03
Electric conductivity (dS m^{-1})	0.41 ± 0.09	6.52 ± 0.23
Organic matter (%)	3.3 ± 0.2	63.9 ± 1.5
N Kjeldahl(%)	0.14 ± 0.01	1.99 ± 0.05
Carbonates (%)	2.2 ± 0.1	n.d.†
Sand (%)	71 ± 1	n.p. §
Silt (%)	15 ± 1	n.p.
Clay (%)	14 ± 1	n.p.
Pseudo-total Pb (mg kg^{-1})	1429 ± 26	1.20 ± 0.20
Pseudo-total Mn (mg kg^{-1})	226 ± 20	541 ± 27
Pseudo-total Cu (mg kg^{-1})	19.6 ± 3.4	60.1 ± 5.5
Pseudo-total Zn (mg kg^{-1})	66.0 ± 6.8	307 ± 21
Pseudo-total Cd (mg kg^{-1})	0.13 ± 0.23	n.d.

†n.d.: not detected; § n.p.: not performed

8.2.2. Isolation of the fungus and preparation of the inocula

A. bisporus was isolated from SAS using the following procedure: the hyphae were washed with sterilized water and inoculated onto rose bengal cloramphenicol agar (Sigma-Aldrich, Spain). After 7 days (d) incubation at 20°C, the mycelium was sub-cultured onto malt extract agar for 10d at 20°C.

Two *A. bisporus* inocula formulations were used. One consisted of the SAS without any treatment. This inoculum contained *A. bisporus* and the inherent microorganism with the ability to degrade PAH (García-Delgado et al., 2015a). The second inoculum used consisted of SAS which had been sterilized in an autoclave at 121ºC, for 30 min, and inoculated with 3, 1 cm diameter agar plugs taken from a 10d-old culture of *A. bisporus* grown on malt extract agar and incubated for 7d at 20°C (Appendix Fig. A2).

8.2.3. Preparation of the remediation microcosms

Regardless of the microcosm type, all experiments were carried out in 1l glass reactors using 50g (dry mass) unsterilized contaminated soil. The three microcosms amended with SAS were carried out using a 4:1 (soil:SAS) mass ratio. The soil moisture content in all treatments was adjusted to 70% of its water holding capacity prior to the beginning of incubation. The microcosms were designed in order to simulate:

- Natural attenuation: the preparation of the soil microcosm (SM) simply involved the adjustment of the soil's moisture content prior to the beginning of the incubation.
- Biostimulation: The polluted soil was amended with sterilized SAS (121°C, 30 min) to yield the SSAS microcosm. This approach was intended to assess the stimulatory effect of a sterilized organic waste on the resident soil microbiota and the effect of the SAS material on Pb availability, without SAS microorganism.
- Bioaugmentation SAS: the polluted soil was amended with SAS without previous treatment to yield the SAS microcosm. This approach was aimed at

assessing the combined effect of both *A. bisporus* and the inherent SAS microbiota on PAH degradation and Pb availability.

- Bioaugmentation Abisp: to prepare this microcosm, sterilized SAS was re-inoculated with *A. bisporus* as described above. The colonized matrix was mixed with the contaminated soil. This bioaugmentation approach, termed the Abisp microcosm, was aimed at determining the effect of *A. bisporus* on PAH biodegradation and Pb availability.

Each microcosm was carried out in triplicate and incubated at 20°C for 0, 28 and 63 days in the dark.

8.2.4. Extraction and analysis of PAH

Total PAH was extracted and analyzed using HPLC-PDA as described in the method of García-Delgado et al. (2013a). Fourteen of the 16 US-EPA PAH were detected and quantified: acenaphthene, (Ace), fluorene, (Flu) phenanthrene, (Phe), anthracene (Ant), fluoranthene (Fla), pyrene (Py), benzo[a]anthracene (BaA), chrysene (Chr), benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF), benzo[a]pyrene (BaP), dibenzo[a,h]anthracene (DBahA), benzo[g,h,i]perylene (BghiP), indeno[1,2,3-c,d]pyrene (IcdP).

8.2.5. Determination of heavy metals pseudo-total content and available Pb

The Pb, Mn, Cu, Zn and Cd pseudo-total content of the soil and SAS were extracted by microwave-assisted digestion with aqua regia according to the method of García-Delgado et al. (2012b). The bioavailable Pb fraction was determined by extracting with $CaCl_2$ 0.01M (1:5, w:v) (Luo et al., 2014a, 2014b). Heavy metal analyses were carried out using an ICP-MS (NexION 300 Perkin-Elmer).

8.2.6. Enumeration of cultivable heterotrophic and PAH-degrading bacteria

Soil bacterial counts were performed using a miniaturized most probable number (MPN) method in 96-well microtitre plates, with eight replicate wells per dilution according to Lladó et al. (2009). To prevent fungal

development, cycloheximide at 100 mg l^{-1} final concentration was added to both growth media. The MPN calculation was carried out using US-EPA MPN Calculator v1.1 software.

8.2.7. Biochemical determinations and eco-toxicity testsLignin-modifying enzymes (LME) were extracted from bioremediation microcosms (3g) at 5˚C for 1h using the buffered solution described by D´Annibale et al. (2006). The resultant aqueous suspension was centrifuged (6000g, 30 min) and the supernatant assayed for LME activities.

Laccase activity was spectrophotometrically determined by following the oxidation of 0.2 mM 2,2´-azino-bis-(3-ethylbenzothiazoline-6-sulphonic) acid in 100 mM sodium acetate buffer (pH 4.5) at 420 nm (ε=36000 M^{-1} cm^{-1}). MnP activity was assayed by the oxidation of 1 mM $MnSO_4$ in 50 mM sodium malonate buffer (pH 4.5), in the presence of 0.1 mM H_2O_2 (ε=11590 M^{-1} cm^{-1}) (Wariishi et al., 1992). One unit of enzyme activity (IU) is defined as the amount of enzyme which produces 1 µmol of product per minute under the assay conditions.

Eco-toxicological assessment of remediation microcosms was carried out using two soil health parameters: total hydrolase activity (THA) by hydrolysis of fluorescein diacetate (Adam and Duncan, 2001) and dehydrogenase activity (DHA) with 2,3,5-triphenyltetrazolium chloride (García-Delgado et al., 2015a).

The percentage reduction in carcinogenic risk assessment (RCRA) of the PAH present in the soil was based on the Nisbet and LaGoy (1992) toxic equivalency factors of PAH for environmental exposure and was determined as follows:

$$RCRA(\%) = \frac{\sum_{i=1}^{13}[PAH_i]_{to} \bullet DR \bullet TEF_i}{\sum_{i=1}^{13}[PAH_i]_{to} \bullet TEF_i} \bullet 100$$

where $[PAH_i]_{to}$ is the initial concentration of PAH, DR and TEF the degradation rate and toxic equivalency factor (Table 8.2).

8.2.8. Scanning electron microscopy (SEM) and X-ray diffraction of *A. bisporus* hyphae.Hyphae of *A. bisporus* collected from Abisp microcosm after 63d incubation were dried at 60ºC for 24h. The dry hyphae were gold-coated and observed using a Philips XL30 environmental scanning electron microscope-

field emission gun. The mineralogical characterization of hyphae was carried out using X-ray diffraction (Panalytical X'Pert Pro, Almelo). The configuration of this apparatus was θ/2θ with an X'Celerator detector. Diffraction profiles were analyzed using X'Pert High Score Plus software and PDF-4 of the International Center for Diffraction Data for crystalline identification.

8.2.9. Statistical analysis

All statistical tests were carried out using the IBM SPSS Statistics v22 software package. One-way analysis of variance was carried out after previously performing a Levene variance homogeneity test. To compare the differences between treatments, the Tukey or Games–Howell *post hoc* test (according to variance homogeneity) at $p \leq 0.05$ was used.

8.3. Results

8.3.1. Fungal activity

SM and SSAS did not develop filamentous fungi during the assay. The soil colonization by *A. bisporus* in the Abisp microcosm was completed from day 7 of incubation until the end of the assay (63d), however colonization in SAS microcosms was not completed and hyphae were not observed at day 63 of incubation (Appendix Fig. A4).

The fungal activity in the microcosms was determined by analysis of LME activities (Fig. 8.1) because of their involvement in the early steps of PAH degradation process. Lignin-peroxidase and Mn-independent peroxidase were not detected in any of the microcosms. Laccase was detected in all the microcosms, but MnP was only detected in the amended microcosms. Abisp showed the highest laccase and MnP activities but declined during the incubation period.

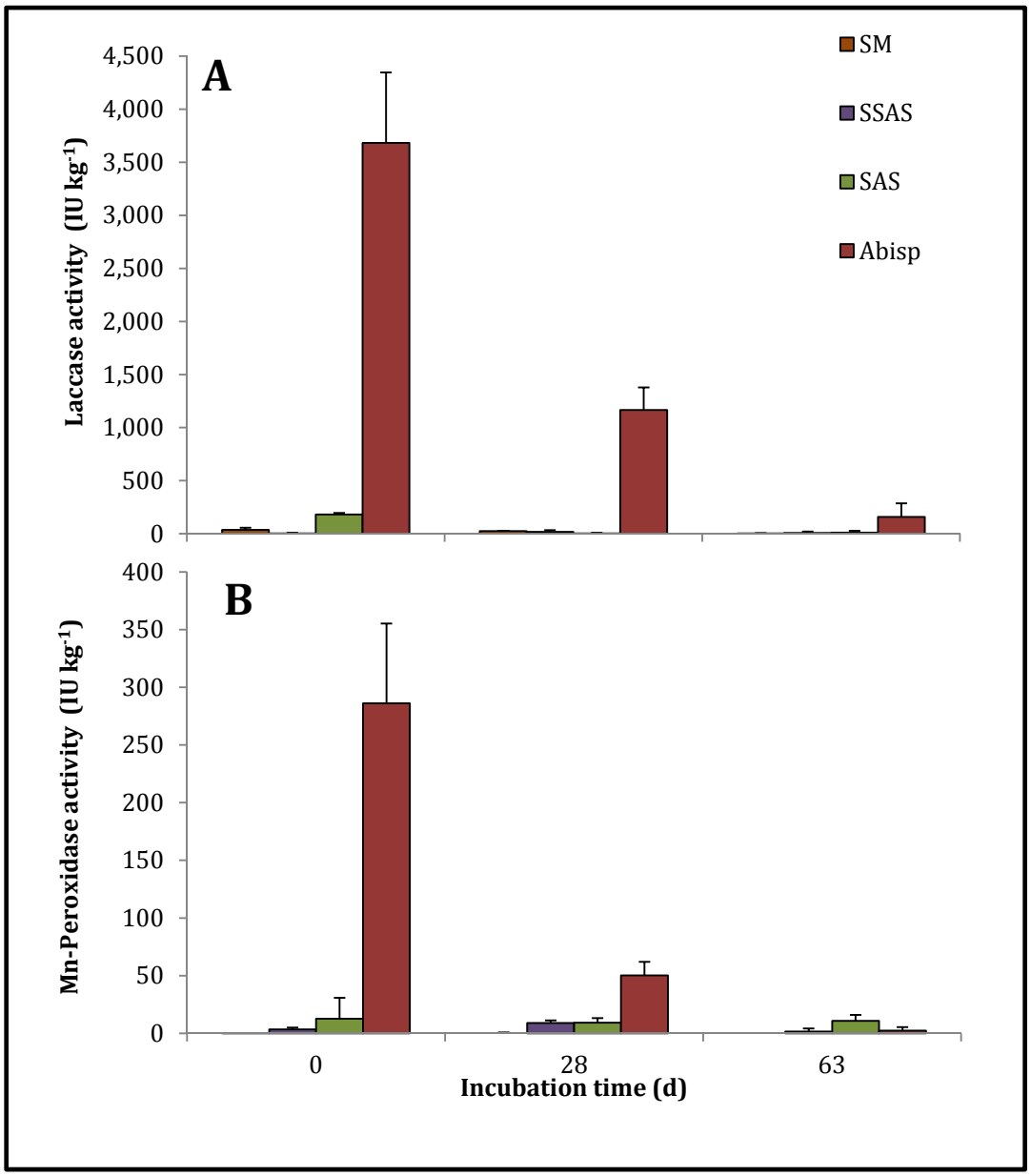


Fig 8.1: Time course of laccase (A) and Mn-peroxidase (B) activities in non amended soil microcosm (SM) or added with sterilized spent Agaricus substrate (SSAS), spent Agaricus substrate (SAS) and sterilized spent Agaricus substrate reinoculated with the *A. bisporus* (Abisp). Data are the mean ± standard deviation of three replicates.

8.3.2. Heterotrophic and PAH-degrading bacteria population

The densities of heterotrophic and PAH-degrading bacteria are shown in Fig. 8.2A and B respectively. The SM developed the lowest heterotrophic and PAH-degrading bacteria populations and no bacterial growth was detected during the assay. The SSAS microcosm increased the heterotrophic bacterial population by one order of magnitude at 28 and 63d.

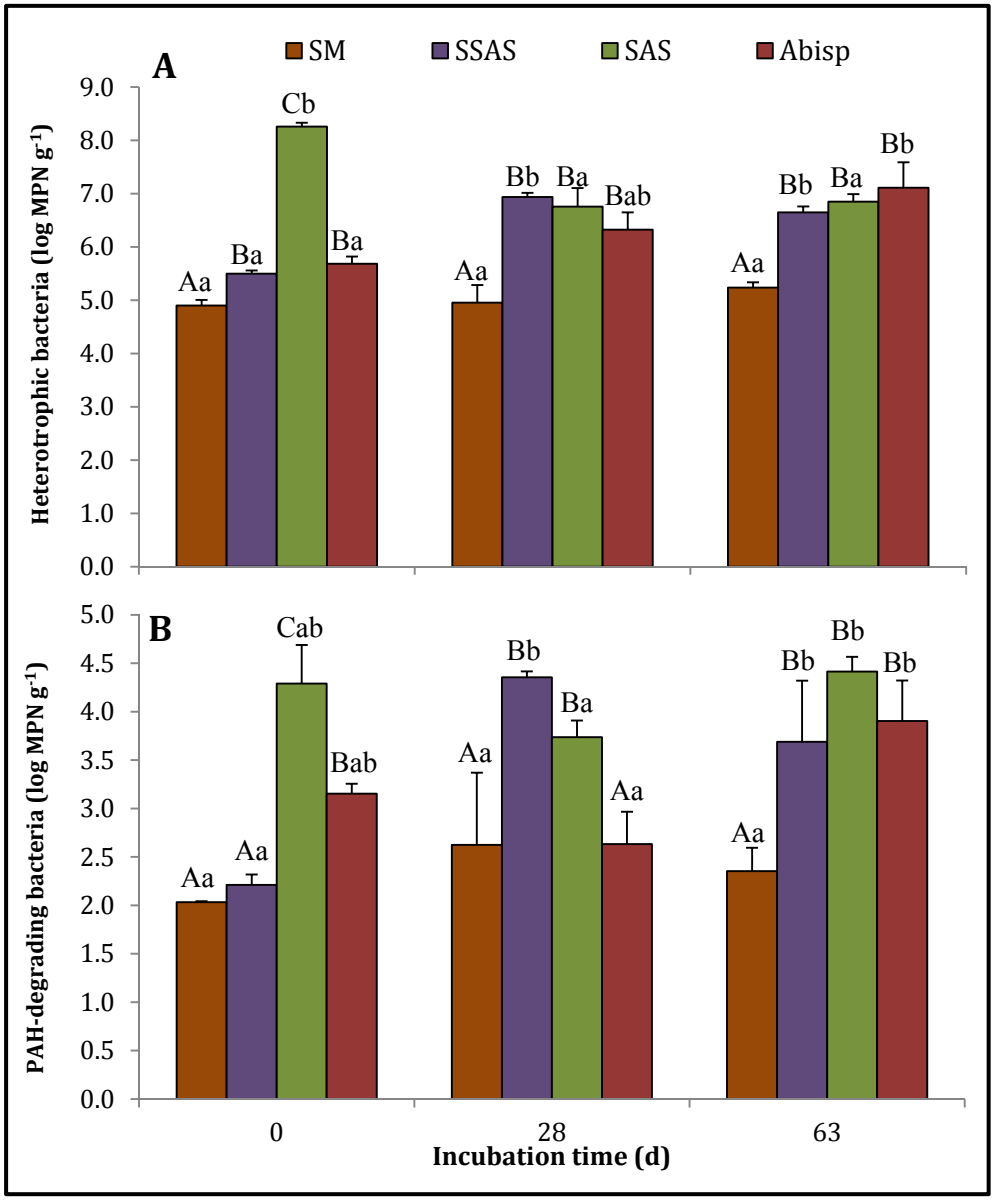


Fig. 8.2: Time course of total heterotrophic (A) and PAH-degrading (A) bacteria in non amended soil microcosm (SM) or added with sterilized spent Agaricus substrate (SSAS), spent Agaricus substrate (SAS) and sterilized spent Agaricus substrate reinoculated with the *A. bisporus* (Abisp). Data are the mean ± standard deviation of three replicates. Different uppercase and lowercase letters indicate significant differences between microcosms at the same incubation time and between incubation times within the same microcosm, respectively ($p \leq 0.05$).

The SSAS also highly stimulated the PAH-degrading bacterial population by about two orders of magnitude during the same time period. The SAS microcosms showed the highest initial heterotrophic and PAH-degrading bacterial populations which was to be expected as a result of the inherent SAS microbial population. The heterotrophic bacterial population increased by more than 3 orders of magnitude with respect to SM, and the PAH-degrading population by more than 2 orders of magnitude. From this we can infer that SAS contains an inherent PAH-degrading bacterial population. The heterotrophic bacterial population decreased significantly from the initial time (1.81×10^8 MPN g^{-1}) to (5.75 and 7.08×10^6 MPN g^{-1}) following 28 and 63d incubation. A similar trend was found in the density of PAH-degrading bacteria after 28d incubation, but then the population returned to its initial values. The Abisp microcosm showed a gradual increase in the numbers of heterotrophic bacteria and achieved the highest population, (1.29×10^7 MPN g^{-1}) at 63d. The density of the PAH-degrading bacteria did not show any significant differences between the initial density and that at the end of the assay.

At the final sampling time (63d), no significant differences were found between the heterotrophic and PAH-degrading bacteria populations in the amended microcosms but lower densities were recorded in the SM.

8.3.3. PAH removal

The initial PAH concentration of the soil, and the degradation rates for each microcosm are shown in Table 8.2. The total PAH concentration of the initial soil was 757±10 mg kg^{-1}. Grouped by rings, the most abundant were the Σ5,6-rings (49%) followed by the Σ4-rings (40%). Therefore, the PAH pollution was mainly caused by high molecular weight PAH (HMW-PAH).

Table 8.2: Initial PAH concentration (mean ± standard deviation), biodegradation rates at 63d incubation respect to initial concentration and reduction in carcinogenic risk assessment (RCRA) for each microcosm: non amendment soil (SM), amendment with sterilized spent *A. bisporus* substrate (SSAS), spent *A. bisporus* substrate (SAS) and sterilized spent *A. bisporus* substrate reinoculated with the fungus (Abisp). Different letters indicates significant differences between microcosms ($p \leq 0.05$) n=3.

	Initial soil (mg kg^{-1})	SM	SSAS	SAS	Abisp	Carcinogenic classification*	TEF†
		PAH degradation rate (%)					
Ace	49.9 ± 0.4	36^{c}	20^{ab}	16^{a}	24^{b}	3	0.001
Flu	0.82 ± 0.05	79^{c}	6^{a}	44^{b}	100^{d}	3	0.001
Phe	28.9 ± 0.6	14^{ab}	0^{a}	22^{bc}	35^{c}	3	0.001
Ant	7.40 ± 0.13	13^{ab}	2^{a}	20^{ab}	32^{b}	3	0.01
Fla	71.0 ± 1.0	8^{a}	0^{a}	22^{b}	31^{b}	3	0.001
Py	95.8 ± 2.3	7^{a}	0^{a}	20^{b}	30^{b}	3	0.001
BaA	56.8 ± 1.3	5^{a}	0^{a}	17^{b}	28^{c}	2B	0.1
Chr	76.4 ± 1.9	4^{a}	0^{a}	17^{b}	27^{c}	2B	0.01
BbF	112 ± 2	2^{a}	0^{a}	10^{ab}	28^{b}	2B	0.1
BkF	32.3 ± 1.7	3^{a}	0^{a}	9^{ab}	22^{b}	2B	0.1
BaP	93.1 ± 0.1	3^{a}	0^{a}	29^{b}	39^{b}	1	1
DBhaA	8.47 ± .035	0^{a}	3^{a}	5^{a}	21^{b}	2A	5
BghiP	74.1 ± 1.8	2^{a}	0^{a}	8^{a}	28^{b}	3	0.01
IcdP	49.5 ± 0.2	4^{a}	0^{a}	12^{ab}	30^{b}	2B	0.1
Σ3rings	87.0 ± 0.7	27^{b}	11^{a}	19^{ab}	30^{b}		
Σ4rings	300 ± 6	6^{a}	0^{a}	19^{b}	29^{c}		
Σ5-6rings	370 ± 5	2^{a}	0^{a}	15^{b}	29^{c}		
ΣPAH	757 ± 10	7^{a}	1^{a}	17^{b}	29^{c}		
RCRA		1.5^{a}	0.7^{a}	20.5^{b}	30.5^{c}		

*IARC 2010: 1 carcinogenic, 2A probable carcinogenic, 2B possible carcinogenic, 3 not classifiable as carcinogenic.

† TEF: Toxic Equivalence Factor

The SM and SSAS microcosms showed a degradation of 27 and 11% respectively for Σ3-rings PAH. However, it did not achieve a significant degradation for the total or HMW-PAH. The SAS microcosm was the second most efficient. The total PAH removal achieved was 17%. The most degradable compounds were Flu (44%) and BaP (29%). No significant differences were found with respect to the efficiency of PAH removal based on the number of rings. The bioaugmented Abisp microcosms achieved the best PAH degradation rates for total (29%) and individual PAH (21 – 100%). The most degradable compounds were Flu (100%) and BaP (39%) as was previously observed in SAS microcosm. The microcosms bioaugmented with *A. bisporus* (SAS and Abisp) were the most

efficient remediation treatments for PAH degradation and, especially for HMW-PAH, the most abundant PAH in this soil. However, the degradation rate for Σ4-rings, Σ5,6-rings and ΣPAH was significantly higher in the Abisp than in the SAS microcosms.

8.3.4. Lead availability

Lead availability and soil pH were monitored at 0, 28 and 63d incubation (Table 8.3). The SM did not show variations in either pH or Pb availability during the assay. Nevertheless, significant changes in pH and Pb availability were observed in the amended microcosms during the time assayed.

The SAS microcosms, where the organic amendment had not been sterilized, showed an increase in the available Pb with respect to the SM but did not produce any change in pH_{H2O} at day 0. During the assay, the SAS produced a slight but significant, acidification of the microcosms together a reduction in the available Pb ($R=0.739$, $p < 0.05$).

In the SSAS and Abisp treatments in which the substrate had been sterilized by autoclaving, there was a significant acidification of the microcosms with the Abisp treatment producing a lower pH. The Pb availability increased in both microcosms at the initial time with respect to SM and reached 1‰ of the total Pb. The Pb in the SSAS decreased during the assay until values similar to those of SAS at 28 and 63d. The Pb availability in Abisp increased 10-fold at 28d with respect to SSAS to achieve the highest value (2.5‰) but at day 63 this value decreased and at the same time the pH increased to 8.61. In fact, the pH_{CaCl2} and available Pb showed a correlation ($R=-0.788$, $p< 0.05$) for this microcosm.

The global effect of Pb availability at the end of the incubation period was a minimal increase in the amended treatments (0.1–0.4‰) with respect to SM.

Table 8.3: pH in water and $CaCl_2$ 0.01M (mean ± standard deviation, n=3) and ‰ available lead ($CaCl_2$ 0.01M) in the microcosms: non amendment soil (SM), amendment with sterilized spent *A. bisporus* substrate (SSAS), spent *A. bisporus* substrate (SAS) and sterilized spent *A. bisporus* substrate re-inoculated *A. bisporus* (Abisp) at 0, 28 ad 63d incubation. Different uppercase and lowercase letters indicate significant differences between microcosms at the same incubation time and between incubation times within the same microcosm, respectively ($p \leq 0.05$).

	Incubation days		
	0	28	63
pH-H_2O			
SM	8.17 ± 0.08^{Ca}	8.31 ± 0.12^{Ca}	7.96 ± 0.01^{Ba}
SSAS	7.38 ± 0.17^{Ba}	7.76 ± 0.06^{Bb}	7.55 ± 0.08^{Aab}
SAS	7.96 ± 0.07^{Cb}	7.70 ± 0.07^{Bab}	7.51 ± 0.17^{Aa}
Abisp	6.92 ± 0.02^{Ab}	6.67 ± 0.06^{Aa}	8.61 ± 0.03^{Cc}
pH-$CaCl_2$			
SM	7.13 ± 0.03^{Ba}	7.42 ± 0.11^{Bb}	7.15 ± 0.05^{Aa}
SSAS	7.31 ± 0.16^{Ba}	7.64 ± 0.07^{Bb}	7.38 ± 0.11^{Aa}
SAS	7.90 ± 0.06^{Cb}	7.59 ± 0.09^{Ba}	7.32 ± 0.22^{Aa}
Abisp	6.76 ± 0.10^{Ab}	6.44 ± 0.06^{Aa}	8.46 ± 0.04^{Bc}
Available Pb (‰)			
SM	0.003^{Aa}	n.d.^{*Aa}	0.004^{Aa}
SSAS	0.980^{Bb}	0.266^{Ba}	0.108^{Ba}
SAS	0.584^{Bb}	0.191^{Ba}	0.156^{Ba}
Abisp	1.06^{Bb}	2.52^{Cc}	0.443^{Ba}

*n.d.: not detected

8.3.5. Assessment of soil eco-toxicity

Fig. 8.3 shows the results of the soil eco-toxicity tests. At day 0, SM and SSAS showed lower THA than the bioaugmented microcosms (SAS and Abisp). At day 28 Abisp and SSAS retained the initial THA but in SAS this activity was reduced dramatically (76%). By contrast SM showed an increase in THA and reached similar values to those of SSAS and SAS. At the end of the assay, after 63d incubation, the SM showed the lowest values. The THA increased in SSAS and it achieved similar values to those recorded in SAS. Finally, Abisp maintained the high HTA values at day 0 and 28d.

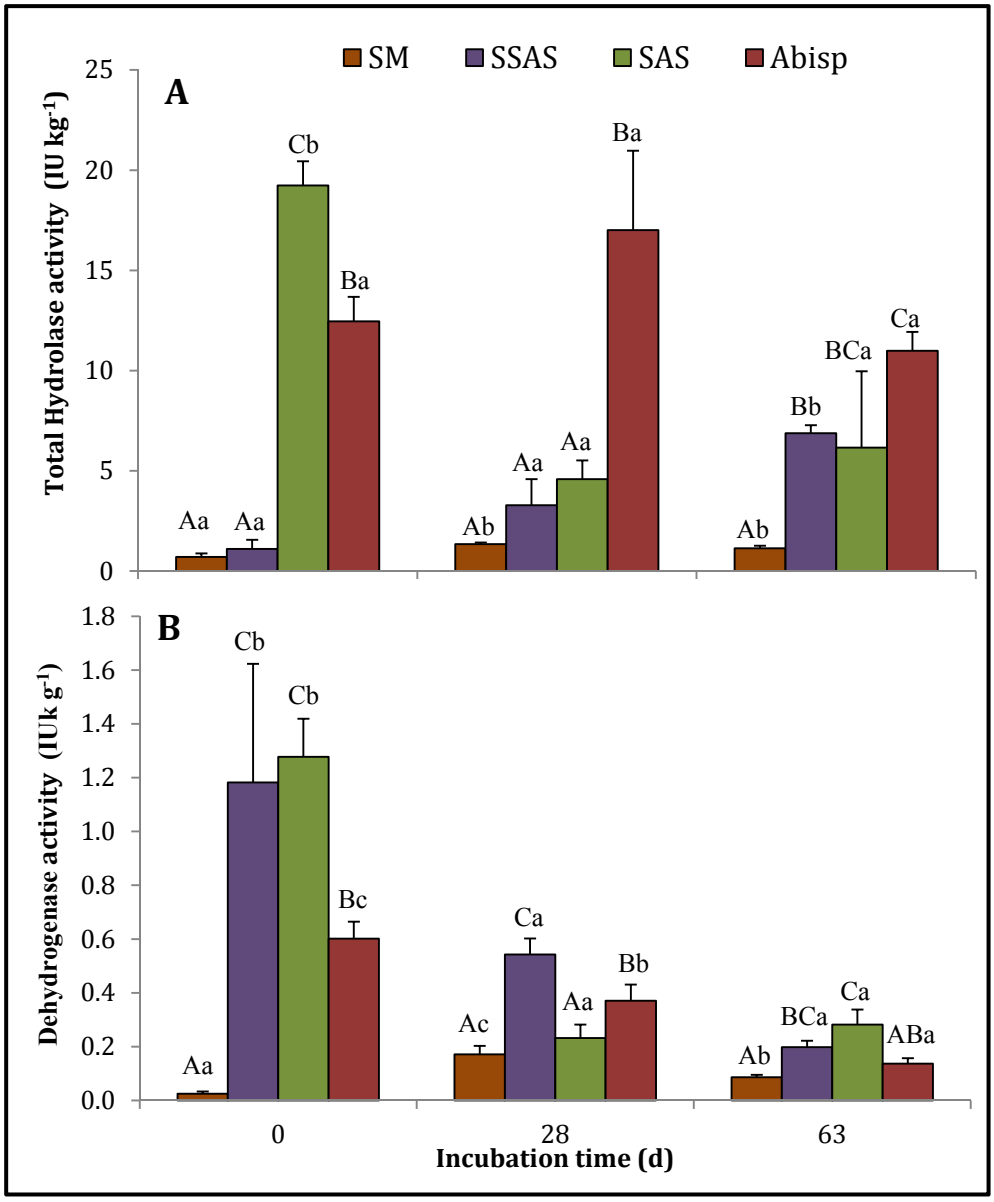


Fig. 8.3: Total hydrolase (A) and dehydrogenase (B) activities in non amended soil microcosm (SM) or added with sterilized spent Agaricus substrate (SSAS), spent Agaricus substrate (SAS) and sterilized spent Agaricus substrate reinoculated with the *A. bisporus* (Abisp). Data are the mean ± standard deviation of three replicates. Different uppercase and lowercase letters indicate significant differences between microcosms at the same incubation time and between incubation times within the same microcosm, respectively ($p \leq 0.05$).

DHA showed a significant decrease during the assay in the amended microcosms (SSAS, SAS and Abisp). By contrast SM presented higher DHA values at 28 and 63d incubation. At 28d, the SSAS microcosms achieved the highest DHA followed by Abisp. SAS reduced DHA by approximately 70% from day 0 to 28d and then retained the activity at 63d. The drastic reduction in THA and DHA from the initial value to 28 and 63d incubation in the SAS microcosm, and the positive

correlation with the reduction in the heterotrophic bacterial population (R = 0.964 and 0.966 respectively, $p<0.01$) offers clear evidence that soil polluted by Pb and PAH, impacted negatively on microbial activity and population. The Abisp showed a continuous decrease in DHA from day 0 to 63d. At the end of the assay SSAS and SAS showed higher DHA than either SM or Abisp which had similar DHA values.

The reduction in the carcinogenic risk assessments (RCRA, Table 8.2) shows very low values for the SM and SSAS microcosms. The bioaugmented treatments, SAS and Abisp, were the most effective remediation strategies for PAH detoxification. With regard to PAH removal and RCRA, Abisp was the most efficient bioremediation treatment in reducing the soil eco-toxicity associated to PAH pollution.

8.4. Discussion

This chapter reports the results of natural attenuation and three different SAS applications that involve one biostimulation, and two biougmentation strategies, to remediate a multi-polluted soil close to a firing range. With regard to the Spanish (BOE, 2005) and Autonomous Community of Madrid (BOCM, 2007) legislation for 'other' soil uses, i.e. not urban or industrial, this soil exceeded the maximum reference levels for Pb (75 mg kg^{-1}) and 9 PAH (Table 8.2), including BaP, which has proved carcinogenic properties, and which exceed 4655 times the reference level. Therefore, this soil can be classified as heavily polluted.

The microcosms that simulate natural attenuation (SM) achieved a low level of PAH biodegradation as a result of a low heterotrophic and PAH-degrading bacteria population and fungal activity. The biodegradation of Σ3-ring PAH showed similar removal rates to the bioaugmented microcosms (SAS and Abisp). Nevertheless, Σ3-ring PAH represents only 11% of the total initial PAH concentration, and according to IARC (IARC, 2010), these compounds do not possess carcinogenic properties. By contrast, PAH which are carcinogenic (PAH with 4,5,6-rings) and mainly BaP achieved biodegradation rates below 5%. Therefore, soil detoxification resulting from PAH degradation was minimum as can be observed in the eco-toxicity tests (Fig. 8.3) and RCRA (Table 8.2). The lowest Pb availability for all the sampling times (Table 8.3) in SM was due to the soil characteristics, i.e. a high pH and 3%

organic matter which is one of the most important factors in the control of Pb mobility (de Santiago-Martín et al., 2014; Tai et al., 2013).

The biostimulated microcosm (SSAS) that involved the sterilization of SAS, produced an increment in the heterotrophic and PAH-degrading bacterial population which was to be expected as a result of the organic matter added to soil. However, PAH removal was unappreciable. This trend could be explained by two possible factors. Firstly, the organic matter of SAS contains a high percentage of aliphatic carbon (about 21%) which could contribute significantly to PAH sorption via hydrophobic interactions (García-Delgado et al., 2013b) and therefore minimize the bacterial biodegradation and probably hinder the organic matter colonization. Secondly, changes in soil chemistry due to the addition of sterilized SAS (SSAS) immediately produced a strong reduction in pH and slight Pb mobilization (0.98‰). The sterilization of organic matter by hydrothermal treatment incremented soluble organic matter and produced LMW- organic acids such as acetic acid (Quitain et al., 2002) which decreased the pH and could act as metal ligands. The available Pb was negatively correlated with the heterotrophic (R=-0.887 $p<0.01$) and PAH-degrading (R=-0.775 $p<0.05$) bacterial population in the SSAS microcosms and could be a factor in the inhibition of PAH degradation as discussed by Olaniran et al. (Olaniran et al., 2013). The reduction in Pb availability during the assay was probably related to microbial consumption of the labile organic matter. de Santiago-Martín et al. (de Santiago-Martín et al., 2014) found a positive relationship between labile organic matter and Pb availability in lettuce and a negative relationship with the recalcitrant organic fraction.

The bioaugmented microcosms (SAS and Abisp) showed different patterns with respect to PAH removal and Pb mobility due to the different microorganisms inoculated and organic amendment manipulation. The SAS microcosms involved the use of SAS without any treatment and produced bioaugmentation with *A. bisporus* and the inherent microbiota with proven PAH degradation capacity (García-Delgado et al., 2013b; 2015a). The other bioaugmented microcosms (Abisp) comprised sterilized SAS which had been reinoculated with *A. bisporus*. The immediate effects of the two bioaugmentation strategies were a very large heterotrophic and PAH-degrading bacterial population in the SAS microcosm, and

in the Abisp microcosm a very high ligninolytic activity, both treatments produced light Pb mobilization. As result, the Abisp microcosm was more efficient at PAH biodegradation than SAS as a result of the combination of the high *A. bisporus* ligninolytic activity (Fig. 8.1) to HMW-PAH removal, and secondly by a growing PAH-degrading bacterial population during the assay (Fig 8.2) which can degrade 3-ring PAH. In fact, comparing PAH removal on the basis of the number of rings, no significant differences were found for biodegradation of Σ3-ring PAH between SAS, Abisp or even SM because the degradation of these compounds can be carried out by bacteria. In contrast, HMW-PAH are typically poorly degraded by bacteria due to their low water solubility and high hydrophobicity which results in mass transfer rates to the bacterial cells that are too low to match the basic cell metabolite requirements (Fernández-Luqueño et al., 2011). In this respect, Abisp was the most efficient microcosm to biodegrade HMW-PAH, mainly BaP, due to its high ligninolytic activity (Fig 8.1). These enzymes are able to degrade PAH with low bioavailabiliy, even beyond their respective bioavailable amount (Covino et al., 2010b).

The availability of Pb in the Abisp microcosms did not appear to inhibit PAH removal, previous studies have reported a total PAH degradation rate of 36% in non Pb polluted soil (García-Delgado et al., 2015a). The fungal defense mechanisms employed to alleviate the toxicity of heavy metals is usually based on their immobilization using extracellular and intracellular chelating compounds. One of the typical metal chelators produced by fungi is oxalate which can be used as a way of immobilizing soluble metal ions or complexes as insoluble oxalates, thus decreasing their bioavailability and increasing tolerance to these metals (Baldrian, 2003). Johansson et al. (Johansson et al., 2008) described the increment of oxalic acid exudation in presence of Pb and Cd by several basidiomycetes. The growth of calcium oxalate hydrate covering the surface of hyphae was observed in this work by SEM and X-ray diffraction of the hyphae (Fig. 8.4). However Pb-oxalate was not detected and therefore oxalate production does not appear to be the most probable Pb detoxification mechanism for *A. bisporus*. In addition, oxalic acid has multiple roles in the decay of wood such as lowering the pH to promote the action of LME and is also implicated in lignocellulose degradation during the initial cellulose depolymerization (Dutton and Evans, 1996). The segregation of oxalic

acid can explain the sharp decrease in pH and the increase in Pb availability in the Abisp microcosms at 0 and 28d when compared to SSAS where the substrate had been sterilized and *A. bisporus* was absent.

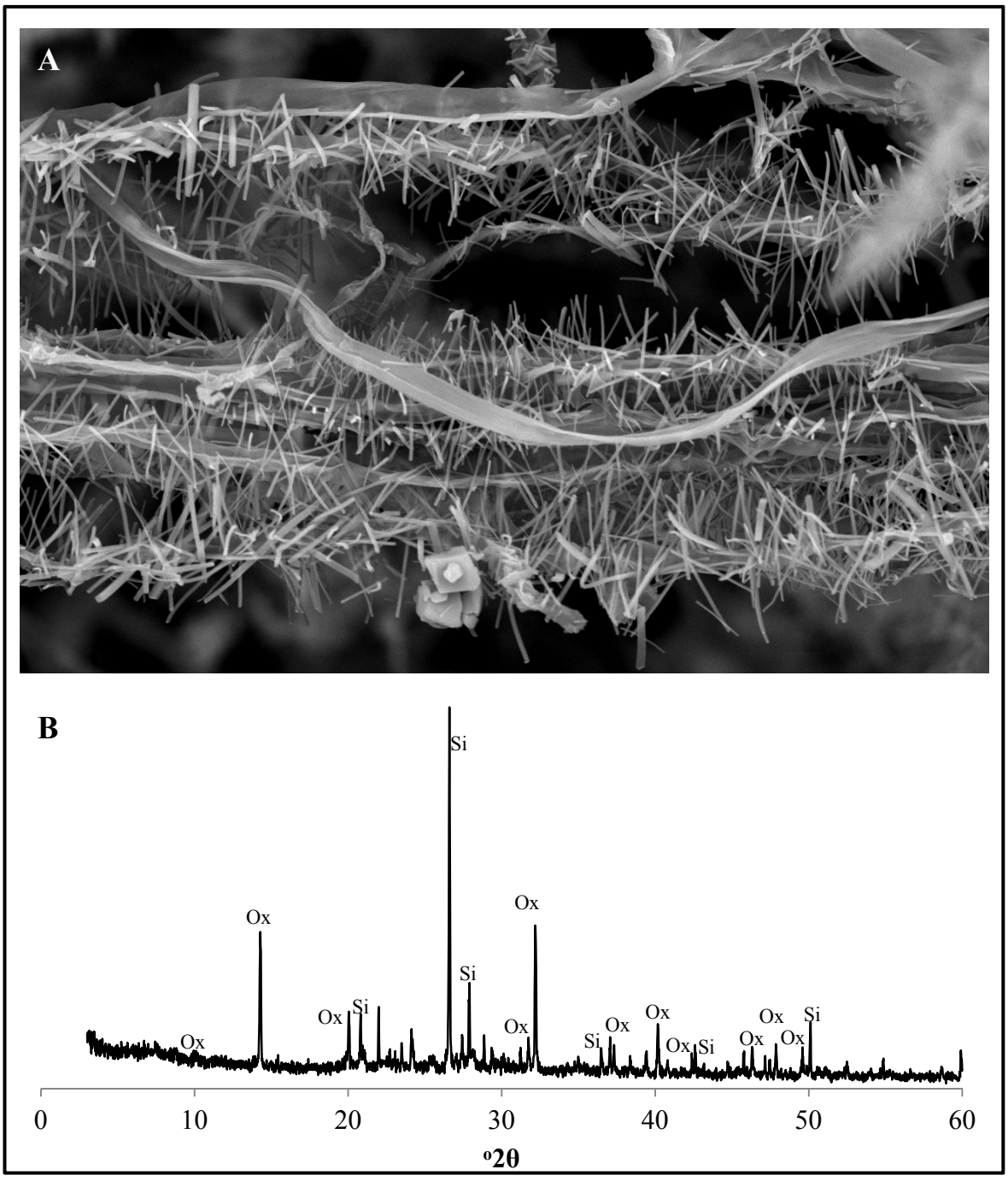


Fig. 8.4: SEM examination (A) and X-ray diffraction spectra (B) of *A. bisporus* aerial hyphae from Abisp microcosm. Ox, calcium oxalate hydrate; Si, silicon oxide.

Despite the significant mobilization of Pb, the concentrations of available Pb were lower than the EC50 values given for the effects of available Pb on the survival and reproduction of the *Eisenia andrei* and *Enchytraeus crypticus* (soil worms) exposed to soils obtained from a shooting range and different reference soils (Luo et al., 2014a, 2014b). Therefore, the risk to soil invertebrates did not increase in the amended microcosms.

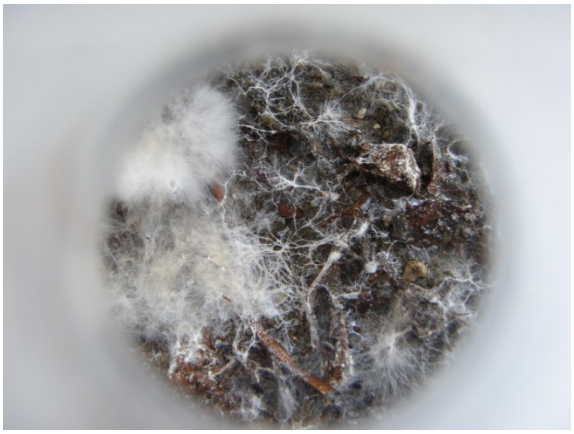
The soil eco-toxicological tests carried out (THA, DHA) showed the pollution impact on soil microbiological activity at 0, 28 and 63d. SM achieved the worst eco-toxicity results, so natural attenuation was not able to detoxify the soil. The three SAS application methods, despite different PAH removal rates or Pb availability, caused a positive effect on soil microbiota activity, probably due to the organic matter and/or microorganisms input. DHA appeared to be related more to the organic matter input, and further degradation over time, than soil remediation, this could be explained by the fact that the DHA value was at its maximum at day 0 when the organic matter had its highest nutrient status.

THA, the other soil eco-toxicological parameter, is a good indicator of total microbial activity (bacteria and fungi) (Adam and Duncan, 2001). At the start of the assay THA was higher in SAS and Abisp (bioagmented microcosms) due to the presence of the inherent SAS microbial population or *A. bisporus* respectively. But at 63d incubation, the THA can be explained by the effects of each remediation treatment. SM showed the lowest THA as a result of the high pollutant content and low bacterial population. The relative high SSAS THA was because Pb mobility had been minimized ($R = -0.763$, $p<0.05$) and the autochthonous soil bacteria enhanced by exogenous organic matter. For Abisp, THA was constant during the three sampling times probably due to a combination of bacterial population growth with time and fungal activity and the significant decrease of PAH concentration.

Focusing on PAH detoxification, the higher RCRA reached by the bioaugmented microcosms was due to the higher removal rates of HMW-PAH, mainly DBahA and BaP, with high toxic equivalency factors (Nisbet and LaGoy, 1992).

8.5. Conclusions

The reuse of spent *A. bisporus* substrate for the biodegradation of PAH in multi-polluted is feasible. However, the SAS application method is a key factor in obtaining the maximum efficiency. The application of sterilized SAS (SSAS microcosms) does not biodegrade PAH in Pb co-polluted soil despite enhancing the bacterial population. The use of SAS without previous treatment provides certain PAH biodegradation by the inoculation of an appreciable inherent microbiota able to degrade PAH, however this was not feasible as inoculum carrier for *A. bisporus* and the heavy pollution of the soil produced toxic effects on microbial community. The SAS sterilization and further *A. bisporus* re-inoculation is adequate as *A. bisporus* inoculum carrier and is the most efficient way of SAS reutilization for PAH biodegradation in Pb co-polluted soil by the high activity of *A. bisporus*. The increment of Pb availability was very low and minimized with time, but the reduction of PAH and its carcinogenic risk is sufficient to consider this method as an effective, cheap and useful soil mycoremediation strategy.



Capítulo 9:
Discusión General

La presente Tesis está dividida en dos grandes bloques de capítulos. En el primer bloque se desarrollan las metodologías de extracción de elementos traza (capítulo 3) y PAH (capítulo 4) que serán utilizados a posteriori. También se muestran los resultados de la localización y selección de suelos potencialmente contaminados para su posterior uso en ensayos de remediación (capítulo 5). Dentro del segundo bloque de capítulos se expone la evaluación del sustrato post-cultivo de champiñón (*A. bisporus*, SAS) como enmienda recuperadora de suelos. En una primera fase se determinó la capacidad del propio sustrato para degradar PAH e inmovilizar metales, así como el efecto de Cd y Pb en las actividades ligninolíticas y en la degradación de PAH (Capítulo 6). En una segunda fase se determinó la utilidad del SAS en la remediación de suelo real contaminado con PAH mediante tres posibles métodos de aplicación (esterilizado, sin tratamiento previo o como inóculo de *A. bisporus* previa esterilización) (Capítulo 7). Por último se evaluó la efectividad de esos tres métodos de aplicación de SAS en un escenario más complicado que el anterior donde la concentración de PAH se elevó considerablemente y además coexistía un contaminante inorgánico (Pb) en elevada concentración (Capítulo 8).

Las enmiendas orgánicas tienen un importante papel en la biorremediación de suelos contaminados tanto como materiales capaces de inmovilizar contaminantes como promotores de la actividad biológica del suelo y por lo tanto de la degradación de contaminantes orgánicos (Bolan et al., 2014; Haritash and Kaushik, 2009; Park et al., 2011; Tyagi et al., 2011). Sin embargo también pueden ser fuente de sustancias contaminantes al suelo, siendo los metales una de las más habituales (Park et al., 2011). Muchos de ellos son esenciales para los seres vivos como el Cu o Zn, pero en altas concentraciones se comportan como contaminantes. Otros como Cd y Pb son tóxicos por naturaleza. Por ello el control de las cantidades totales añadidas al suelo ha de ser exhaustivo, más aun teniendo en cuenta que son sustancias no biodegradables con tiempos de residencia muy elevados en el suelo (Smith, 2009). Durante la evaluación de las propiedades químicas y mineralogía de las enmiendas orgánicas llevada a cabo en el capítulo 3, se observó que la fracción inorgánica de las enmiendas orgánicas tiene una gran peso en la composición total del material (20 – 75% m:m). Esta fracción mineral a su vez contiene un importante porcentaje de la concentración total de metales que se reflejó en la

extracción secuencial BCR y en la mayor concentración obtenida en las digestiones totales en microondas que incluían HF. La enmienda con menor fracción orgánica fue SMC, material con una composición similar al SAS desde el punto de vista físico-químico aunque no biológico, debido al re-compostaje al que ha sido sometido. Esta enmienda presenta un alto porcentaje de la concentración de metales en las fracciones 3 y 4 del fraccionamiento BCR (Fig. 3.2.), lo que dificulta potencialmente su biodisponibilidad.

Dentro de las tres metodologías de digestión pseudo-total (ISO 11466, microondas con agua regia (AR) o microondas con ácido nítrico (N)) se observó similar eficiencia en la extracción de casi todos los metales. En los casos de Ni y Cr, su recuperación mediante digestión en microondas con ácido nítrico se mostró claramente inferior porque estos metales se encuentran preferentemente en la fracción de aluminosilicatos que este ácido apenas ataca (Fig. 3.4). Debido a los buenos resultados y el menor tiempo de digestión se propone el método de digestión en microondas con agua regia como la metodología de digestión pseudo-total.

Sin embargo la inclusión de HF en la mezcla de ácidos para atacar a la fracción silícica de las enmiendas orgánicas se mostró totalmente necesaria para realizar una digestión total. En este caso el procedimiento de digestión en vaso abierto con HF-HNO_3-$HClO_4$ era capaz de digerir completamente la matriz de las muestras pero los resultados analíticos mostraron claramente menores recuperaciones que las digestiones en microondas con HF e incluso a ISO 11466, debido a pérdidas de analitos durante la digestión. Las digestiones en microondas con NF y ARF se mostraron eficientes en el ataque a los aluminosilicatos (Fig. 3.4). De hecho se observó correlación entre el incremento de concentración en las digestiones con y sin HF y la fracción residual de la extracción BCR (Tabla 3.6). Sin embargo el HF producía precipitados de CaF_2 y $CaAlF_5$ en muestras con alto contenido en Ca o Al y especialmente en aquellas con presencia de $CaCO_3$ (Fig. 3.1). La inclusión de HCl en la mezcla de ácidos inhibía la formación de CaF_2 por competencia entre F^- y Cl^-, sin embargo no inhibía la formación de $CaAlF_5$. La formación de estas fases sólidas presentaba ciertos problemas por fenómenos de co-precipitación en el caso de Fe, Ni y Zn. En determinadas muestras con altos

contenidos en $CaCO_3$, Ca y/o Al. La mezcla NF mostró signos de co-precipitación de Fe en CaF_2 y Zn para las muestras SMC y HWC mientras que la mezcla ARF mostró sistemáticamente menores recuperaciones de Ni.

A pesar de estas pérdidas por co-precipitación, el uso de HF en la digestión en microondas (ARF y NF) aumenta la recuperación de metales respecto a la extracción ISO 11466 entre el 10 y 30%. Esto significa que en la actualidad se está añadiendo dosis totales de metales apreciablemente mayores que las determinadas con agua regia. Es cierto que son fracciones poco móviles pero también han de ser tenidas en cuenta ya que tras aplicaciones continuadas, este aporte de metales no recuperado puede llegar a tener consecuencias medioambientales a largo plazo. A lo largo de la tesis no se ha tenido en cuenta únicamente el contenido total de metales y su (bio)disponibilidad ha sido estudiada mediante diferentes extractantes.

Por todas estas razones se propone la digestión en microondas con HF-HNO_3 como método de digestión total para enmiendas orgánicas.

La puesta a punto de la extracción de PAH tanto en suelos como en SAS se realizó por comparación de dos técnicas (ultrasonidos y agitación) y cuatro extractantes (acetona:diclorometano, acetona:hexano, metanol y acetona). Se eligieron estas dos técnicas por varias razones: no requieren grandes equipos, la cantidad de disolvente por muestra no es alta, el tiempo de extracción es razonable y se pueden procesar un elevado número de muestras simultáneamente. La extracción con ultrasonidos, independientemente del extractante, obtuvo menor número de PAH validados en el suelo de referencia (Tabla 4.1). Entre los PAH no validados se encontraba sistemáticamente BaP, que debido a su probada carcinogenicidad es uno de los PAH más importantes en los posteriores estudios de biorremediación. Estas razones llevaron a descartar la técnica de ultrasonidos. Con la extracción por agitación, de los cuatro extractantes, acetona:diclorometano y acetona:hexano fueron los que no obtuvieron concentraciones menores que los valores certificados. Incluso acetona:diclorometano sobreestimó la concentración de 3 PAH (Tabla 4.1). Finalmente se determinó que la extracción mediante agitación con acetona:hexano era la más adecuada por su validación mediante el

suelo certificado y la versatilidad para la extracción de PAH del SAS tanto fresco como seco (Tabla 4.3).

Una vez seleccionados los métodos de extracción y análisis para metales y PAH se procedió a la localización de suelos contaminados. Entre los emplazamientos muestreados se observó que los mayores niveles de concentración de PAH se localizaban en suelos contaminados por creosota (Tabla 5.4). Sin embargo no se encontró co-contaminación por metales debido a anteriores actuaciones de recuperación de los suelos y a que en la composición actual de la creosota se excluyen metales y As. La ausencia de contaminación por metales fue útil como se vio en el capítulo 7 ya que permitió el estudio de la biorremediación de PAH en muestra real de suelo históricamente contaminado pero en ausencia de contaminación por metales. En el suelo cercano al campo de tiro se esperaban encontrar altas concentraciones de Pb (Tabla 5.2), como así ocurrió y únicamente trazas de PAH pero sorprendentemente la concentración de PAH fue muy elevada (Tabla 5.4). Hasta el momento no se ha reportado en la bibliografía un suelo afectado por contaminación procedente de un campo de tiro con niveles de PAH comparables a los de este suelo.

En la primera de las evaluaciones de SAS como enmienda útil en la biorremediación de metales y PAH (capítulo 6) se realizaron diversos ensayos con el fin de determinar la capacidad de degradación de PAH y retención de metales y efecto de estos, tanto en las actividades ligninolíticas de *A. bisporus* como en el proceso de degradación de PAH. Mediante isotermas de adsorción y en el posterior ensayo de degradación de PAH en presencia de Cd y Pb se observó alta capacidad de adsorción de estos metales por parte del SAS (Fig. 6.1 y 6.2), lo que hace pensar en su utilidad para la detoxificación de suelo contaminado por metales. También se observó la inhibición de la actividad de la enzima laccasa a altas concentraciones de Cd y Pb en el ensayo de interacción (Tabla 6.2) aunque en el ensayo de degradación de PAH se constató mayor actividad (Fig. 6.3). Esto sugiere que Cd y Pb inhiben la actividad de la enzima pero promueven la segregación de laccasa y MnP en *A. bisporus*. Por último se demostró la capacidad de degradación de PAH por la microbiota de SAS (Fig. 6.4) aunque con el diseño experimental y las determinaciones realizadas no fue posible discernir el papel del hongo *A. bisporus*

y del resto de microorganismos presentes en el SAS. Esta diferenciación sí se pudo realizar en los siguientes ensayos.

El siguiente paso (capítulo 7) fue la utilización de SAS para la degradación de PAH en suelo real procedente de una planta de creosotado de madera. Se aplicó este residuo agrícola en remediación de suelo contaminado bajo tres formas diferentes, esterilizado para determinar el efecto bioestimulador de la enmienda orgánica (SSAS), sin tratamiento previo como inóculo de *A. bisporus* y su microbiota (SAS) y esterilizado con posterior re-inoculación de *A. bisporus* como inóculo del hongo en el suelo (Abisp). Estas tres formas de aplicación de SAS se compararon con un proceso de atenuación natural como control. Por un lado se confirmaron los resultados obtenidos en el anterior capítulo, SAS es útil como enmienda promotora de la degradación de PAH y por otro se conocieron mejor las implicaciones que conllevan las diferentes formas de aplicación de SAS en la microbiología del suelo y sus consecuencias en la degradación de PAH.

El uso del sustrato esterilizado (SSAS) produjo la esperada bioestimulación de la microbiología del suelo que se reflejó en un considerable aumento de la población bacteriana total y especialmente en las bacterias degradadoras de PAH (Fig. 7.1) que a su vez incrementó la degradación de PAH de bajo peso molecular (3 anillos) respecto a la atenuación natural (Tabla 7.2). Por el contrario la degradación de HMW-PAH no se vio incrementada. Esto es común cuando se utilizan bacterias como degradadores de PAH. Gong et al. (2015) inocularon *Mycobacterium* sp. en suelo contaminado con PAH y obtuvieron alta degradación para PAH de 3 anillos mientras que los PAH entre 4 y 6 anillos no fueron degradados. La aplicación de SAS sin tratamiento previo se mostró eficaz como inóculo de bacterias heterótrofas y bacterias capaces de degradar PAH, así como de *A. bisporus* principalmente durante las primeras semanas de incubación aunque las actividades ligninolíticas obtenidas fueron bajas (Fig. 7.2). Esta forma de aplicación de SAS produjo degradación tanto de PAH de alto como de bajo peso molecular (Tabla 7.2). El último de los métodos de aplicación del sustrato fue la esterilización y posterior inoculación de *A. bisporus* (Abisp) y consiguió que el hongo colonizase el suelo por completo, a pesar de no incrementar el contenido de ergosterol a lo largo del ensayo (Fig. 7.1). Abisp fue el tratamiento que consiguió la mayor

actividad ligninolítica (principalmente laccasa) durante todo el ensayo (Fig 7.2). La población bacteriana fue menor que en el caso de los anteriores tratamientos durante el ensayo pero al final del tiempo de incubación (63 días) esta población se igualó (Fig 7.1). Como resultado de la combinación de la actividad bacteriana y ligninolítica, Abisp consiguió degradación de PAH de alto y bajo peso molecular (Tabla 7.2). En este tratamiento cabe destacar la mayor degradación, en comparación con SSAS y SAS, de BaP y DBahA clasificados como carcinogénico (grupo 1) y probablemente carcinogénico (grupo 2A) para humanos respectivamente (IARC 2010).

El análisis de la comunidad microbiana mediante DGGE (Tabla 7.3) reveló que la bioestimulación con el sustrato esterilizado tuvo impacto positivo en la comunidad bacteriana aumentando su riqueza y variedad pero no en la fúngica que presentó valores similares al suelo sin enmendar. El mismo resultado se obtuvo en el tratamiento de bioaumentación Abisp, sin embargo con la aplicación de SAS aumentaron tanto los parámetros bacterianos como los fúngicos lo que confirmó la inoculación de microbiota exógena al suelo con el sustrato post-cultivo.

La mayor reducción en la evaluación de riesgo carcinogénico del suelo, mayor actividad deshidrogenasa y menor mortalidad de *F. candida* (Tabla 7.6) junto con la mayor degradación de HMW-PAH apuntan a la esterilización y posterior re-inoculación de *A. bisporus* (Abisp) como la mejor forma de aplicación de este residuo al suelo contaminado por PAH para su biodegradación.

El capítulo 8 muestra un diseño experimental muy similar al anterior pero en este caso el escenario de contaminación es diferente. El suelo procedente de las inmediaciones del campo de tiro contiene 1429 mg kg^{-1} de plomo y 757 mg kg^{-1} de PAH de los que 370 mg kg^{-1} corresponden a PAH de 5 y 6 anillos. Mientras que en el ensayo del capítulo 7, el suelo procedente de la planta de creosotado de madera no se encontraba contaminado por Pb, la concentración total de PAH era de 111 mg kg^{-1} y los PAH más abundantes eran los de bajo peso molecular. Por lo tanto, en el capítulo 8 se da un salto importante en la magnitud de la contaminación del suelo a remediar.

La aplicación de SSAS al suelo de campo de tiro aumentó la población bacteriana total en un orden de magnitud y la degradadora de PAH en dos (Fig.

8.2). Sin embargo esta bioestimulación fue menor que en el suelo de creosota, donde la población bacteriana total aumentó hasta tres órdenes de magnitud y la degradadora de PAH cerca de 3,5 órdenes de magnitud (Fig. 7.1). En este caso, la bioestimulación de la población bacteriana no se tradujo en un incremento de la tasa de degradación de PAH respecto de la atenuación natural (Tabla 8.2). Por lo tanto la mayor contaminación de este suelo probablemente inhibió tanto el desarrollo bacteriano como su funcionalidad. De hecho entre la población bacteriana y el Pb disponible se estableció correlación negativa para este microcosmos.

Los dos tratamientos de bioaumentación con *A. bisporus*, SAS y Abisp, obtuvieron tasas de degradación de PAH significativamente mayores que la atenuación natural o la bioestimulación (Tabla 8.2). Sin embargo no parece que en los dos tratamientos el papel de *A. bisporus* y las bacterias fuera el mismo. La bioaumentación con SAS produjo una notable aportación de bacterias degradadoras de PAH pero *A. bisporus* no fue capaz de colonizar este suelo en contraste con el suelo de la planta de creosota. Como consecuencia, la actividad ligninolítica de este tratamiento fue baja (Fig. 8.1). El tratamiento Abisp produjo una completa colonización del suelo por parte de *A. bisporus,* además de alta actividad ligninolítica (Fig. 8.1). Al final del ensayo la población bacteriana degradadora de PAH de Abisp no mostraba diferencias significativas respecto a SSAS y SAS (Fig. 8.2). Como consecuencia de la mayor actividad fúngica en Abisp y similar población bacteriana que en SAS, Abisp produjo mayor tasa de degradación de HMW-PAH mientras que no hubo diferencias significativas en los PAH de 3 anillos (Tabla 8.2). Entre los HMW-PAH cabe destacar que, igual que en el suelo contaminado con creosota y libre de Pb, la mayor tasa de degradación correspondía a BaP y también la elevada degradación de DBahA en comparación con los demás microcosmos. Respecto de la degradación de PAH y detoxificación del suelo, la aplicación del sustrato post-cultivo esterilizado con re-inoculación de *A. bisporus* vuelve a ser el método de aplicación más eficiente.

Como se plasma en las revisiones realizadas por Baldrian (2003) y Olaniran et al. (2013), sobre bioaumentación con hongos ligninolíticos o bacterias en suelos contaminados simultáneamente con compuestos orgánicos y metales, es poco

común considerar la fracción (bio)disponible de los metales. En la mayoría de los estudios de biorremediación con hongos, simplemente se reporta el contenido total de metales y utilizan ese valor como parámetro indicador de la inhibición en la degradación de contaminantes orgánicos. Sin embargo las características de cada suelo (textura, m.o., pH,…) modifican notablemente la movilidad de los metales aunque la concentración total sea similar y eso produce que las concentraciones reportadas que inhiben la degradación de compuestos orgánicos sea muy amplia (Thavamani et al., 2011). Por lo tanto la concentración total de un metal en un determinado suelo no es el parámetro más adecuado para determinar su grado de toxicidad. En cambio en los trabajos que evalúan enmiendas orgánicas como inmovilizadoras de metales pesados y/o bioestimuladoras de la microbiología del suelo es común que se determine el efecto en la movilidad/biodisponibilidad de los metales (Beesley et al., 2010) y de los PAH (Covino et al., 2010a, 2010b; Gomez-Eyles et al., 2011; Wu et al., 2013).

En los ensayos de esta memoria de Tesis, no sólo se ha prestado atención al contenido total de metales y PAH, que es muy importante, sino también a la fracción disponible o potencialmente móvil ya que es la fracción biológicamente más activa. En el ensayo de remediación de suelo procedente de la industria de creosotado de madera (capítulo 7) se valoró el efecto del SAS en la biodisponibilidad de los PAH. Se determinó que la utilización de esta enmienda bajo las tres formas de aplicación probadas disminuía la disponibilidad de los PAH mediante dos vías, degradación de las fracciones más disponibles y adsorción en SAS debido al gran carácter alifático de su carbono orgánico (21% del total). En un principio puede parecer beneficiosa la adsorción de PAH en las enmiendas orgánicas para dificultar su movilidad en el suelo pero esto dificulta notablemente la degradación de PAH por bacterias. En este sentido, Wu et al. (2013) mostró como la adsorción y desorción de PAH en la materia orgánica de compost limitan y favorecen su degradación respectivamente. En el caso de hongos ligninolíticos parece que la biodisponibilidad de los contaminantes no es un factor limitante debido a que las enzimas ligninolíticas son capaces de difundir por la matriz y actuar sobre ellos (Haritash and Kaushik, 2009). En este sentido Covino et al. (2010a, 2010b) observó tasas de degradación de PAH por encima de su disponibilidad en micorremediación con hongos ligninolíticos. Esta puede ser la

razón por la que el tratamiento Abisp no mostrara correlación negativa entre degradación de PAH en el suelo de creosota y ciertas propiedades físico-químicas de los PAH relacionados con su disponibilidad en suelos como son el peso molecular, coeficiente de adsorción en carbono orgánico e hidrofobicidad y el bajo coeficiente de correlación con la solubilidad en agua (Tabla 7.4). En los otros tres tratamientos donde el papel de las bacterias era mucho más marcado, la degradación de PAH sí mostraba correlación negativa con la hidrofobicidad de PAH y positiva con la solubilidad en agua.

El efecto de la disponibilidad de metales en la degradación de PAH mediante SAS se evaluó en los capítulos 6 y 8. En el capítulo 6, los estudios se realizaron añadiendo artificialmente PAH y Cd o Pb sobre el SAS. En el capítulo 8 se evaluó un escenario de biorremediación más realista donde la fuente de Pb y PAH fue suelo real procedente de las inmediaciones de un campo de tiro. Claramente se trata de dos escenarios diferentes pero complementarios. En los ensayos de contaminación artificial se evaluó la capacidad del material para retener metales y el efecto de estos en el proceso de degradación de PAH. SAS se mostró como un material con alta capacidad de adsorber Cd y Pb. Tras la aplicación de 500 μmol kg^{-1} de Cd o Pb en el sustrato, a los 28 días sólo se hallaba disponible el 27 y 28% (15 y 30 mg kg^{-1} respectivamente) de ambos metales pero esta fracción fue suficiente para que se observara disminución en la degradación de PAH. En el capítulo 8, donde la fuente de Pb era un suelo real, se determinaron las implicaciones del método de aplicación de SAS en la movilidad del Pb. Los tres tratamientos producían una ligera movilización del Pb (< 0,1%) al inicio del ensayo. Sin embargo SSAS y SAS minimizaron esta movilización a los 28 días de incubación mientas que Abisp tendió a aumentarla junto con un notable descenso del pH. Estas diferencias de comportamiento entre tratamientos se deben a la actividad del hongo que tiende a segregar ácido oxálico. Los diferentes efectos de la movilización de Pb en la degradación de PAH se deben claramente a la diferente microbiota implicadas en este proceso. En SSAS los principales organismos encargados de la degradación de PAH fueron las bacterias que aunque aumentaron su población (Fig. 8.2) no tuvieron una actividad metabólica alta durante el ensayo según los resultados de actividad deshidrogenasa e hidrolasa (Fig. 8.3). En el caso de SAS ocurrió algo similar, las actividades deshidrogenasa e hidrolasa fueron muy altas al inicio del

ensayo pero decayeron en los siguientes muestreos, en este caso la inhibición en la degradación de PAH no fue tan marcada porque *A. bisporus* también participó en el proceso. En el tratamiento Abisp, el hongo parece ser el principal responsable de la degradación de PAH y comparando con los resultados de degradación de PAH en los capítulos 6 y 7 no parece que el Pb tuviera un claro efecto negativo.

La aparente contradicción en los resultados de adsorción de Pb en los capítulos 6 y 8 no es tal y tienen justificación por varios factores. En primer lugar el material con el que se realiza la capa de cobertura durante el cultivo de champiñón ha variado durante el desarrollo de los trabajos de Tesis. Al inicio la cobertura se realizaba con una mezcla de suelo calizo y turba rubia pero debido a la producción de champiñones de mejor calidad con coberturas de turba negra, en pocos años se ha sustituido la mezcla de suelo calizo - turba rubia por la cobertura con turba negra. Claramente esta modificación afecta a las propiedades del SAS para retener metales aunque los componentes del sustrato inicial sean los mismos. En segundo lugar la fuente de Pb en el capítulo 6 fue una disolución de nitrato de Pb mientras que en el capítulo 8 era un suelo real con una disponibilidad muy baja y la materia orgánica soluble del SAS pudo actuar como complejante de Pb.

En resumen, durante la presente Tesis se han desarrollado los métodos de extracción de metales y PAH tanto de suelo como de SAS y se ha mostrado la utilidad del sustrato post-cultivo de champiñón en biorremediación de suelos contaminados con PAH tanto en presencia como en ausencia de metales. Estos estudios han arrojado luz sobre varios métodos de aplicación de este residuo agrícola en procesos tanto de bioestimulación como de bioaumentación y las implicaciones que estos tienen para la microbiología del suelo. A su vez se ha constatado la versatilidad del sustrato post-cultivo de champiñón para la biodegradación de PAH de alto y bajo peso molecular y el papel de su microbiota en la degradación de este tipo de compuestos. Por todo ello la aplicación de sustrato post-cultivo de champiñón en biorremediación de suelos contaminados con PAH se propone como una aplicación factible que puede proporcionar a este residuo un gran valor.

Capítulo 10:
Conclusiones / Conclusions

De los resultados obtenidos en el presente trabajo y su discusión se pueden extraer las siguientes conclusiones:

- ✓ En la digestión total de enmiendas orgánicas para extracción de metales, la fracción inorgánica tiene una gran importancia y el uso de HF es necesario para realizar el ataque total. El contenido en calcio de la enmienda orgánica determina la formación de precipitados (CaF_2 y/o $CaAlF_5$) durante la digestión total en microondas conllevando riesgo de co-precipitación de metales. La digestión en microondas con HNO_3 y HF fue el método más adecuado para la extracción total mientras que la digestión con agua regia fue el mejor método como extracción pseudo-total.
- ✓ La metodología de extracción de PAH más adecuada, tanto para suelo como para SAS húmedo o seco, fue la agitación con acetona:hexano (1:1).
- ✓ De los emplazamientos muestreados, los suelos afectados por cresosota fueron los que presentaban mayor contaminación por PAH pero bajas concentraciones de metales, mientras que el suelo adyacente al campo de tiro mostró multicontaminación por PAH y Pb.
- ✓ El sustrato post-cultivo de champiñón (*A. bisporus*) sin tratamiento previo es capaz de degradar PAH y simultáneamente inmovilizar Cd y Pb. La presencia de estos metales disminuyen la degradación de PAH.
- ✓ El modo de aplicación del sustrato post-cultivo de champiñón (SAS) en la bioremediación de suelos contaminados con PAH es determinante tanto en los microorganismos implicados en el proceso de degradación de PAH como en la efectividad del mismo.
    - o El uso de SAS previamente esterilizado (SSAS) estimula la microbiota autóctona del suelo contaminado con PAH tanto en presencia como en ausencia de Pb pero sólo se muestra útil en la degradación de PAH cuando no hay co-contaminación con Pb. Mediante bioestimulación, los PAH con 3 anillos son degradados en gran medida mientras que los PAH de alto peso molecular no se degradan de forma efectiva con este método de aplicación.

- La aplicación de SAS sin tratamiento previo (SAS) a suelo contaminado con PAH fue útil como inóculo de bacterias con capacidad degradadora de PAH. En el caso de ausencia de co-contaminación por Pb también se observó capacidad de colonización de *A. bisporus* mediante este método de aplicación. La bioaumentación con la microbiota presente en el SAS se muestra eficaz como degradadora de PAH tanto de alto como de bajo peso molecular.
- *A. bisporus* es capaz de colonizar suelo contaminado con PAH, tanto en presencia como en ausencia de Pb, mediante la aplicación de SAS esterilizado y posteriormente re-inoculado con *A. bisporus* (Abisp). Este método de aplicación se mostró muy útil en la biodegradación de PAH de alto peso molecular por acción de *A. bisporus*, especialmente para benzo[a]pireno y dibenzo[a,h]antraceno que son los dos PAH con mayor significación carcinogénica.

✓ La reutilización de SAS como enmienda recuperadora de suelos contaminados con PAH disminuye la toxicidad del suelo y aumenta su población y actividad microbiana.

Como conclusión general, el sustrato post-cultivo de champiñón (*A. bisporus*) se muestra como un residuo agrícola con alto potencial para biorremediación de suelos contaminados por hidrocarburos aromáticos policíclicos.

The main conclusions obtained in this Doctoral Thesis are:

- ✓ The extraction of trace elements from organic amendments is influenced for the mineralogy and the chemical composition of the amendment. The use of HF is mandatory for total digestion. The Ca content of the amendment promotes the formation of precipitates (CaF_2 and/or $CaAlF_5$) during the microwaves-assisted extraction which increases the risk of metal co-precipitation. Microwave-assisted digestion with a mixture HNO_3-HF is recommended for complete digestion of organic amendments, while aqua regia is proposed for pseudo-total digestion.
- ✓ The acetone:hexane (1:1), orbital shaking has been shown to be an adequate methodology for PAH extraction from soil and either dry and fresh SAS.
- ✓ The most PAH polluted soils were found in the creosote wood treatment plant. The surrounding soil of shooting range showed multi-pollution by PAH and Pb.
- ✓ Spent *A. bisporus* substrate (SAS) without previous treatment is able to degrade PAH and to immobilize Cd and Pb simultaneously. The presence of Cd and Pb decreases the PAH degradation.
- ✓ The SAS application method for bioremediation of PAH polluted soil decides the microorganisms involved in the PAH degradation process and its effectivity.
    - o The use of sterilized SAS (SSAS) stimulates the autochthonous microbiota of the PAH polluted soil with or without Pb. The biostimulation process is useful for PAH biodegradation when the PAH polluted soil is not co-contaminated with Pb. This metal prevent the PAH degradation in this conditions. The soil microbiota biostimulation promotes the degradation of 3-rings PAH but this process is not effective for HMW-PAH degradation.
    - o The application of SAS without previous treatment (SAS) to PAH polluted soil is situable for PAH-degrading bacteria input. In the case of

non Pb polluted soils, *A. bisporus* is able to colonize the soil. The bioaugmentation with SAS is effective for LMW and HMW-PAH.

- *A. bisporus* is able to colonize PAH (and Pb) polluted soil applying sterilized SAS inoculated with *A. bisporus* (Abisp). This method of application is very effective for HMW-PAH degradation because *A. bisporus* activity, especially for benzo[a]pyrene and dibenzo[a,h]anthracene which present proved carcinogenic properties.

✓ The SAS reuse for bioremediation of PAH polluted soils decreases the toxicity of the soil and increases its microbial population and activity.

In summary, the agricultural waste spent mushroom (*A. bisporus*) substrate shows high potential to be used in bioremediation of Polycyclic Aromatic Hydrocarbons polluted soils.

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Anexo / Appendix

Fig A1: Aspect of the experiment (chapter 6) at 21 (S3) and 28 (S4) days of incubation.

Fig. A2: Abisp treatment after *A. bisporus* incubation for 10 days at 20 ºC and before the addition of polluted soil.

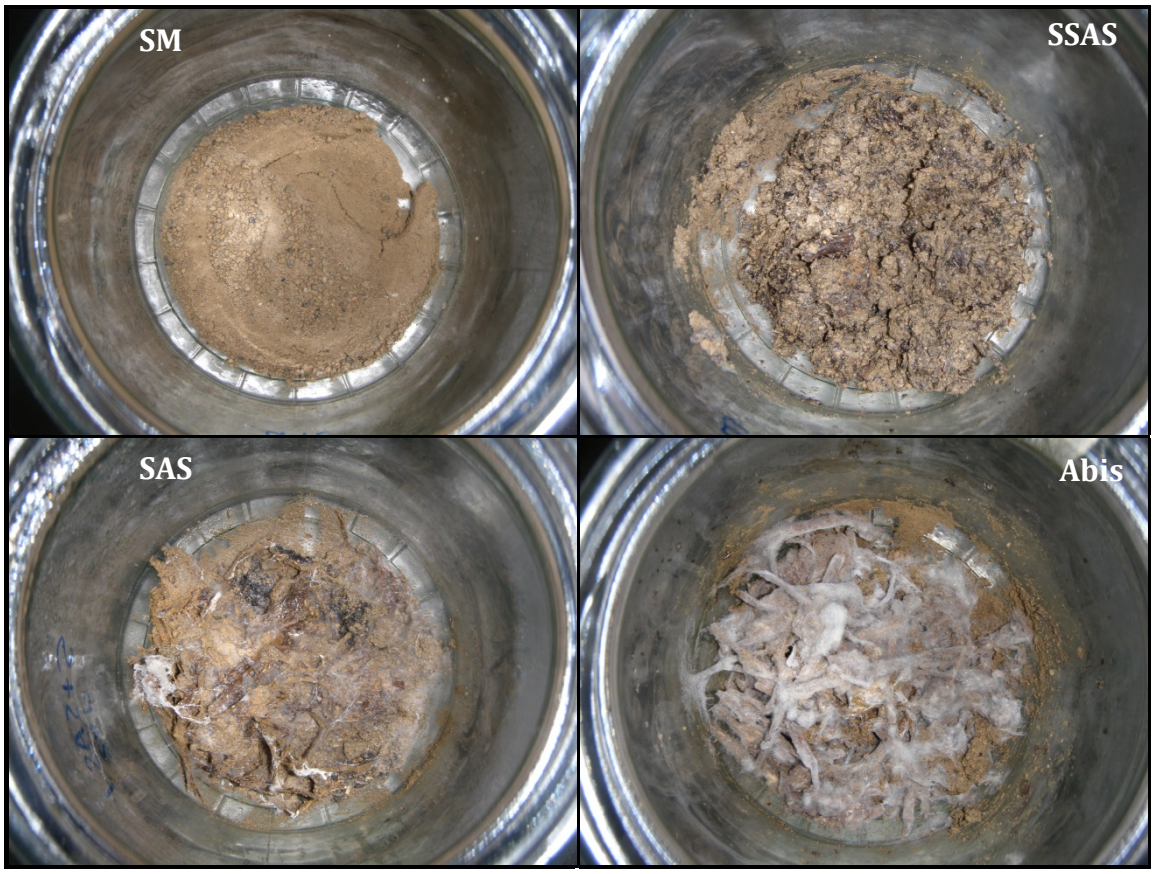

Fig. A3: Bioremediation microcosms of the creosote polluted soil, non amended soil microcosm (SM), amended with sterilized spent *Agaricus* substrate (SSAS), spent *Agaricus* substrate (SAS) and sterilized spent *Agaricus* substrate reinoculated with the fungus (Abisp), at the end of the assay (63 days of incubation).

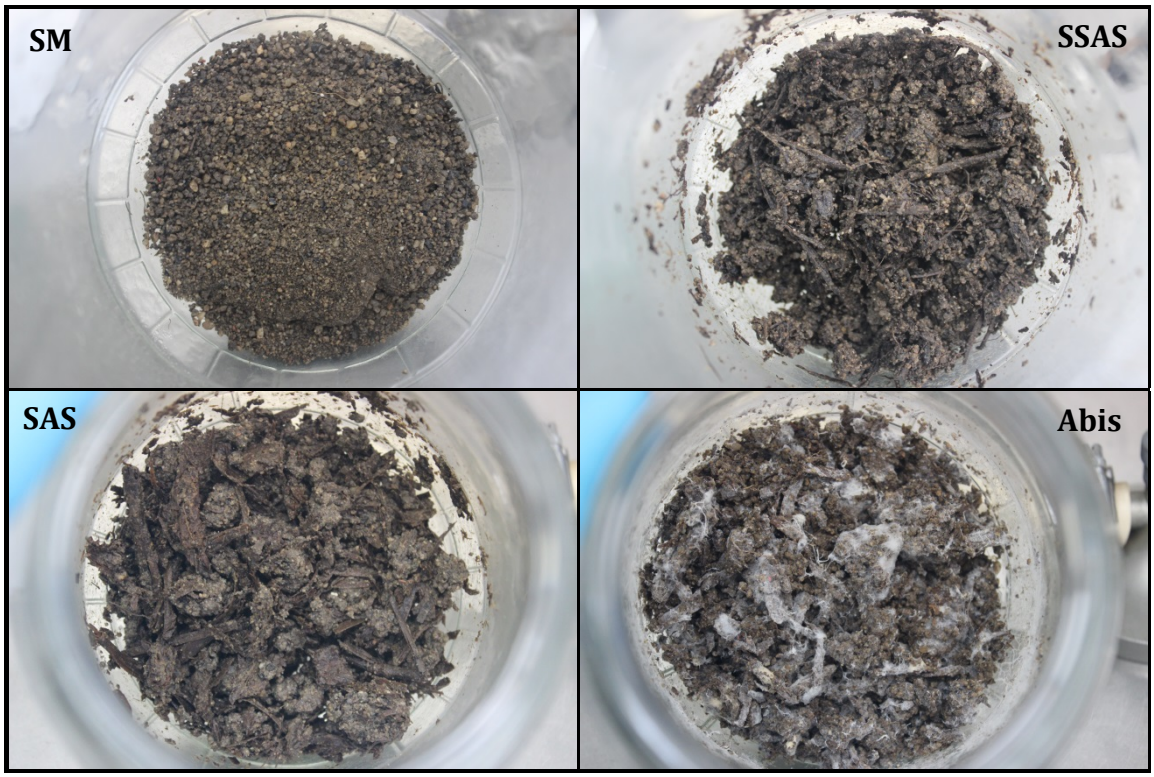

Fig. A4: Bioremediation microcosms of the shooting range soil, non amended soil microcosm (SM), amended with sterilized spent *Agaricus* substrate (SSAS), spent *Agaricus* substrate (SAS) and sterilized spent *Agaricus* substrate reinoculated with the fungus (Abisp), at the end of the assay (63 days of incubation).

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Influence of chemical and mineralogical properties of organic amendments on the selection of an adequate analytical procedure for trace elements determination

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ABSTRACT

Six digestion procedures were tested to improve extraction methods for determination of trace elements in various organic amendments with high inorganic fractions. These procedures were tested in terms of pH, $CaCO_3$, organic matter, elemental analysis, BCR sequential extraction and X-ray diffraction analysis. Aqua regia extraction (ISO 11466), total digestion HF–HNO_3–$HClO_4$ and four microwave-assisted digestions (i.e., HNO_3, HCl–HNO_3, HNO_3–HF and HCl–HNO_3–HF) were used. The effect of acid mixtures on microwave-assisted digestion of mineral fractions was assessed by Si and Al analysis and X-ray diffraction in the solid residues obtained. Microwave HF acid mixtures obtained highest trace element recoveries for all tested metals except Al. CaF_2 and $CaAlF_5$ precipitates were also detected using X-ray diffraction in the residues after microwave digestions with HF acid mixtures of amendments with high calcium content. A decision flowchart was suggested to determine the best acid mixture according to the amendment and the metals to be analyzed.

1. Introduction

Composted organic wastes are commonly used as organic amendment, fertilizer or growing media in agriculture and degraded soil recovery. The composts supply organic matter, nutrients and microorganisms, which improve the physico-chemical and microbiological soil properties and the fertility status. However, organic wastes can be an important source of trace elements in soils. During the stabilization process of organic matter and nitrogen, trace elements are concentrated in the compost matrix due to mass loss [1]. A major limitation of compost application to soil is thus the potential for high trace elements content.

The incorporation of trace elements after repeated applications of organic amendments in soils can have phytotoxic effects and also affect soil microorganisms [2]. In addition, once trace elements are applied, they have very long residence times in soil [3]. An exhaustive control of trace elements in agricultural soils is therefore necessary to prevent soil degradation and trace elements incorporation in the food chain. Fast, repeatable and robust methods for trace elements analysis are necessary to control organic amendments samples with different compositions and from multiple origins.

Regulations and guidelines for compost applications are currently based on total metal loadings. Traditionally, the literature and international directives have described compost as materials based in organic components, so analytical procedures to digest them and determine trace elements content are usually focused on the total oxidation of the organic matter. However, organic amendments can be composed of diverse organic wastes and inorganic materials including soils, sediments and inorganic residues, such as lime, gypsum, clay and silica.

The inorganic fraction may be relatively large in some organic amendments. In numerous countries, composted and co-composted wastes are being generated with appreciably high mineral fractions. For example, sewage sludge produced as a by-product of municipal wastewater treatment is composed of approximately 50% organic and 50% inorganic material [4]. Because the analysis of the trace elements concentrations in these waste materials is crucial for monitoring and risk assessment, the methods of sample mineralization must be able to liberate the trace elements associated with the inorganic fractions of compost.

In the literature, various methods for compost digestion have been described: dry ashing [5], heated mixture of acids [6], microwave-assisted wet digestion [7] and various acid mixtures

Abbreviations: SMC, spent mushroom compost; GWS, green waste + sewage sludge compost; BP, black peat; HWC, horticultural waste compost; AR, HCl–HNO_3 acid mixture; ARF, HCl–HNO_3–HF acid mixture; N, HNO_3 acid mixture; NF, HNO_3–HF acid mixture; A, anorthite; Al, aluminum hydroxides; C, calcite; CF, CaF_2; D, dolomite; F, $AlCaF_5$; Fe, iron oxides; G, gypsum; H, hallosite; I, illite; K, kaolinite; M, mica; P, $AlPO_4$; Q, quartz; W, whewelite.

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for wet digestions (e.g., HNO_3–HCl [8–10], HNO_3–$HClO_4$ [11–13], HNO_3, [10,13,14], HNO_3–HF–H_2O [15], HNO_3–H_2O_2 [16], and HNO_3–HF–$HClO_4$ [13]). Not all acid mixtures completely attack the inorganic fraction, so hidrofluorhidric acid is sometimes necessary for complete digestion. Hseu [6] and Sandroni and Smith [14] reported that HNO_3 produced the best recoveries for metal analysis except Al, which required HF.

Microwave-assisted acid extraction has proven to be a suitable method for the digestion of complex matrices, such as soils, sediments, biosolids and compost [14,17–19]. This procedure presents great advantages over traditional wet digestion, such as reduced duration of digestion, smaller quantities of acids, improved detection limits, less frequent contamination and loss of volatile analytes and increased reproducibility and accuracy.

Sequential extraction procedures have been used to determine the chemical forms of trace elements present in sediments and soils, and also in organics amendments, such as green waste, biosolids and municipal solid waste composts [1,3,8,20,21]. Such information is traditionally considered to be valuable for predicting metal mobility, bioavailability and leaching rates when composts are applied to soils [22]. Information from sequential extraction can also be considered in the selection of the optimal method for the extraction of trace elements present in composts. However, metal fractionation using sequential extractions is largely operational because the reagents are not completely selective and re-adsorption and redistribution of metal ions after release can occur [23]. For this reason, better understanding of trace elements speciation in the amendments can be obtained by combining different analytical methods, such as basic characterization, sequential extraction and mineralogical analysis, to better understand the chemical forms of trace elements and the mineralogical composition of organic amendments for selecting the most adequate analytical procedures for trace elements determination.

The purposes of this work were as follows: (1) to evaluate the Cr, Mn, Fe, Ni, Cu, Zn, Cd and Pb content in four organic amendments with high inorganic fractions using different digestion methods. These methods included the conventional aqua regia extraction ISO 11466 [9], an open-vessel HF–HNO_3–$HClO_4$ total digestion, and four microwave-assisted methods (i.e., HNO_3 and HCl–HNO_3 as acid extractable and HNO_3–HF and HCl–HNO_3–HF as total digestion) and (2) to select the most appropriate microwave-assisted acid digestion method for trace elements in amendments according to their chemical and organic compositions, sequential extraction data and mineralogical changes in residues after microwave-assisted acid digestion.

2. Experimental

2.1. Samples and characterization

The following organic amendments were used: (1) Spent mushroom compost (SMC). Initial compost is produced from wheat straw (55%), grape marc (15%) and poultry litter (30%) (m/m); this is commonly used as a mushroom (*Agaricus bisporus*) cultivation medium. After cultivation, waste is composted for 3 months. (2) Green waste + sewage sludge (3:1, m/m) (GWS). This mix is composted in tunnels with forced air for 14 days and matured in the open air. Composting is completed in approximately 6 months. (3) Natural black peat (BP), obtained from northern Spain. (4) Horticultural waste compost (HWC), obtained from greenhouse production, sieved at <25 mm and composted in the open air for 90 days.

Accuracy of analysis was evaluated using a Certified Reference Material: sewage sludge LGC6181 from LGC Standards (UK). The extractable metal content refers to metals soluble in hot aqua regia using method based on ISO 11466 [9].

All samples were air-dried for 14 days, and electrical conductivity (EC) and pH were determined in water extracts (1:5, v/v) using a conductivity meter (Crison CM 2200, Barcelona, Spain) and a pH electrode (Orion 720A, Beverly, MA, USA), respectively. Organic matter content was determined by mass loss-on-ignition at 450 °C over 4 h. Dry samples were milled and sieved to 100 μm. $CaCO_3$ content was analyzed by calcimetry. Total carbon, nitrogen, hydrogen and sulfur were analyzed using combustion (LECO CHNS-932 analyzer, USA).

A mineralogical characterization of the raw amendments samples and their solid residues remaining after microwave-assisted digestion was carried out by X-ray diffraction (Panalytical X'Pert Pro, Almelo, The Netherlands). The configuration of this apparatus was θ/2θ with an X'Celerator detector. Diffraction profiles were analyzed using X'Pert High Score Plus software and PDF-4 of the International Center for Diffraction Data for crystalline identification.

2.2. Reagents

All reagents used in sample digestion were better than analytical grade and supplied by Panreac (Spain), including Hiperpur HNO_3 (69%), Hiperpur HCl (35%), Hiperpur HF (48%) and Hiperpur $HClO_4$ (70%). Reagents used for sequential extraction were of analytical grade or better and supplied by Merck (Germany): acetic acid glacial, ammonium acetate, hydroxylammonium chloride, Suprapur hydrogen peroxide (30%). Ultrapure water was obtained from a Milli-Q water purification system (Millipore Corporation, Spain) and used throughout the work. All glassware and plasticware used were washed with 5% (v/v) nitric acid and rinsed with ultrapure water.

2.3. BCR sequential extraction procedure

The optimized BCR sequential extraction procedure [23] was applied to the organic amendment samples. This extraction procedure consists of three steps: Step 1 (exchangeable and weak acid soluble fractions) with acetic acid (0.11 M, 16 h). Step 2 (reducible fraction; Fe–Mn oxides), with hydroxylammonium chloride (0.5 M, pH 1.5, 16 h). Step 3 (oxidizable fraction; organic matter and sulfides) with H_2O_2 (8.8 M, 2 × 1 h, 85 °C) followed by extraction with 1.0 M ammonium acetate. Additionally, a fourth step was added to dissolve the final residue. The residual resistant fraction represented metals that were strongly associated with crystalline structures of minerals and which were unlikely to be released under conditions that are normally encountered in the environment. This residual fraction is typically extracted with aqua regia (ISO 11466), but HF–HNO_3–$HClO_4$ total digestion was performed in this work instead.

2.4. Aqua regia extraction (ISO 11466)

The aqua regia extraction was based on the ISO 11466 [9] procedure. Briefly, 3 g (±0.1 mg) of sample was placed in a 250 mL Pyrex digestion tube; 21 mL of 35% HCl and 7 mL of 69% HNO_3 were added, then the sample was covered with a watch glass and pre-digested at room temperature for 16 h. Subsequently, the suspension was digested for 2 h under reflux conditions. After cooling, the suspension was filtered through an ashless Whatman 42 filter, diluted to 50 mL with 0.5 M HNO_3, and stored in polyethylene bottles at 4 °C for analyses. Blanks were also treated using the same procedure.

2.5. Total digestion (HF–HNO_3–$HClO_4$)

For this method, 1 g (±0.1 mg) of sample was placed into a Teflon open vessel. A 10 mL volume of 48% HF was then added and

Table 1
Acid volumes (mL) used in microwave-assisted digestion procedures.

	HCl	HNO_3	HF
Mixture AR	9.00	3.00	–
Mixture ARF	8.25	2.75	1.00
Mixture N	–	12.00	–
Mixture NF	–	11.00	1.00

pre-digested at room temperature over 16 h. Next, the suspension was heated until dryness on a sand bath; 10 mL of 69% HNO_3 and 5 mL of 37% $HClO_4$ were then added and the reaction was heated until approximately 0.5 mL final volume remained. The final volume was then made up to 50 mL with 0.5 M HNO_3 and stored in polyethylene bottles at 4 °C for later analyses. Blank digestions were also performed using the same protocol.

2.6. Microwave-assisted acid digestion

A pressurized closed-vessel microwave system (CEM Mars X Press, USA) was used to digest the samples. Microwave polyfluoroacetylene (PFA)-teflon vessels were cleaned before each digestion using 12 mL of 69% HNO_3, heated for 20 min at 200 °C and then rinsing with ultrapure water. After the microwave-assisted digestion procedure was optimized, the following conditions were employed: samples were accurately weighed to 250 mg (±0.1 mg) in microwave vessels. They were then subjected to four different digestion procedures using various acid combinations (Table 1) using the same solid sample/reagent volume ratio (250 mg to 12 mL). The digestion program consisted of a 15-min gradual increase to 200 °C, a 15-min digestion step at 200 °C and 1200 W and then a cooling stage. Acid mixtures used were selected according to previous reports for soil and compost trace elements analysis [10,13,18,24,25]. Blanks were processed in a method identical to the samples.

2.7. Trace and major elements analysis

Analyses were conducted on a ICP-MS spectrometer (Perkin Elmer Sciex Elan 6000) equipped with an autosampler (AS 91, Canada). The isotopes used for metals analyses were ^{52}Cr, ^{55}Mn, ^{56}Fe, ^{58}Ni, ^{63}Cu, ^{64}Zn, ^{114}Cd and ^{208}Pb. In addition, Al and Si (isotopes measured ^{27}Al and ^{28}Si) were analyzed for monitoring microwave-assisted digestion of silicates and aluminosilicates. Limits of quantitation were calculated over 10 measurements of the chemical blank from each acid mixture of microwave-assisted digestion as $10\sigma/b$, where σ is signal standard deviation and b is linear coefficient of the calibration graph.

2.8. Statistical analysis and chemical speciation software

SPSS 15.0 software was used for statistical analysis. Significant differences between different microwave-assisted acid digestions, aqua regia extraction (ISO 11466) and HF–HNO_3–$HClO_4$ total digestion were compared using 1-way ANOVA with Tukey's post-hoc test. Statistical significance was defined as $p < 0.05$. The V MINTEQ v.3.0 software was used to confirm Ca chemical species in microwave HF acid mixtures.

3. Results and discussion

3.1. Basic characterization of organic amendments

After a first approach based on basic chemical properties (Table 2), the selected organic amendments could be classified into three types:

1) SMC and HWC are composted agricultural wastes with high $CaCO_3$ and EC (10.4 and 11.6 dS m^{-1}), very low organic matter and C content (close to threshold values for organic amendments, as required by Spanish law RD 824/2005 [26]), and C/N values of approximately 10. These two materials have similar percentages of S.
2) GWS and LGC1681 present sewage sludge in their composition. They have remarkably high organic matter (50–60%), with almost 25% of their content coming from C. They also have several interesting similarities in pH, lack of carbonates and high EC. An important difference between these materials is elemental composition, as sewage sludge LGC6181 has higher values for N (low C/N) and S.
3) Natural black peat (BP), which has a lower pH value (4.86) and a low EC, but the highest organic matter (81%), C content and C/N.

3.2. Mineralogical characterization

All organic amendments were found to have quartz as the main mineral through X-ray diffractometry (Fig. 1). In addition to quartz, SMC showed calcite and gypsum. HWC showed calcite, dolomite, mica, anorthite and whewellite ($CaC_2O_4{\cdot}H_2O$) as secondary minerals. Whewellite also appeared in GWS, as did mica and anorthite. The presence of whewellite in HWC and GWS indicated the vegetal origin of these composts, as many plants contain calcium oxalate phytolites in their leaves, bark and wood as monoclinic whewellite crystals [27].

The secondary mineralogical composition of the reference standard material LGC1681 was gypsum, aluminum hydroxides and iron oxide. This was not surprising, as gypsum has been reported to precipitate during wastewater and sludge treatment [28]. In contrast, BP showed mica as a secondary mineral. Poorly crystalline iron hydroxides, such a ferrihydrite, might also have been present in SMC and BP due to the presence of a weak and broad XRD peak at 2.58 Å, but this was difficult to identify in bulk XRD analysis.

3.3. Distribution of trace elements in organic amendments according to the BCR procedure

The BCR extraction results are shown in Fig. 2. As can be seen, Cr, Fe, Ni and Pb were mainly associated with the residual fraction, suggesting that these metals were primarily contained in silicates and other resistant minerals. Other works obtained similar results

Table 2
Basic chemical characteristics and elemental analysis of organic amendments.

	pH	$CaCO_3$ (%)	EC[a] (dS m^{-1})	OM[b] (%)	C (%)	N (%)	H (%)	S (%)	C/N
SMC	7.02	28.1	10.4	25.2	12.4	1.37	1.73	0.21	10.7
GWS	7.19	–	2.00	58.1	26.2	1.41	3.49	0.04	23.9
BP	4.86	–	0.6	80.8	37.5	0.87	4.68	0.06	53.9
HWC	9.00	18.1	11.6	32.6	19.2	1.82	2.47	0.17	10.4
LGC1681	6.57	–	6.0	53.0	24.6	3.54	4.14	1.08	8.7

[a] Electrical conductivity.
[b] Organic matter.

for sewage sludge [20], municipal solid waste compost [21], and deinking paper fiber and green waste compost [1]. A particularity of LGC6181, and sewage sludge in general, was the great mobility of Ni compared to other trace elements [1,3,20]. The majority of Fe in the amendments was associated with the residual fraction (26–84%), followed by the oxidizable fraction (9–47%). This observation implied that Fe occurred in silicates and organic matter as sulfides with very little in the poorly crystalline hydroxides. Alonso et al. [20] found similar distribution patterns of Fe in sewage sludges.

Mn appeared in all four fractions with no preference for any particular one. In addition, most of the Cu was released by H_2O_2 extraction (70–85%), implying a great tendency to be bound in the organic fraction. Furthermore, a significant proportion of Cu (approximately 25%) was present in the residual fraction, suggesting a presence in resistant minerals. The majority of Cd was released during hydroxylamine–HCl extraction (17–71%), suggesting that Cd was primarily contained in reducible hydroxides and oxides. Very little Cd was present in the residual fraction (2–10%), except for HWC (24%). Finally, Zn was mainly associated with the reducible fraction (47–50%), except amendments with high $CaCO_3$ contents, and the organic fraction (26–74%); its residual fraction was variable from 4 to 56%.

The results from the sequential extraction procedure indicated that HF would be recommended for total digestion together with a strong oxidant acid in order to completely dissolve the organic and residual fractions of the amendments of interest.

3.4. Comparison of open system digestions

Table 3 shows the results of aqua regia extraction (ISO 11466) and total digestion ($HF–HNO_3–HClO_4$) of organic amendments. The precision of the methods was assessed by the percentage relative standard deviation (%RSD). For both methods, %RSD was good, generally <10% for all the metals and amendments. Pseudo-total digestion according to ISO 11466 obtained equal or higher concentrations of Cr, Mn, Ni, Cu, Zn, Pb and Cd (except for SMC) than total digestion. Only Fe concentrations were higher in the extracts from tri-acid total digestion for all amendments, suggesting the release of Fe included in the aluminosilicate phase during this treatment. These results indicated that open total digestion $HF–HNO_3–HClO_4$ produced losses specially Ni, Cu and Zn; similar results were obtained by other authors [24,29], suggesting the use of closed digestion methods.

3.5. Accuracy of open system digestions

The accuracy of the aqua regia extraction (pseudo-total digestion) and tri-acid total digestion was assessed with the analysis of the certified reference material LGC6181 which certified

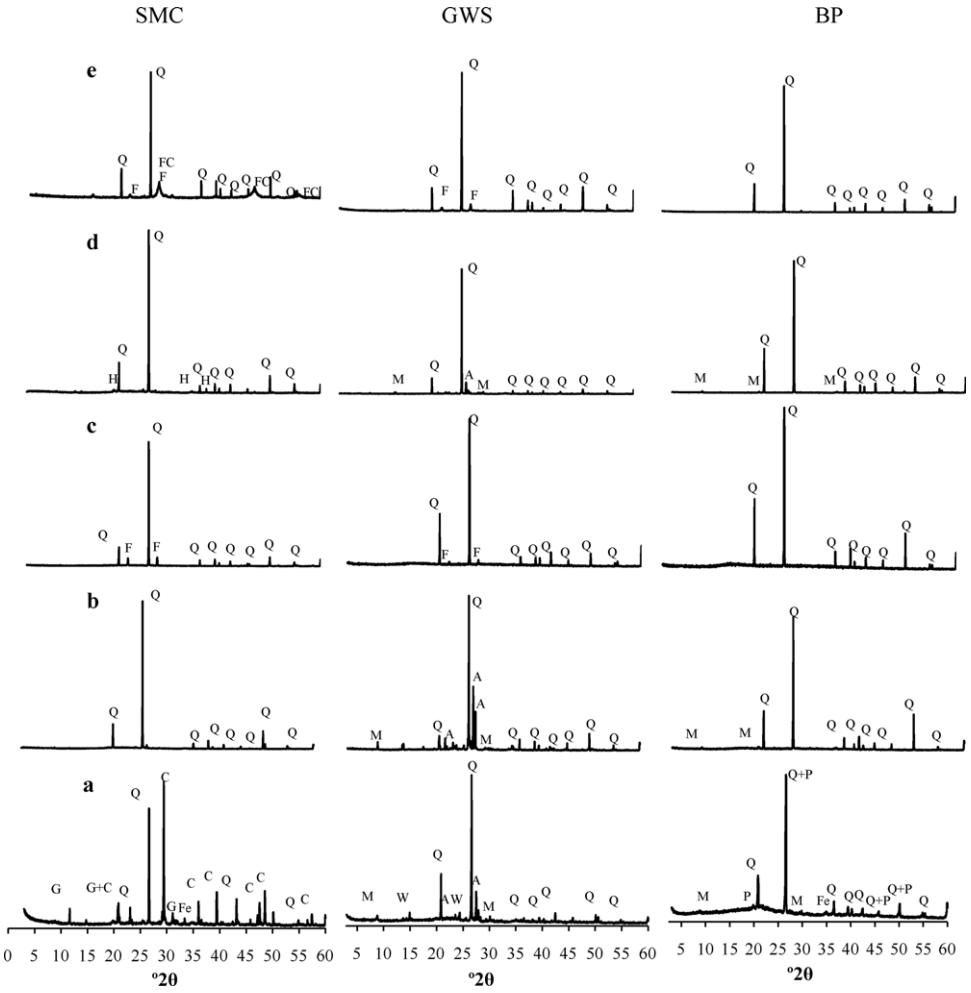

Fig. 1. X-ray diffractograms of SMC, GWS and BP (a) and their residues after four different acid mixture microwave-assisted digestion methods (b: AR; c: ARF; d: N; e: NF). A, anorthite; Al, aluminum hydroxides; C, calcite; CF, CaF_2; D, dolomite; F, $AlCaF_5$; Fe, iron oxides; G, gypsum; H, hallosite; I, illite; K, kaolinite; M, mica; P, $AlPO_4$; Q, quartz; W, whewelite.

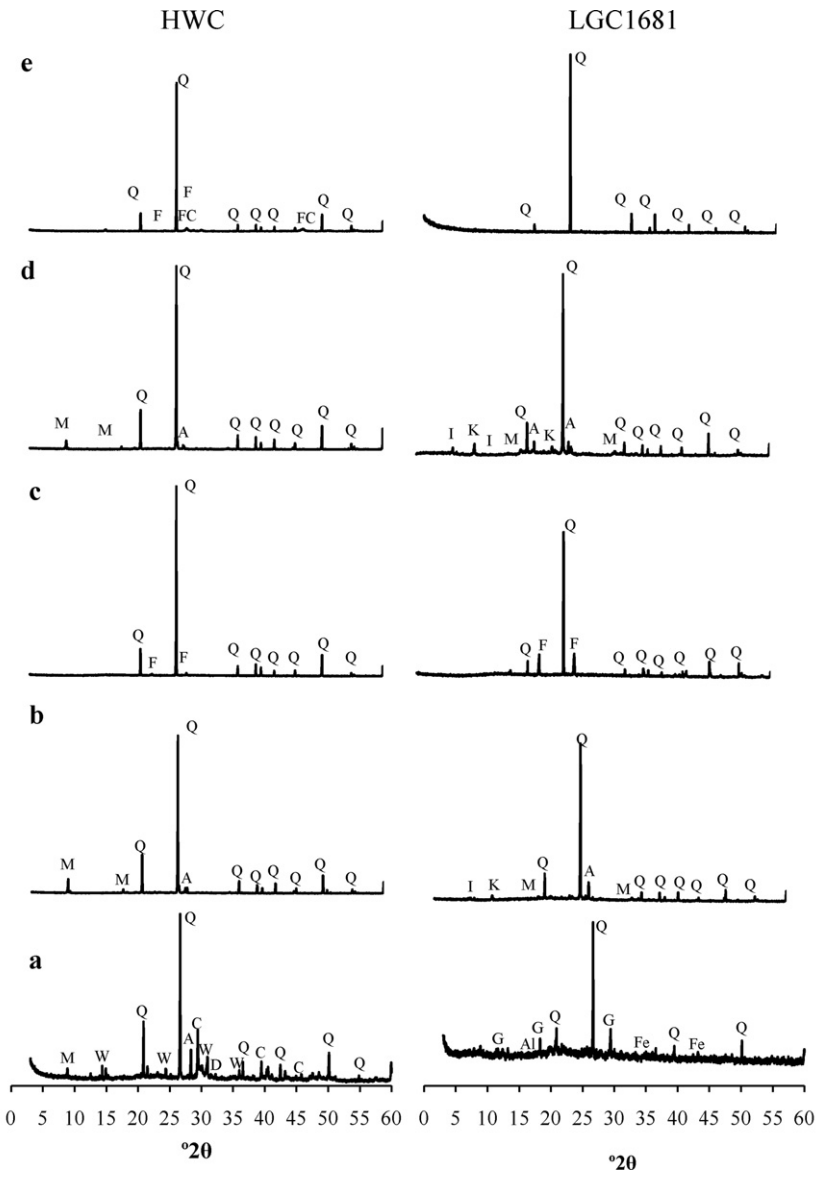

Fig. 1. (*Continued*).

values corresponding to the extractable metal contents (Table 4). The recovery of each metal was calculated based on the mean value of CRM LGC6181 [(measured concentration (mg kg^{-1})/mean certified value (mg kg^{-1})) × 100]. The accuracy was better for the aqua regia extraction (recovery ranged from 97% to 107%) compared to total digestion (recovery ranged from 39% to 101%). For both methods precision was very satisfactory, with %RSD < 5%. According to the ERM [30] aqua regia extraction using protocol ISO 11466 produces results that agree perfectly with certified values. However, HF–HNO_3–$HClO_4$ total digestion results only agreed for Cr and Mn.

Table 3
Multi-elemental analysis of four organic amendments using aqua regia extraction (ISO 11466) and HF–HNO_3–$HClO_4$ total digestion. Results are expressed in mg kg^{-1}. Letters indicate significant differences between digestion methods for each material and metal. RSD is given in brackets; $n = 3$.

	SMC		GWS		BP		HWC	
	Aqua Regia	HF–HNO_3–$HClO_4$	Aqua Regia	HF–HNO_3–$HClO_4$	Aqua Regia	HF–HNO_3–$HClO_4$	Aqua Regia	HF–HNO_3–$HClO_4$
Cr	83.0 (5)	86.6 (1)	355 (5)	383 (2)	99.8^a (1)	87.0^b (0.1)	322 (0.1)	324 (3)
Mn	318^a (6)	268^b (1)	237 (2)	225 (3)	22.2^a (2)	19.0^b (7)	372^a (1)	319^b (4)
Fe	8848^b (6)	11496^a (12)	8014^b (4)	9737^a (3)	2493^b (2)	3507^a (1)	9098^b (1)	11795^a (4)
Ni	36.5^a (6)	28.4^b (2)	130^a (4)	104^b (3)	29.5 (1)	28.3 (2)	122^a (2)	83.0^b (4)
Cu	30.1^a (7)	20.5^b (1)	94.4^a (4)	79.0^b (10)	3.96 (6)	3.55 (12)	153^a (2)	100^b (3)
Zn	139^a (6)	79.0^b (1)	120^a (5)	80.7^b (3)	45.5^a (23)	9.26^b (4)	248^a (2)	123^b (4)
Cd	0.144^b (5)	0.162^a (1)	0.273 (2)	0.263 (4)	0.147 (1)	0.140 (1)	0.453^a (7)	0.397^b (3)
Pb	10.1^a (3)	5.42^b (6)	20.2 (4)	21.4 (4)	6.13 (4)	6.11 (14)	21.0 (2)	20.5 (4)

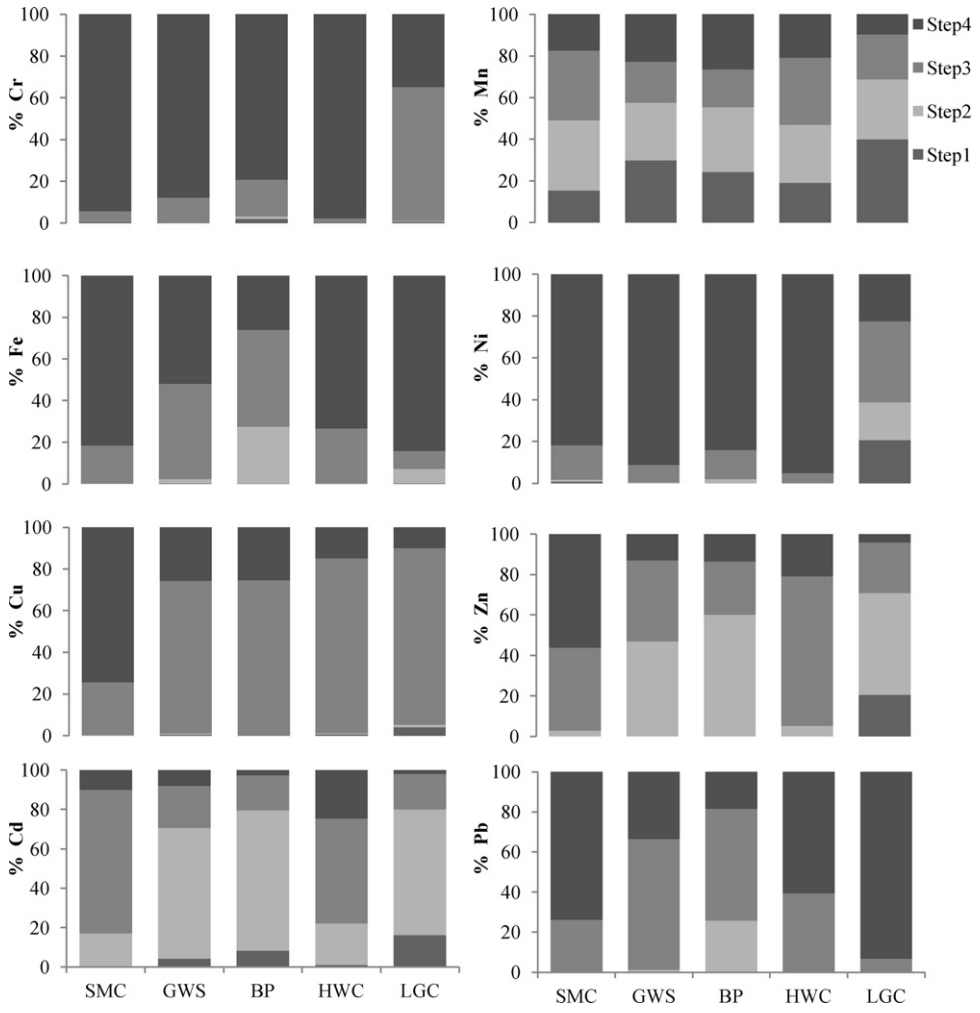

Fig. 2. Distribution of fractions for BCR sequential extraction of Cr, Mn, Fe, Ni, Cu, Zn, Cd and Pb of organic amendments. Step 1 is exchangeable, water and acid soluble fraction, Step 2 is reducible fraction, Step 3 is oxidizable fraction and Step 4 is residual fraction. Results are mean percentages for $n = 3$.

Due to these results, only the aqua regia extraction using ISO 11466 is considered adequate for multi-elemental analysis for this kind of organic amendments.

For further comparisons, concentrations of trace elements of amendments obtained with aqua regia extraction using ISO11466 are considered as 100% recovery.

3.6. Quality control of microwave-assisted closed vessel digestions

The quality control of the microwave-assisted acid digestions was performed by comparison with the LGC6181 (Fig. 3). Reproducibility of four acid mixtures was very good with %RSD in most cases lower than 5%. However, recovery for the microwave-assisted AR procedure was lower than aqua regia extraction (ISO 11466) for all the metals with respect to certified values. Additionally, microwave-assisted N digestion led to similar results of those for LGC6181 with the exception of Zn. Nitric acid microwave-assisted digestion has been considered an alternative for aqua regia extraction for samples with high organic matter content [13].

Total digestion procedures using HF (ARF and NF) showed higher concentrations than AR and N for all trace elements and major recoveries up to100% for Cr, Mn, Fe and Pb, between 111 and 123% (Fig. 3). These results could be attributed to more complete

Table 4
Multi-elemental analysis of Certified Reference Material LGC6181 using aqua regia extraction (ISO 11466) and HF–HNO_3–$HClO_4$ total digestion. Results are expressed as recovery percentage (Rec) of certified values and relative standard deviation (RSD). Letters indicate significant differences between digestion methods for each metal; $n = 3$.

	LGC6181	Aqua regia		HF–HNO_3–$HClO_4$	
	Certified (mg kg^{-1})	Rec (%)	RSD (%)	Rec (%)	RSD (%)
Cr	78 ± 8	105[a]	0.4	96[b]	1
Mn	454 ± 23	102	2	101	3
Fe	40300 ± 2300	100[a]	1	88[b]	3
Ni	45 ± 3	107[a]	2	81[b]	1
Cu	354 ± 18	100[a]	2	71[b]	2
Zn	1100 ± 50	102[a]	2	92[b]	4
Cd	5.8 ± 0.3	103[a]	2	78[b]	2
Pb	105 ± 8	97[a]	3	39[b]	10

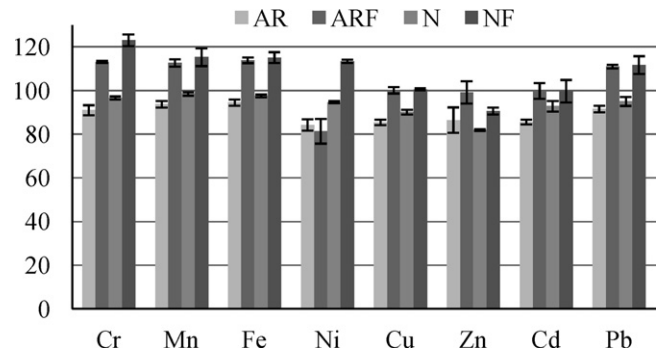

Fig. 3. Trace elements microwave-assisted acid digestion recoveries (%) of four acid mixtures (AR, ARF, N, NF) obtained from LGC1681. Bars indicate standard deviation; $n = 3$.

digestion of silicate components present in the sewage sludge LGC6181. These higher recoveries meant that ISO 11466 (extractable aqua regia metals) did not completely digest the test material, so the acid mixtures ARF and NF were more effective in sewage sludge matrix digestion and therefore in metal extraction strongly associated with mineral matter.

Limits of quantitation (LOQ) of microwave-assisted acid digestions (Table 5) were significantly lower than those obtained in the literature for determining and monitoring trace elements of organic amendments [31]. Especially remarkable were the low LOQ values for Cd (0.002–0.006 mg kg^{-1}). The use of HF in general increased LOQ, only Cd in ARF mixture and Ni and Zn in NF mixture got LOQ lower. For pseudo-total digestions, AR mixture produced LOQ major than N except Cu and Zn. For total digestion ARF mixture produced major LOQ except Mn. As general pattern, the mixture of acids produced an increase of LOQ values.

3.7. *Effects of microwave-assisted acid mixture digestions on the inorganic matrix of amendments*

Visual observation of the solid residues remaining following the four microwave-assisted acid digestions showed remarkable differences. HF acid mixture digestion on amendments with high $CaCO_3$ contents (SMC, HWC) created a white precipitate, which was more abundant in SMC. Samples characterized by the absence of $CaCO_3$ content (BP, GWS, LGC6181) digested with HF acid mixtures showed a minor quantity of solid residue. In these amendments, the acid mixture NF looked to be most effective because very little residue remained after microwave-assisted digestion.

X-ray diffractograms of organic amendments and their residues after the four microwave-assisted acid mixtures digestions are shown in Fig. 1. In general, similar patterns were observed in the mineralogical composition of the samples after the digestion procedures. Interestingly, all residues after all microwave-assisted acid digestions showed residual quartz. In addition, calcite, dolomite, gypsum, whewellite, Al hydroxides and Fe oxides were attacked by the four acid mixtures assayed and not detected in the residues. Acid mixtures, ARF and NF, completely digested silicate compounds, such as mica, ilite, kaolinite and anorthite.

Table 5
Limits of quantitation (LOQ, mg kg^{-1}) of acid mixtures after ICP-MS analysis.

	AR	ARF	N	NF
Al	2	5	1	3
Si	179	188	128	147
Cr	0.2	0.2	0.03	0.03
Mn	0.03	0.06	0.01	0.08
Fe	9	9	3	7
Ni	0.2	0.3	0.1	0.07
Cu	0.08	0.1	0.08	0.07
Zn	0.5	0.8	0.8	0.3
Cd	0.006	0.004	0.002	0.002
Pb	0.04	0.06	0.02	0.02

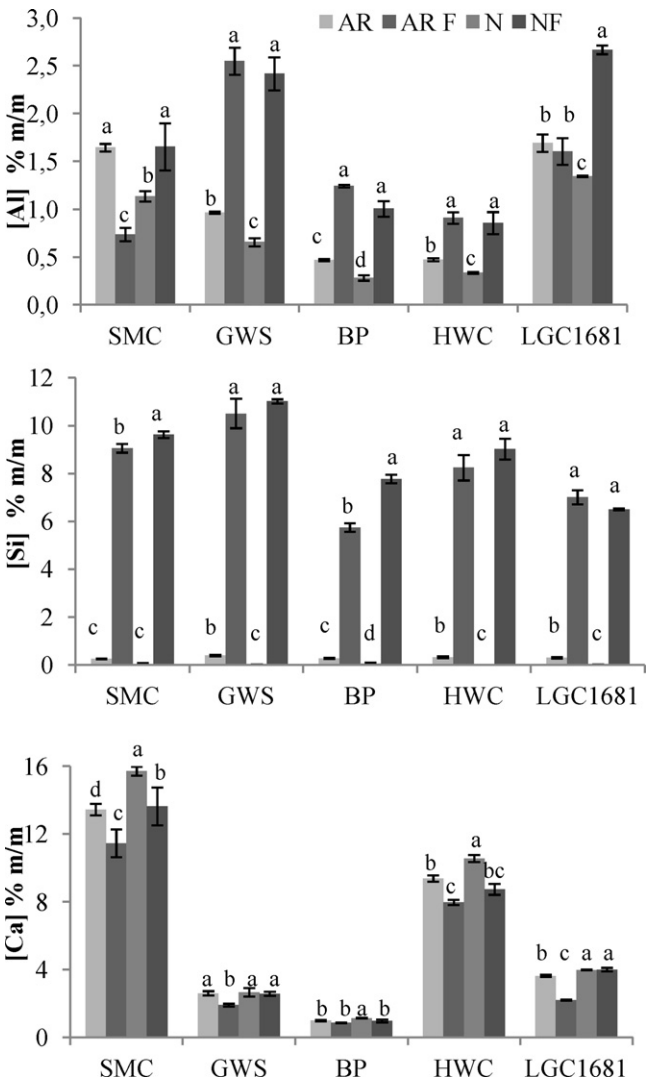

Fig. 4. Concentrations of Al, Si and Ca from four acid mixture microwave-assisted digestions (AR, ARF, N, NF). Bars indicate standard derivation ($n = 3$), and different letters indicate significant differences at $p < 0.05$.

XRD patterns of the residues, except those obtained in BP, after ARF and NF microwave-assisted digestion (Fig. 1c and e, respectively) showed three peaks at 3.15, 3.93 and 1.82 Å for $CaAlF_5$, with different intensities for each material. This result suggested the formation of calcium fluoroaluminate during both ARF and NF digestions. These reflections were more intense for SMC and LGC6181, and a significant decrease in recoveries of Al (56 and 40% respectively) and Ca (18 and 45%) employing ARF vs NF was also observed. These results suggested an important $CaAlF_5$ formation stage probably due to the high Ca and Al content of these materials (Fig. 4). However, $AlCaF_5$ was not detected for BP residues due to the low content of Ca and Al. In support of these observations, Marin et al. [18] reported Ca–F and Al–F complexes in calcareous soils after microwave-assisted HF digestion.

Additional broad peaks occurred at 1.93, 3.15 and 1.65 Å in the samples with high $CaCO_3$ content (SMC and HWC) only after NF microwave-assisted digestion. These reflections corresponded to CaF_2 and their intensities were higher in SMC than HWC, in agreement with the higher SMC Ca content (Fig. 4). The lack of CaF_2

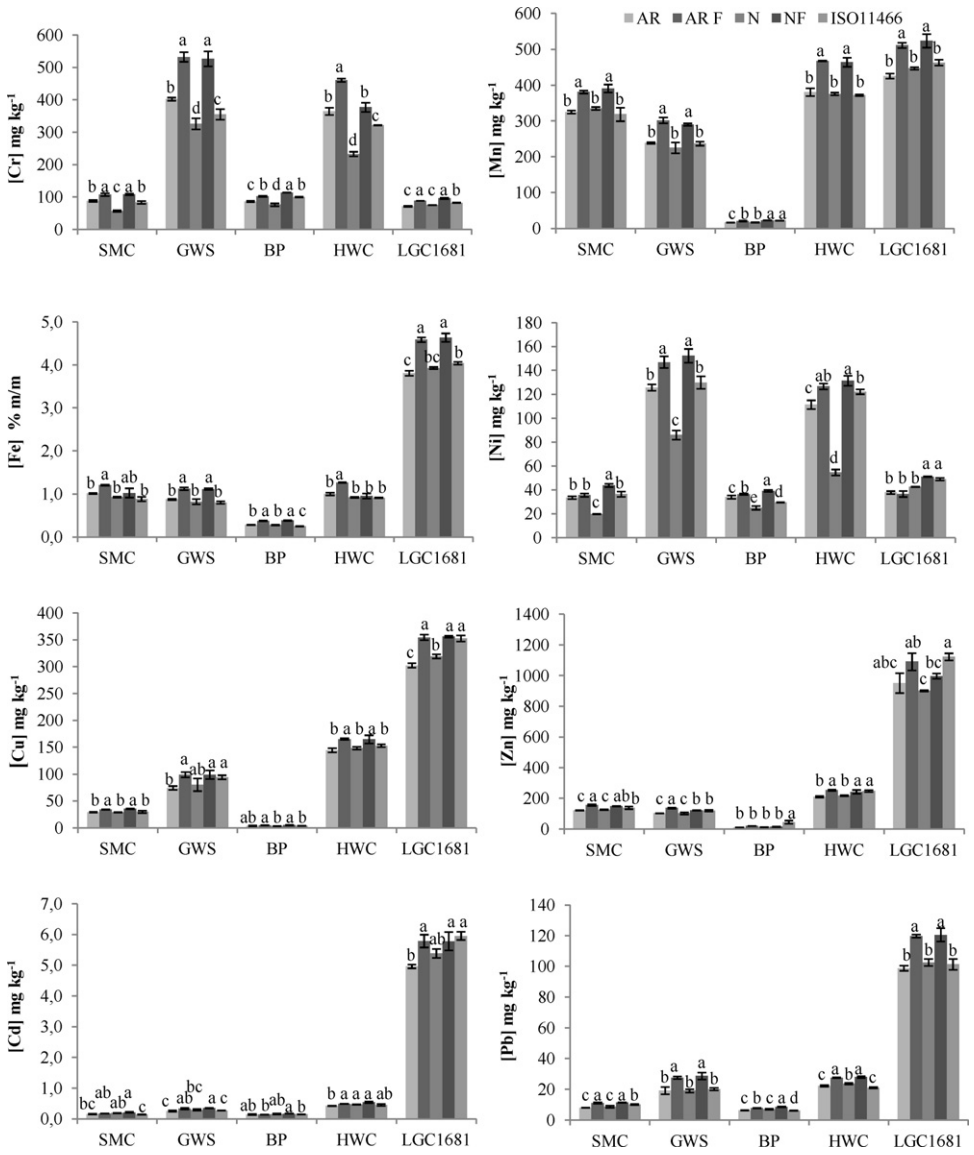

Fig. 5. Comparison of trace elements concentrations of four acid mixture microwave-assisted digestions (AR, ARF, N, NF) and ISO 11466. Bars indicate standard derivation ($n = 3$), and different letters indicate significant differences at $p < 0.05$.

in the solid residue after ARF digestion could suggest inhibition of formation by HCl. This hypothesis was confirmed by chemical speciation models performed in Visual Minteq 3.0. This model revealed that $CaCl^{+}_{(aq)}$ was the predominant Ca species under ARF digestion conditions.

Decomposition of aluminosilicates in each microwave-assisted acid mixture digestion was determined through the aluminum and silicon concentrations (Fig. 4). As can be seen, microwave ARF and NF digestions liberated higher concentrations of Al and Si than AR and N. Similar results were obtained by Sandroni and Smith [14] for Al in sewage sludge when employing HF. However, aluminum concentrations of SMC decreased with ARF and did not increase with NF, while Al concentrations of LGC6181 with ARF did not increase due to the formation of $CaAlF_5$. Furthermore, microwave-assisted AR digestion liberated higher Si and Al concentrations than microwave-assisted N digestion, because hot aqua regia is capable of dissolving tri-octahedral clays, primary and secondary salts and hydroxy oxides of amorphous aluminosilicates [32]. Silicon

Table 6
Linear correlation coefficients (r) between trace elements content of the residual fraction of BCR sequential extraction and metal concentration increase when HF was employed ($n = 15$).

	Cr	Mn	Fe	Ni	Cu	Zn	Cd	Pb
ARF-AR[a]	0.935***	0.827***	0.954***	0.917***	0.816***	0.529*	0.629*	0.916***
NF-N[b]	0.911***	0.850***	0.844***	0.974***	0.749***	0.565*	0.638*	0.798***

[a] Increase of metal concentration using acid mixtures ARF vs AR.
[b] Increase of metal concentration using acid mixtures NF vs N.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

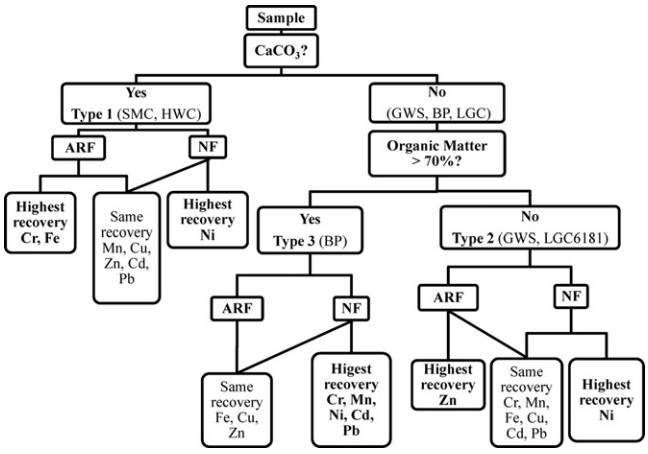

Fig. 6. Decision flowchart for the selection of a total digestion method for organic amendments according to chemical characteristics.

concentrations obtained with HNO_3 microwave extraction digestions were smaller than the quantitation limit except for black peat.

3.8. Comparison of microwave-assisted digestion of organic amendments

Microwave acid mixture digestion procedures were compared to each other and with the ISO11466 method that was previously checked in Section 3.5. Results are shown in Fig. 5.

Microwave-assisted AR and N digestions liberated similar concentrations of trace elements to ISO11466, except for Cr and Ni, which were extracted mainly in the residual fraction during the BCR procedure. For these metals, microwave-assisted AR digestion released significantly larger amounts than microwave-assisted N digestion. Florian et al. [7] reported similar behavior for microwave nitric acid and HCl:HNO_3 (3:1) digestions with respect to Ni and Cr.

Acid mixtures ARF and NF (with HF) more efficiently attacked aluminosilicates of samples, as is shown by the lack of aluminosilicate compounds in the XRD of residues after microwave-assisted HF digestions (Fig. 1) and higher Si recoveries (Fig. 4). Therefore, microwave-assisted HF digestions were more efficient and obtained higher trace elements concentrations than AR, N and ISO11466.

The increase in recoveries obtained when HF was used had a positive correlation with trace elements extracted in the fourth step of BCR (Table 6). This correlation indicated that metals extracted with HF were absorbed into aluminosilicate compounds, while AR and N were not able to extract them. Consequently, HF must be included for total acid digestion of organic amendments with high inorganic fractions. However, underestimation of certain element concentrations has been reported due to their trapping in Ca–F and Al–F complexes in calcareous soils after microwave-assisted HF digestion [18]. For comparison, our results showed a significant decrease in Fe concentrations when CaF_2 was formed after NF microwave-assisted digestion of amendments (SMC and HWC) rich in carbonates. In addition, Zn showed lower recoveries in GWS and LGC6181 with NF than ARF. However, ARF microwave digestions had lower Ni recoveries for all materials tested. These results suggested a co-precipitation phenomenon for CaF_2 and $CaAlF_5$ detected by XRD analysis.

Fig. 6 summarizes the results obtained from the different microwave-assisted digestion procedures. This flowchart is proposed as a procedure decision tree for organic amendment digestion for trace elements analysis.

In the literature, there are few reference materials other than sewage sludge to be consulted for the validation of digestion methods for compost or organic amendments. The different behavior of organic amendments shown in this paper indicates that Certified Reference Materials other than sewage sludge are necessary for future method development.

4. Conclusion

Microwave-assisted HF acid mixtures were more effective in attacking the aluminosilicate fraction and increased trace elements and iron recoveries. Additionally, this study demonstrated the formation of precipitates in the residues of organic amendments rich in calcium minerals after microwave-assisted digestion with HF mixtures (i.e., $CaAlF_5$ formation after microwave ARF and NF digestion, and CaF_2 formation only after microwave NF digestion).

As was noted in the text, Al, Fe, Zn and Ni could be underestimated by microwave-assisted HF acid mixture digestion in amendments with high Ca content as a consequence of their trapping in calcium fluoroaluminates or calcium fluoride precipitates.

Based on the analytical results and the required time of extraction, microwave-assisted digestion with a mixture of 11 mL of HNO_3 (69%) and 1 mL of HF (48%) is recommended for complete digestion of organic amendments, while a mixture of 9 mL of HCl (37%) and 3 mL of HNO_3 (69%) is proposed for pseudo-total digestion.

Acknowledgements

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Methodology for Polycyclic Aromatic Hydrocarbons Extraction from Either Fresh or Dry Spent Mushroom Compost and Quantification by High-Performance Liquid Chromatography–Photodiode Array Detection

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Polycyclic aromatic hydrocarbons (PAH) are a family of compounds classified as persistent organic pollutants, which are hazardous for environmental and human health. White rot fungi are organisms that are able to remediate PAH from polluted soils. Spent mushroom compost (SMC) is employed for soil bioremediation and environmental research. In this study, four solvents [acetone/dichloromethane (CH_2Cl_2) 1:1 mixture; acetone/hexane 1:1 mixture; methanol, and acetone], which are among those already used for PAH solvents, were chosen to be combined with two extraction procedures (ultrasound and orbital shaking). All extracted PAH were quantified by high-performance liquid chromatography (HPLC)–photodiode array detection (PDA). Certified soil CRM141, containing 16 PAH included in the U.S. Environmental Protection Agency priority list, was used for methodology validation. The orbital shaking procedure was selected because all detected and quantified PAH were within the CRM141 prediction interval, and there was less variability for all checked solvents than in the ultrasonic procedure. Once the orbital shaking methodology was selected, fluorene, phenanthrene, anthracene, and pyrene standards were added to fresh (60% moisture content) and dry SMC. The orbital shaking procedure was carried out over 24 h to avoid PAH degradation, and 1 month later to provide PAH interaction with solid matrix in this period of time. The PAH orbital shaking extraction over dry SMC showed large recovery percentages for all tested solvents. The PAH extracted from fresh SMC showed biodegradation after 24 h. Acetone/CH_2Cl_2 (1:1), methanol, and acetone solvents employed over fresh SMC recovered less than solvents over dry SMC. Acetone/hexane (1:1) recovered more than 80% and did not show any decrease in recovery over fresh SMC. Results indicate that it is not necessary to include a drying step prior to PAH extraction if the acetone/hexane (1:1) solvent mixture is employed. The recommended methodology for PAH extraction includes orbital shaking of fresh compost with acetone/hexane (1:1) solvent mixture and quantification by HPLC-PDA.

Keywords Orbital shaking, PAH, persistent organic pollutants, soil pollution, ultrasonic extraction

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Introduction

Polycyclic aromatic hydrocarbons (PAH) are a family of aromatic hydrocarbons with two or more condensed benzene rings formed during incomplete combustions. The PAH are persistent organic pollutants with toxic, mutagenic, and carcinogenic properties (IARC 2010) and consequently their presence in soil is hazardous for the environment and human health. These compounds are deposited on soils because of their low vapor pressure and water solubility. Today, soil bioremediation techniques, such as white rot fungi application, are being researched. These organisms are able to biodegrade PAH because of unspecific enzymatic activities such as laccase, peroxidase, manganese peroxidase, and lignin peroxidase (Gramss et al. 1999). One of the ways in which the fungus is supplied to the soil is through spent mushroom compost (SMC) (Reid, Fermor, and Semple 2002), so it is necessary to validate analytical procedures for detection and quantification of PAH extracted from SMC.

The PAH extraction from their matrix is a critical step of the analytical sequence. The PAH extraction by Soxhlet is the method most commonly used for soils, sediments, and sewage sludge, but because of high solvent volumes and time consumption other methods have been developed such as ultrasound (Santos, Aparicio, and Alonso 2007; Sun 1998), shaking (Song et al. 2002), and microwave-assisted extraction (MAE) (Camel 2000; Villar et al. 2004). Pressurized-liquid extraction (PLE) (Zuloaga et al. 2000) and supercritical fluid extraction (SFE) (Miège, Dugay, and Hennion 2003) can be used with good results although substantial outlay for equipment is required. Ultrasonic and shaking extraction methods are good alternative techniques that require moderate amounts of both time and solvent volumes (Song et al. 2002).

Sample moisture is a controversial point of PAH extraction. Some authors have reported either PAH losses or PAH extraction improvement depending on the sample moisture content, technique, and solvent (Song et al. 2002; Shu and Lai 2001).

The aims of this work were (a) to validate an extraction analytical procedure for quantification of PAH from soil and (b) to select a solvent for PAH extraction from fresh and dry spent mushroom compost (SMC).

Materials and methods

Chemicals and Reagents

Acetonitrile, acetone, and hexane (alkane mixture), high-performance liquid chromatography (HPLC) grade, were obtained from Panreac (Barcelona, Spain). Dichloromethane Chromasolv (HPLC) was obtained from Sigma-Aldrich (France). Ultrapure water was obtained from a Milli-Q water system (Millipore Corporation, France). Standard solutions mixture of 16 U.S. Environmental Protection Agency PAH [naphthalene (Naph), acenaphthylene (Acy), acenaphthene (Ace), fluorene (Flu), phenanthrene (Phe), anthracene (Ant), fluoranthene (Fla), pyrene (Py), benzo[a]anthracene (BaA), chrysene (Ch), benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF), benzo[a]pyrene (BaP), dibenzo[a,h]anthracene (DBahA), and benzo[g,h,i]perylene (BghiP)], at a range of concentration 100–2000 $\mu g\ mL^{-1}$ in methanol/ methylene chloride (1:1) were provided by Supelco (Bellefonte, USA). Standard solutions for single Flu at 5000 $\mu g\ mL^{-1}$ in methanol, Phe at 5000 $\mu g\ mL^{-1}$ in methanol, Ant 1000 $\mu g\ mL^{-1}$ in acetone, and Py at 1000 $\mu g\ mL^{-1}$ in methanol were also provided by Supelco. Appropriate dilutions of the standards with acetonitrile or acetone were made to HLPC standards or PAH spiked in SMC, respectively.

Apparatus

An ultrasonic cleaning bath, Selecta (Barcelona, Spain), with a 50–60 KHz operating frequency was used for soil PAH extraction by the ultrasonic method. An orbital shaker, Selecta, was used for PAH extraction from both soil and SMC by mechanical shaker. The HPLC system was a Waters 2695 separation module coupled with a Waters 996 photodiode array detector (PDA).

Soil and Spent Mushroom Compost Samples

Certified reference material (loamy-clay soil CRM141) fortified with 16 PAH was used for analytical procedure validation. Limits of detection (LD) and quantification (LQ) of soil samples were determined on an unpolluted agricultural soil (texture sandy-loam, pH 7.3, and 1.4% of organic matter) as 3 Standard Deviation (SD) and 10 SD of signal noise of extracts, respectively.

Mushroom compost was produced from wheat straw (55%), poultry litter (30%), and grape marc (15%). After composting for 21 days, a soil with high calcium carbonate content was added up to 30% of final mixture. After being used to grow *Agaricus bisporus* for 100 days, the compost was labelled as SMC. Dry SMC was obtained after drying at room temperature and grinding an aliquot. Moisture content of fresh SMC was 60% (w/w). One ml of acetone, Flu, Phe, Ant, and Py standard solutions at 125 $\mu g\ mL^{-1}$ were added on 5 g of both fresh and dry SMC to achieve 25 $\mu g\ g^{-1}$. Spiked dry SMC was aged in the dark at room temperature for 30 days. Fresh SMC was extracted 24 h after PAH solution was applied to permit acetone evaporation and to minimize PAH biodegradation.

The PAH Extraction Procedures in Soil CRM141

Two ultrasonic and orbital shaking extraction methods were tested with four solvents, acetone/dichloromethane (1:1), acetone/hexane (1:1), methanol, and acetone. Three replicates per tested solvent were performed.

Ultrasonic extraction procedure was as follows: 0.5 g of sample plus 10 mL of each tested solvent were immersed in an ultrasonic bath for 30 min with occasional manual shaking to avoid sample caking.

Orbital shaking extraction procedure was as follows: 5 g of sample plus 25 mL of each tested solvent were shaken for 2 h at 200 rpm. The solution was left for 30 min before decanting.

After the extraction step, solutions were filtered through nylon syringe filters with a 0.45-μm pore size (Whatman International, Maidstone, UK). One mL of each resultant solution was dried by N_2 flow. Residue was redissolved in 1 mL of acetonitrile solvent. The PAH detection and quantification from the resultant extract were performed by HPLC-PDA.

Effect of Evaporation of Extracts

Solvent change of extracts was necessary because all solvents were not miscible with the mobile phase of HPLC or appropriate for the cartridge column used. The aim of this test was to assess the effect of drying acetone/hexane (1:1) extracts with N_2 flow on PAH analysis. One mL of diluted standard solution of each of the 16 PAH (10 $\mu g\ mL^{-1}$ of Acy; 5 $\mu g\ mL^{-1}$ of Naph and Ace; 1 $\mu g\ mL^{-1}$ of Flu, Fla, BbF, DBahA, and BghiP; and

0.5 μg mL^{-1} of Phe, Ant, Py, BaA, Chr, BkF, BaP, and IcdP) in acetone/hexane (1:1) was evaporated under N_2 flow to dryness (approximately 10 min). Residue was redissolved in 1 mL of acetonitrile. The PAH detection and quantification from resultant extract were performed by HPLC-PDA. Results were compared with the same 16 PAH diluted standard solutions in acetonitrile without the drying step. Three replicates for each one were performed.

The PAH Extraction from Dry and Fresh Spent Mushroom Compost

According to the results obtained from soil PAH extraction procedures, orbital shaking extraction was chosen as the extraction method for PAH extraction from both dry and fresh SMC. The previously described four solvents were tested as well to assess the moisture content effect on the PAH extraction effectiveness. Dry and fresh SMC without any added PAH was processed in parallel, considered as blank solutions, so both LD and LQ were determined from them.

Liquid Chromatography Conditions

The analytical cartridge column was a Supelcosil LC-PAH, 250 × 3 mm, 5 μm. The cartridge column was protected by a Supelguard LC-18 20 × 3 mm guard column. The mobile phase was an acetonitrile–water gradient at a flow rate of 0.5 mL min^{-1}. The gradient elution program was 0–5 min: 60% acetonitrile and 40% water, then a linear gradient elution from 60% acetonitrile at 5 min to 100% acetonitrile at 15 min followed by isocratic elution for 20 min (Santos, Aparicio, and Alonso 2007). The column run temperature was fixed at 28 °C. Sample injection volume was 20 μL. The chromatograms were monitored at 254 nm and processed by Empower software (Waters, Milford, Mass., USA). Each PAH was identified by both time retention and ultraviolet (UV) spectrum by comparison with standards.

Statistical Analysis

Statistical analyses were performed with SPSS statistical software (version 19.0; SPSS Inc., Chicago, Ill.). Data were statistically evaluated by one-way analysis of variance (ANOVA). Levene's variance homogeneity test was performed before ANOVA, and Duncan or Games–Howell post hoc tests at $P \leq 0.05$ were used, as parametric and nonparametric appropriate tests, respectively.

Means comparison was used to find differences when recovery rates from extraction methods for each solvent were compared.

Nonparametric Kruskal–Wallis test was applied to compare the number of PAH validated against both solvent and extraction method.

Results and discussion

Validation of PAH Extraction Methods

Recovery rates for all combinations of two extraction procedures, ultrasonic and orbital shaking, and four solvents, acetone/dichloromethane (1:1), acetone/hexane (1:1), methanol, and acetone, each tested on CRM 141 certified material for the 16 PAH, are shown in Table 1. The Naph and Acy might be not detected because they

Table 1
Soil CRM 141 certified values and average recoveries (%) of acetone/dichloromethane, acetone/hexane, methanol, and acetone with ultrasonic and orbital shaking extraction (RSD is in parentheses)

	CRM 141	Acetone/dichloromethane		Acetone/hexane		Methanol		Acetone	
Compound	($\mu g\ kg^{-1}$)	Ultrasonic	Shaking	Ultrasonic	Shaking	Ultrasonic	Shaking	Ultrasonic	Shaking
Naph	188 ± 40.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Acy	176 ± 45.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ace	693 ± 174	106[a] (21)	91[a] (27)	297 (22)	51[a] (84)	70[a] (87)	157 (17)	117[a] (20)	37 (140)
Flu	338 ± 111	96[a] (10)	99[a] (11)	86 [a](25)	68[a] (53)	71[a] (31)	136 (2)	88[a] (26)	69[a] (33)
Phe	719 ± 221	116[a] (9)	120[a] (3)	107[a] (11)	108[a] (9)	109[a] (4)	134 (3)	117[a] (14)	108[a] (7)
Ant	393 ± 130	63 (27)	100[a] (2)	69[a] (11)	91[a] (9)	66 (4)	105[a] (1)	72[a] (17)	75[a] (11)
Fla	176 ± 40.3	168 (24)	109[a] (12)	78[a] (9)	103[a] (9)	101[a] (22)	123 (3)	117[a] (20)	83[a] (8)
Py	331 ± 62.0	112[a] (14)	110[a] (12)	93[a] (7)	101[a] (14)	82 (6)	107[a] (29)	96[a] (25)	93[a] (5)
BaA	409 ± 83.0	103[a] (10)	116[a] (3)	106[a] (4)	118[a] (16)	107[a] (8)	116[a] (3)	110[a] (13)	111[a] (3)
Chr	316 ± 52.0	133 (11)	129 (4)	136 (7)	128[a] (14)	123 (4)	129 (0)	134 (16)	122 (2)
BbF	364 ± 48.6	89[a] (4)	117 (2)	88[a] (10)	117[a] (17)	79 (18)	113[a] (5)	89[a] (16)	112[a] (4)
BkF	253 ± 43.9	94[a] (22)	112[a] (5)	103[a] (5)	113[a] (16)	97[a] (9)	106[a] (1)	110[a] (12)	108[a] (5)
BaP	198 ± 25.8	33 (87)	102[a] (11)	71 (4)	102[a] (14)	51 (15)	85 (9)	55 (16)	82 (10)
DBhaA	451 ± 70.4	97[a] (8)	113[a] (7)	111[a] (6)	116[a] (17)	81[a] (17)	94[a] (5)	110[a] (11)	111[a] (6)
BghiP	618 ± 109	82[a] (37)	109[a] (6)	96[a] (6)	110[a] (19)	79 (14)	88[a] (1)	101[a] (10)	108[a] (2)
IcdP	394 ± 52.0	100[a] (13)	115 (4)	105[a] (5)	115[a] (16)	80 (15)	91[a] (5)	107[a] (10)	112[a] (3)
Meanrecovery		99ns	110ns	110ns	103ns	85b	113a	102ns	95ns
Mean RSD		16a	8b	9ns	22ns	18a	4b	16a	8b

Notes. n.d. indicates no detection of the compound in HPLC analysis; ns indicates no significant differences. Different letters indicate significant differences at $P \leq 0.05$; $n = 3$.

[a]Recovery validated according IRMM (2010) criteria.

were below detection limit (DL) for any solvent and extraction method. Means of 14 PAH recovery rates and percentages of relative standard deviation (RSD) of each solvent were compared for orbital shaking and ultrasonic extraction methods. For acetone/dichloromethane, acetone/hexane, and acetone, no significant differences were found between ultrasonic and orbital shaking extraction when recovery rates were analyzed, but for methanol, orbital shaking showed greater recovery than ultrasonic extraction. The RSD of ultrasonic extraction were significant better than the RSD of orbital shaking for the acetone/dichloromethane solvent mixture, methanol, and acetone solvents.

According to the IRMM (2010) protocol for comparison of the result of a measurement with a certified value, orbital shaking extraction with acetone/hexane solvent mixture was the only method in which the 14 PAH results agreed with the certified values. Many of the individual PAH results that did not agree with the certified value were overestimations; only Ant and BaP showed systematic underestimation. However, no significant differences were found among the numbers of PAH validated for ultrasonic and orbital shaking extraction for any solvent. The same behaviour was observed when the four solvents were compared using ultrasonic extraction. However, when orbital shaking extraction was entered as a factor, significant differences were found at $P = 0.056$ between acetone/hexane (14 validated PAH) and methanol (eight validated PAH) (Table 1).

The PAH Recoveries of Standard Solutions Evaporated to Dryness

The PAH recovery percentages and RSD percentage from 16 PAH standard solutions in acetone/hexane (1:1) are shown in Table 2. Recovery rates were calculated as percentages of the values obtained without applying the drying step. Large recovery rates were found

Table 2
Recovery rates and relative standard deviations (RSD) of 16 PAH standard solutions in acetone/hexane (1:1) evaporated to dryness with N_2 flow; $n = 3$

Compound	Aromatic rings	Recovery (%)	RSD (%)
Naph	2	20	25
Acy	2	76	2
Ace	2	77	7
Flu	2	88	4
Phe	3	96	3
Ant	3	98	3
Fla	3	102	3
Py	4	102	4
BaA	4	103	2
Chr	4	103	2
BbF	4	103	3
BkF	4	103	3
BaP	5	102	3
DBhaA	5	103	2
IcdP	5	103	3
BghiP	6	103	2

for PAH with more than two aromatic rings. Among the PAH with two aromatic rings, satisfactory recovery rates for Acy (76%), Ace (77%), and Flu (88%) were found (Miège, Dugay, and Hennion 2003) and significant losses were found when the dryness step was applied only for the most volatile PAH, Naph.

Regarding the RSD values in general, low RSD were obtained for all tested PAH. As expected, Naph presented the largest RSD (25%), surely correlated with great losses. No more than 7% RSD was found for the other 15 PAH. This behavior is in accordance with characteristic PAH vapor pressure values, which decrease as the number of aromatic rings increase (WGPAH 2001). According to these results, if N_2 flow is passed for 10 min to take the solution to dryness, 15 of 16 PAH may be properly detected and quantified by HPLC-PDA.

Moisture Effects of PAH Extraction from SMC

Because of the large values of PAH recoveries and/or minor RSD found for orbital shaking extraction, four solvents with orbital shaking were used to assay PAH extraction from both dry and fresh SMCs. Recovery rates for each solvent are shown in Table 3. Recovery rates of Flu, Phe, Ant, and Py from fresh SMC were significantly less than those from dry SMC when using acetone/dichloromethane, methanol, and acetone solvents. Methanol, as the most polar solvent, presented a mean 48% recovery decrease from fresh SMC. However, significantly greater recoveries of Flu and Phe were found for the acetone/hexane solvent mixture. No significant differences were found for Py. In the case of Ant, it showed a significant recovery decrease of 5%. Nevertheless, acetone/hexane solvent mixture recovery for Ant in fresh SMC (82%) was considered adequate (Santos, Aparicio, and Alonso 2007). These results indicate that for moist samples, polar solvents should be discarded because poor PAH recovery rates were achieved and the mixture with nonpolar solvent is necessary for PAH extraction yield improvement.

Chromatograms from fresh SMC spiked with Flu, Phe, Ant, and Py presented two peaks that did not appear in dry SMC spiked with the 4 PAH (Figure 1) or in fresh unspiked SMC. Possible PAH biodegradation could occur during the 24 h between spiking and extracting the PAH. By comparing PAH recoveries obtained in dry SMC and losses of Flu in the dryness step of the extraction with nitrogen flow, the degradation at 24 h was low.

Table 3

Flu, Phe, Ant, and Py average recoveries using acetone/dichloromethane, acetone/hexane, methanol, and acetone with orbital shaking extraction of fresh and dry spent mushroom compost (SMC) (RSD is shown in parentheses)

	Acetone/ dichloromethane		Acetone/hexane		Methanol		Acetone	
Compound	Fresh	Dry	Fresh	Dry	Fresh	Dry	Fresh	Dry
Flu	59 b (7)	73 a (11)	81 a (2)	63 b (3)	24 b (19)	73 a (4)	59 b (7)	72 a (3)
Phe	82ns (7)	87ns (7)	106 a (2)	91 b (3)	46 b (13)	91 a (4)	78 b (5)	87 a (1)
Ant	65 b (4)	83 a (7)	82 b (2)	87 a (3)	35 b (13)	87 a (3)	56 b (4)	83 a (1)
Py	84 b (6)	95 a (7)	108ns (2)	104ns (3)	50 b (11)	95 a (4)	79 b (4)	96 a (1)

Notes. ns, no significant differences. Letters indicate significant differences for each solvent between fresh and dry SMC at $P \leq 0.05$; $n = 4$.

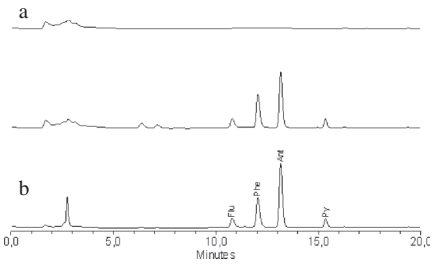

Figure 1. HPLC-PDA chromatograms at 254 nm of acetone/hexane (1:1) orbital shaking extraction of spent mushroom compost (SMC): (a) fresh SMC without PAH spiking, (b) fresh spiked SMC, and (c) dry spiked SMC. Samples were spiked with 25 mg kg^{-1} of Flu, Phe, Ant, and Py.

Limits of Detection and Quantification

The LD and LQ were determined from extraction of soil and dry and fresh SMC using orbital shaking extraction with acetone/hexane (1:1) (Table 4). The LD and LQ of soil and dry SMC were similar and presented values between 1 and 35 μg kg^{-1} DW (Table 4). The greatest LD and LQ were found in fresh SMC except for Chr. According to these results, only in mildly polluted SMC is it recommended to include the drying step.

Conclusion

The PAH extraction with acetone/hexane (1:1) solvent mixture, orbital shaking, and quantification by HPLC-PDA has been validated for 14 of the 16 PAH included in the

Table 4
Limits of detection (μg kg^{-1} DW) and quantification (μg kg^{-1} DW) of PAH in soil, fresh SMC, and dry SMC extracted with acetone/hexane (1:1) using orbital shaking; $n = 3$

	Soil		Fresh SMC		Dry SMC	
Compound	LD	LQ	LD	LQ	LD	LQ
Naph	1	3	9	29	3	9
Acy	6	20	76	252	11	35
Ace	3	10	18	62	9	31
Flu	<1	1	2	7	1	3
Phe	1	3	1	3	1	2
Ant	<1	1	<1	1	<1	1
Fla	<1	1	3	10	<1	1
Py	1	3	3	10	<1	1
BaA	<1	1	1	5	1	2
Chr	3	11	3	9	4	14
BbF	1	2	16	54	3	10
BkF	<1	1	2	7	1	3
BaP	<1	1	10	33	2	5
DBhaA	1	2	4	14	<1	1
BghiP	<1	1	11	37	1	2
IcdP	<1	1	2	6	<1	1

CRM141 reference soil. In addition, PAH extraction from fresh SMC with acetone/hexane and orbital shaking showed adequate recovery rates. Therefore, acetone/hexane (1:1), orbital shaking, and quantification by HPLC-PDA has been shown to be an adequate methodology for PAH analysis from soil and dry and fresh SMC.

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RESEARCH ARTICLE

Cadmium and lead bioavailability and their effects on polycyclic aromatic hydrocarbons biodegradation by spent mushroom substrate

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Abstract Bioremediation of mixed metal–organic soil pollution constitutes a difficult task in different ecosystems all around the world. The aims of this work are to determine the capacity of two spent mushroom substrates (*Agaricus bisporus* and *Pleurotus ostreatus*) to immobilize Cd and Pb, to assess the effect of these metals on laccase activity, and to determine the potential of spent *A. bisporus* substrate to biodegrade four polycyclic aromatic hydrocarbons (PAH): fluorene, phenanthrene, anthracene, and pyrene, when those toxic heavy metals Cd and Pb are present. According to adsorption isotherms, spent *P. ostreatus* and *A. bisporus* substrates showed a high Pb and Cd adsorption capacity. Pb and Cd interactions with crude laccase enzyme extracts from spent *P. ostreatus* and *A. bisporus* substrates showed Cd and Pb enzyme inhibition; however, laccase activity of *A. bisporus* presented lower inhibition. Spent *A. bisporus* substrate polluted with PAH and Cd or Pb was able to biodegrade PAH, although both metals decrease the biodegradation rate. Spent *A. bisporus* substrate contained a microbiological consortium able to oxidize PAH with high ionization potential. Cd and Pb were immobilized during the bioremediation process by spent *A. bisporus* substrate. Consequently, spent *A. bisporus* substrate was adequate as a multi-polluted soil bioremediator.

Keywords PAH · Bioremediation · Multi-pollution · Ligninolytic enzymes · Heavy metals · Persistent organic pollutants

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Introduction

Many industrial-polluted soils simultaneously hold inorganic and organic pollutants, such as heavy metals and polycyclic aromatic hydrocarbons (PAH) (Beesley et al. 2010; Thavamani et al. 2011). However, many soil remediation research studies are focused on organic or inorganic pollutants individually, but not as a mixture. The mixture of different kinds of pollutants complicates the soil remediation process, even more so if bioremediation is the selected remediation technique (Megharaj et al. 2011). This environmentally friendly technique includes the use of plants, bacteria, algae, or fungi as organisms and compost, as well as manure or straw as organic matter sources to immobilize heavy metals or enhance the activity of PAH-degrading organisms (Hwang and Zhao 2007; Haritash and Kaushik 2009; Tandy et al. 2009).

Over the past few years, a growing interest has been shown in assessing the bioremediation potential of fungi. One of the more studied fungi for organic pollutant biodegradation is white rot fungi. It is a group with the capacity to degrade lignin by extracellular enzymes. Ligninolytic enzymes are mainly lignin peroxidase (LiP; EC 1.11.1.14), manganese-dependent peroxidase (MnP; EC 1.11.1.13), versatile peroxidase (VP; EC 1.11.1.16), and laccase (Lac; EC 1.10.3.2) (Wong 2009). These enzymes are nonspecific and oxidize a wide variety of organic compounds. The PAH oxidation mechanism by fungal ligninolytic enzymes is similar to the degradation of non-phenolic lignin (Peng et al. 2008). The white rot fungi most studied for PAH degradation were *Pleurotus ostreatus* and *Phanerochaete chrysosporium* (Haritash and Kaushik 2009), instead of other successfully used fungi, such as *Irpex lacteus* and *Lentinus tigrinus* (Covino et al. 2010). However, the presence of heavy metals affects fungi soil penetration, consequently affecting PAH biodegradation (Baldrian et al. 2000) and playing an important

role in the regulation of extracellular enzyme activities. In this sense, Cu increases Lac induction and its stabilization, Cd increases Lac activity, and Pb and Hg decrease Lac activity in *P. ostreatus* (Baldrian and Gabriel 2002).

Agaricus bisporus is able to biodegrade organic xenobiotics present in soil, such as phenolic compounds (Trejo-Hernández et al. 2001) and PAH (Li et al. 2010; Reid et al. 2002). Spent *A. bisporus* substrate (SAS) is a source of mycelia and ligninolytic enzymes, especially Lac (Trejo-Hernández et al. 2001). A large amount of SAS is generated; just in Europe, more than 3.5×10^6 t are produced every year. The accumulation of SAS causes important environmental pollution in mushroom production areas due to salts and soluble organic carbon leaching during SAS storage (Guo et al. 2001). Some authors have researched how to reduce the amount of SAS produced (Royse 2010) or how to reuse it (Pardo-Giménez and Pardo-González 2008) so as to minimize the adverse environmental impact of SAS.

SAS used as *A. bisporus* inoculum carrier can be useful for soil bioremediation, producing simultaneous effects, such as the immobilization of heavy metals which alter fungi development, an increase in the activities of ligninolytic enzymes, and consequently, the enhancement of PAH biodegradation.

The aims of this study were as follows: (1) to assess the Cd and Pb immobilization properties of spent *A. bisporus* substrate and spent *P. ostreatus* substrate; (2) to evaluate the effects of Cd and Pb on laccase activity of *A. bisporus* and *P. ostreatus* from crude extracts of spent *A. bisporus* substrate and spent *P. ostreatus* substrate, compared with a purified laccase enzyme from *T. versicolor*; and (3) to determine the potential of spent *A. bisporus* substrate to biodegrade PAH in the presence of Cd or Pb, and its effects on Cd and Pb bioavailability.

Materials and methods

The selection of spent substrates was made bearing in mind the most significant edible fungi cropped around the world (*P. ostreatus* and *A. bisporus*) and, therefore, the substrate wastes that present the highest environmental implications. *A. bisporus* substrate was produced from wheat straw (55 %), poultry litter (30 %), and grape marc (15 %). After composting for 21 days, gypsum was added. During the crop, a mixture of sphagnum blond peat and soil with high calcium carbonate content as casing was added to up to 30 % (*m*/*m*) of the final mixture (Pardo-Giménez and Pardo-González 2008). After *A. bisporus* harvest, the compost was labeled as spent *A. bisporus* substrate (SAS) (pH=6.04, %C=27, %N=1.7). The *P. ostreatus* substrate component is 100 % wheat straw (Michael et al. 2011). After the *P. ostreatus* harvest, spent substrate was labeled as spent *P. ostreatus* substrate (SPS) (pH=6.95, %C=42, %N=0.7). The SAS and SPS samples were provided by commercial farms (Cuenca, Spain) which traditionally use these substrates.

In order to describe the most important functional groups contained in the organic matter of SAS and SPS, they were subjected to cross-polarization and magic angle spinning nuclear magnetic resonance (CP-MAS ^{13}C NMR) in a Bruker AV-400-WB apparatus (Billerica, MA, USA) operating at 100.61 MHz (rotor spin rate 10 kHz, contact time 3.5 ms, acquisition time 4 s, 18,000 scans). Samples were packed in 4 mm zirconia rotors with Kel-F caps. The pulse sequence was applied with a 1-h ramp. Chemical shift (δ) regions were divided into the main resonance regions according to Tapia et al. (2010) and the characterization of organic matter shown on Table 1. The highest spectra region corresponded to the *O*-alkyl group for both SPS and SAS, attributable above all to cellulose and hemicellulose. However, SPS showed more *O*-alkyl carbon than SAS, probably because SPS was only wheat straw and SAS contains other sources of carbon such as poultry litter in its initial composition. Attending to the aliphatic/*O*-alkyl ratio that is an indicator of the evolution of organic matter (Tapia et al. 2010), SAS displayed organic matter more mature than SPS (ratio values 0.55 and 0.06, respectively). This result is consistent, considering that SAS had suffered a more intense composting process than SPS before the mushroom harvest. SAS showed a higher alkyl and carboxyl region than SPS. These higher amounts of alkyl and carboxyl groups may be related to the composting process, which probably produced fatty acids and humic acids with aliphatic structure. The high presence of carboxyl acidic groups can increase the cation exchange capacity of the material and would induce the formation of heavy metal complexes.

Table 1 Relative areas (percentage of total area) of the chemical shift (δ) regions in ^{13}C cross-polarization and magic angle spinning nuclear magnetic resonance (CP-MAS ^{13}C NMR) spectra of spent *P. ostreatus* (SPS) and *A. bisporus* (SAS) substrates

Chemical shift (ppm)	Type of C bond	SPS % total area	SAS
$0<\delta\leq45$	Alkyl (aliphatic)	4.4	21.4
$45<\delta\leq60$	*N*-alkyl	3.9	7.3
$60<\delta\leq93$	*O*-alkyl	67.8	39.2
$93<\delta\leq110$	Di-*O*-alkyl	14.6	10.0
$110<\delta\leq140$	Aromatic	1.8	4.2
$140<\delta\leq160$	Phenolic	2.7	2.8
$160<\delta\leq190$	Carboxyl	3.9	14.6
$190<\delta\leq220$	Amide-ketonic	0.9	0.5

Heavy metal adsorption isotherms in SAS and SPS

Cd and Pb adsorption isotherms were constructed according to Chen et al. (2005). SAS and SPS were air-dried, milled, and sieved at 0.2 mm. The sample (0.0100 g) was suspended in 10 ml of $Cd(NO_3)_2$ or $Pb(NO_3)_2$ solution at 0, 10, 25, 50, 75, 100, 150, and 300 mg l^{-1} adjusted to pH 6.5. After 24 h of interaction at 25 °C, suspension was centrifuged at 3,000 rpm for 15 min and the remaining supernatant was filtered through an ashless Whatman 42 filter. Interactions were made per triplicate. The solutions obtained were analyzed by a PerkinElmer AA800 atomic absorption spectrometer. Data obtained were adjusted to the Langmuir adsorption model according to the equation: $q_e = \frac{q_{max} \cdot a_L \cdot C_e}{1+(a_L \cdot C_e)}$ where q_e is the heavy metal amount adsorbed (in milligram per gram), q_{max} is the maximum amount of heavy metal adsorbed (in milligram per gram), a_L is the adsorption constant (l mg^{-1}), and C_e is the heavy metal concentration after interaction (in milligram per liter).

Extraction and quantification of ligninolytic enzyme activity

Enzymatic extraction was carried out per triplicate with a modified method by Trejo-Hernández et al. (2001): 50 g of SAS or SPS was mixed with 1 l of 0.1 M Tris–HCl buffer (pH 7.5) and 1 h shaken at 4 °C, then centrifuged at 5,000 rpm, and the supernatant was used directly as the enzyme source and was labeled as enzymatic crude extract.

Lac activity was evaluated spectrophotometrically by following the oxidation of 0.2 mM 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) in 100 mM sodium acetate buffer (pH 4.5) at 420 nm (ε= 36,000 M^{-1} cm^{-1}). MnP activity was assayed by the oxidation of 1 mM $MnSO_4$ in 50 mM sodium malonate buffer (pH 4.5), in the presence of 0.1 mM H_2O_2. Manganic ions, Mn^{3+}, form a complex with malonate, which absorbs at 270 nm (ε=11,590 M^{-1} cm^{-1}) (Wariishi et al. 1992). One unit of enzyme activity (IU) is defined as the amount of enzyme which produced 1 μmol of product per minute under assay conditions.

Heavy metals–laccase enzyme interaction

Increasing concentrations of Cd and Pb were assayed to assess the effect on Lac activity from low to very high bioavailable Cd and Pb. $Cd(NO_3)_2$ or $Pb(NO_3)_2$ were added to SAS and SPS crude enzymatic extracts, and purified laccase of *Trametes versicolor* provided from Sigma-Aldrich, as Lac model, dissolved in 0.1 M Tris–HCl buffer (pH 7.5). Final metal concentrations were 0 (control) 5, 50, and 500 μM. Interactions were incubated per triplicate at 25 °C in the dark with orbital shaking (160 rpm). Lac activity was analyzed at 1 h, 24 h, and 7 days later, according to Baldrian and Gabriel (2002).

Biodegradation of PAH by SAS in the presence of Cd and Pb

The assay was conducted per quadruplicate, in a growth chamber in the dark at 20 °C and 80 % relative air humidity. Twenty grams of fresh SAS (56 % moisture content) and fresh SAS sterilized twice in autoclave at 120 °C for 45 min as an abiotic control were incubated in 100 ml Erlenmeyer flask for 28 days. In order to assess the PAH biodegradation rate of SAS with and without Cd or Pb, the following treatments were assayed: fresh SAS without PAH, sterilized SAS with PAH, fresh SAS with PAH, fresh SAS with PAH plus 500 μmol kg^{-1} Cd, and fresh SAS with PAH plus 500 μmol kg^{-1} Pb. PAH were fluorene 98 % (Flu), phenanthrene 98 % (Phe), anthracene 97 % (Ant), and pyrene 98 % (Py) provided for Sigma-Aldrich. PAH were added as acetone HPLC-grade (Panreac) solution (1 ml) to achieve a final concentration in SAS of 100 mg kg^{-1}. Acetone was added to treatment without PAH, to equal the initial solvent content in SAS. The samples were placed 24 h in a chemical hood to acetone evaporation after acetone solution addition. Cd and Pb were added as $Cd(NO_3)_2$ $4H_2O$ and $Pb(NO_3)_2$ (Panreac) aqueous solution (2 ml). For treatments without heavy metals, $Ca(NO_3)_2$ $4H_2O$ (Panreac) was added to equal the nitrate content of SAS. Cd and Pb concentrations were selected according to results obtained in the adsorption assay and Lac–heavy metals interactions in order to get an appreciable heavy metals bioavailability and previously published works (Baldrian and Gabriel 2002). PAH concentration was based on previously published works (Márquez-Rocha et al. 2000).

To assess the quick and long-term effect of SAS in heavy metals bioavailability, ligninolytic activity, and PAH biodegradation, both soluble and bioavailable Cd and Pb content, Lac and MnP activities, and total PAH were determined, respectively, on days 0, 7, 21, and 28.

Cd and Pb availability was determined per quadruplicate by extracting with water as soluble fraction or 0.05 M EDTA-Na_2 pH 7 as bioavailable fraction in soils (Zhang et al. 2002). One gram of dry and milled SAS was extracted for 1 h with 10 ml of Milli-Q water (Millipore Corporation) or 0.05 M EDTA, respectively. Heavy metals analyses were carried out by atomic absorption spectrometry (PerkinElmer AA800).

Lac and MnP were extracted and activities determined per quadruplicate as described in the "Extraction and quantification of ligninolytic enzymatic activity" section.

PAH analysis and metabolites identification

PAH extraction and determination were carried out per quadruplicate according to García-Delgado et al. (2013). Five

grams of fresh SAS sample was extracted with 25 ml of acetone/hexane (1:1) in an orbital shaker for 120 min, and then the solution was left for 30 min for solution decanting. After the extraction step, the solutions were filtered through a nylon syringe filter with 0.45 μm pore size (Whatman International). One milliliter of each resulting solution was taken to dryness by N_2 flow. The residue was redissolved in 1 ml of acetonitrile. PAH were analyzed by a HPLC Waters 2695 Separation Module coupled with a Waters 996 photodiode array detector (PDA). The analytical cartridge column was a Supelcosil™ LC-PAH 250×3 mm, 5 μm. The cartridge column was protected by a Supelguard™ LC-18 20×3 mm guard column. The mobile phase was acetonitrile/water gradient at a flow rate of 0.5 ml min^{-1}. The gradient elution program was 0–5 min: 60 % acetonitrile and 40 % water, then a linear gradient elution from 60 % acetonitrile at 5 min to 100 % acetonitrile at 15 min followed by isocratic elution for 20 min. The column run temperature was set at 28 °C. Each PAH was identified both by time retention and by the UV spectrum by comparing with standards and was determined at 254 nm. PAH metabolites were identified according to Aranda et al. (2010).

Statistical analysis

All statistical tests were carried out using the Statistical Product and Service Solutions 15.0 software package. One-way analysis of variance after previously performing a Levene variance homogeneity test was carried out. To compare the differences between treatments, the Duncan or Games–Howell post hoc test (according to variance homogeneity) at $p<0.05$ for metal–laccase interaction and $p<0.1$ for PAH biodegradation assay was used.

Results and discussion

Spent *P. ostreatus* and *A. bisporus* substrates Cd and Pb adsorption isotherms

Results of Cd and Pb adsorption on SAS and SPS were modeled according to the Langmuir equation (Fig. 1). SAS Langmuir curve fits for Cd and Pb were good with 0.973 and 0.960 R^2 values, respectively (Fig. 1a). Maximum amounts of adsorbed heavy metals (q_{max}) were 33.9 and 19.7 mg g^{-1} for Cd and Pb, respectively.

SPS showed Langmuir curve fits for Cd and Pb with 0.947 and 0.982 R^2 values, respectively (Fig. 1b). Maximum amounts of adsorbed heavy metals (q_{max}) were 16.6 mg g^{-1} for Cd and 51.5 mg g^{-1} for Pb.

SAS presented higher Cd affinity than Pb; however, SPS showed higher Pb adsorption capacity. The different behavior towards Pb adsorption could be partially explained according

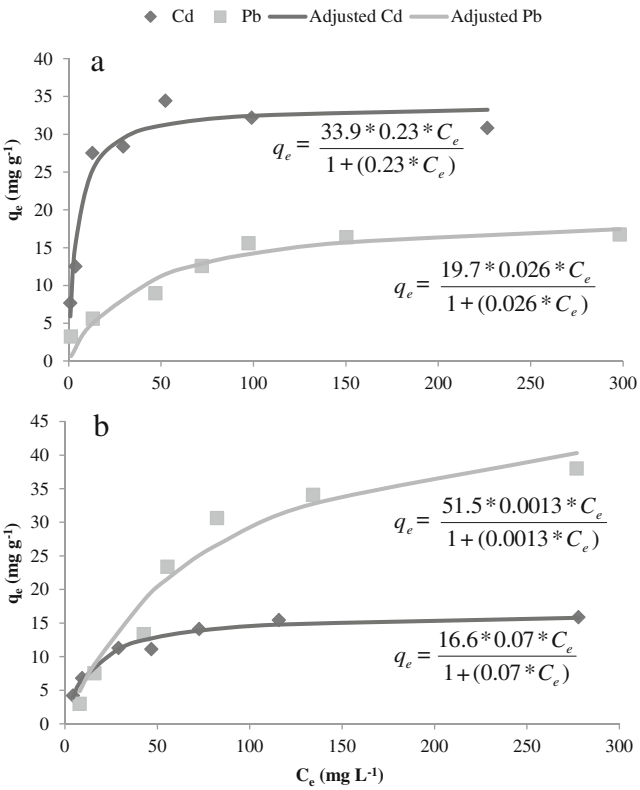

Fig. 1 Adsorption of Langmuir isotherms (25 °C) of Cd^{2+} and Pb^{2+} by spent *A. bisporus* substrate (**a**) and spent *P. ostreatus* substrate (**b**) where q_e is the heavy metal amount adsorbed (in milligram per gram) and C_e is the heavy metal concentration after interaction (in milligram per liter). $n=3$

to the CP-MAS ^{13}C NMR *O*-alkyl content of substrates (Table 1), which was higher for SPS than for SAS. Cation Pb^{2+} formed a complex with cellulose and precipitation of the hydrolysis product of the Pb^{2+} complex (Zhou et al. 2005). Additionally, the pH value of SPS (6.95) was higher than SAS (6.04) and it would help in heavy metal precipitation such as hydroxides.

The higher SAS Cd adsorption could be justified by two factors: firstly, the SAS composition that was more complex (organic and inorganic components) than SPS (only wheat straw), and that underwent the composting process that produced more stabilization of organic matter and higher carboxylic acids amount according to CP-MAS ^{13}C NMR analysis. The second reason is that SAS contained inorganic compounds such as clays, Fe and Mn oxides, $CaCO_3$, and $CaSO_4$ (Tapia et al. 2010, García-Delgado et al. 2012) which may be responsible for heavy metal adsorption or precipitation phenomena.

Throughout the bioremediation process, cellulose, hemicellulose, and lignin components of SPS and SAS will be gradually degraded and then adsorption properties of heavy metals will be altered. However, SAS is a more stable material than SPS and holds more stabilized organic matter and inorganic components that probably will produce smaller changes

throughout time. As such, all of these reasons suggest that heavy metal adsorption properties would be maintained longer on SAS than on SPS.

In view of the high Cd and Pb adsorption capacity of SAS and SPS, these agricultural wastes could be reused as organic amendments of polluted soils to immobilize Cd and/or Pb. Both SAS and SPS could be used in polluted soils where heavy metals extraction in not possible and, therefore, immobilization will be the best option to minimize their bioavailability, and hence, the environmental damage or heavy metals entering the food chain.

Interaction of heavy metals with laccase enzyme obtained from *T. versicolor*, SAS (*A. bisporus*) and SPS (*P. ostreatus*)

Interactions of Lac–heavy metals were made to determine the inhibition or increase of Lac activity in presence of Cd or Pb from different sources of this ligninolytic enzyme. Purified Lac from *T. versicolor* was used as model of purified enzyme. Table 2 presents the results of percentage of Lac activity after 1 h, 24 h, and 7 days of incubation with Cd or Pb. The substrate SAS shows higher Lac activity (8,670±460 U kg^{-1}) than SPS (3,540±86 U kg^{-1}). Initial Lac activity quantified in extracts for *T. versicolor*, SAS (*A. bisporus*), and SPS (*P. ostreatus*) were 0.25±0.02, 0.43±0.02, and 0.177±0.004 IU ml^{-1}, respectively. *P. ostreatus* Lac activity showed the highest decline (49–77 % of initial activity) at 24 h. For *T. versicolor*, the reduction was about 0–54 % and *A. bisporus* Lac activity reduction was between 8 and 31 %. However, on the 7th day, *A. bisporus* Lac activity was not detected and *P. ostreatus* Lac activity showed a high reduction (>90 %) except in treatments with heavy metal at 500 μM. *T. versicolor* Lac was the most resistant at 7 days and showed a reduction of 42–69 % in comparison with initial activity.

The different behavior towards time and heavy metals found between purified Lac from *T. versicolor* and crude Lac of SAS and SPS could be explained by two reasons. Firstly, because enzyme purification could increase the life of enzymes during the incubation time due to the lack of microbial content and therefore lower microbial degradation than crude extracts of SAS and SPS, and secondly, according to Baldrian (2006), there is considerable heterogeneity in the composition and properties of Lac from different fungi. In this sense, *T. versicolor* and *A. bisporus* Lac were negatively affected by Cd and its increasing concentrations produced a decrease in Lac activity. However, *P. ostreatus* only showed this tendency at 1 h sampling because at 24 h and 7 days increasing Cd concentrations increase Lac activity. Baldrian and Gabriel (2002) found similar results for interactions of *P. ostreatus* Lac extracts at Cd 50 μM or at higher concentrations. Lead strongly inhibited Lac activity and showed a clear trend to reduce activity, according to Pb concentration that increases for all fungi tested, with the exception of Lac from *P. ostreatus* that showed a positive effect with Pb (500 μM) at 24 h. However, the Lac activity remaining at 24 h of SAS extracts was higher at any concentrations of Cd and Pb.

Higher initial Lac activity of SAS (*A. bisporus*) and its lower inhibition towards Cd and Pb than SPS (*P. ostreatus*) at 24 h may indicate that SAS could be more effective as a Lac source for PAH biodegradation in the presence of Cd or Pb. In addition, Li et al. (2010) reported that crude extracts from SAS were excellent for PAH detoxification in liquid medium.

PAH biodegradation by spent *A. bisporus* substrate in the presence of Cd and Pb

According to previous results of heavy metals adsorption capacity and Lac–heavy metals interactions, a biodegradation assay, in solid phase, of PAH in presence of Cd and Pb with SAS was carried out. The aim of the assay consisted in the assessment of the SAS potential to immobilize Cd and Pb and to biodegrade PAH simultaneously.

Cd and Pb immobilization during PAH biodegradation

Figure 2 shows percentages of water (Fig. 2a) and 0.05 M EDTA (pH = 7.0) (Fig. 2b) soluble by both Cd and Pb during the PAH biodegradation process. Water soluble contents were lower than 6 and 1 % of Cd and Pb added (500 μmol kg^{-1}), respectively. No differences were found

Table 2 Percentage of laccase activity (mean±standard deviation) of purified enzyme of *T. versicolor* and crude extracts from spent *A. bisporus* (SAS) and *P. ostreatus* (SPS) substrates after incubation with Cd and Pb in Tris–HCl 0.1 M pH 7.5 buffer (n=3)

T. versicolor					SAS			SPS		
Treatment		1 h	24 h	7 days	1 h	24 h	7 days	1 h	24 h	7 days
Control		100±8	102±4	46±14	100±5	82±1	1±1	100±2	33±1	3±3
Cd	5 μM	91±3	91±5	58±5	107±3	92±8	n.d.	95±2	25±1	3±4
	50 μM	80±12	86±3	56±6	96±5	90±2	n.d.	91±4	23±2	21±6
	500 μM	75±8	79±5	52±6	90±4	79±5	5±2	81±5	51±1	25±4
Pb	5 μM	91±10	86±4	46±5	85±3	77±4	n.d.	101±4	29±6	2±2
	50 μM	93±5	78±7	48±14	81±5	69±3	n.d.	96±16	27±5	6±7
	500 μM	74±8	46±8	31±5	74±3	75±3	2±1	83±5	45±2	21±21

n.d. not detected

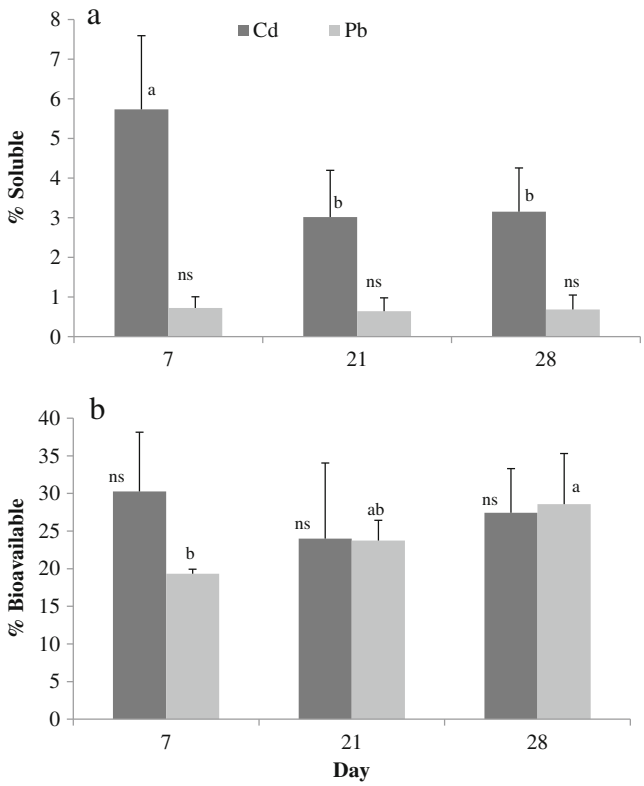

Fig. 2 Percentage of soluble (water extractable) (**a**) and bioavailable (0.05 M EDTA-Na_2 pH 7 extractable) (**b**) Cd and Pb of spent *A. bisporus* substrate spiked with 500 μmol kg^{-1} of Cd or Pb. Significant differences were provided at $p<0.1$. *Bars* indicate standard deviation. $n=4$. *ns* indicates no significant differences

in water-soluble Pb during the three sampling times. However, Cd decreased its water soluble content by about 50 % from the 7th to the 21st day (Fig. 2a). Metals extracted with EDTA (bioavailable fraction) were lower than 30 % of Cd and Pb added. On day 7, Cd-EDTA and water-soluble percentages were higher than Pb; however, on the 21th and 28th days, their bioavailable fractions were similar. Therefore, Cd immobilization, according to water soluble content, was slower than Pb. No differences were found for bioavailable Cd during the three sampling times, but the bioavailable Pb fraction increased gradually from 19 to 29 % throughout the 28 days. These results may be explained according to the expected evolution of cellulose and carboxylic acid content of SAS because Pb have a high tendency to be adsorbed onto cellulose materials (Wong 2009). During the biodegradation process, cellulose was degraded; therefore, the Pb could be desorbed. On the other hand, lignin, hemicelluloses, and other organic compounds after degradation increased the amount of carboxylic groups in their structure that were able to fix Cd and therefore decreased its solubility. Not only compost chemistry affected Cd and Pb adsorption; fungi are able to immobilize heavy metals mainly attributable to defense mechanisms that produce extracellular compounds such as oxalic acid, melanins, and phenolic molecules associated with the cell wall (Baldrian 2003; Jarosz-Wilkolazka et al. 2006).

The low heavy metal bioavailability regarding the total content due to adsorption or precipitation phenomena indicates that the mere measurement of total heavy metal content in a solid medium would not be adequate for making a realistic interpretation, and heavy metal bioavailability must be considered.

Cd and Pb effects over laccase and Mn-peroxidase activity of spent A. bisporus substrate during PAH biodegradation

Initial values for SAS enzymatic activity showed Lac as a major enzyme, about 23 times higher than MnP (Fig. 3a, b). Neither LiP nor VP was detected using veratryl alcohol and ABTS in the presence of H_2O_2 methods (Tanaka et al. 1999), respectively. Lac and MnP patterns were similar; both enzymes decrease their activities throughout the assay period. Lac activity reduction went from 7,860 to 950 IU kg^{-1} and MnP activity dropped from 350 to 1.5 IU kg^{-1}. Baldrian et al.

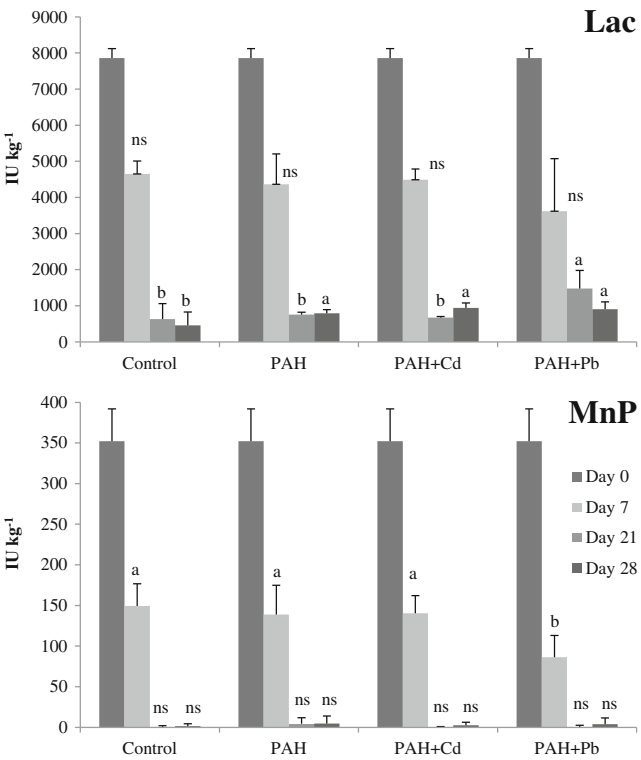

Fig. 3 Laccase and Mn-peroxidase activities (in unit per gram) during the PAH biodegradation process by spent *A. bisporus* substrate with and without Cd or Pb 500 μmol kg^{-1}. Control treatment was spent *A. bisporus* substrate without PAH, Cd, or Pb. Significant differences were provided at $p<0.1$. Different letters indicate significant differences between treatments in the same sampling time. *Bars* indicate standard deviation. $n=4$. *ns* indicates no significant differences

(2000) obtained a similar decrease for Lac activity throughout the 30 days for a soil bioremediation assay using *P. ostreatus*. Cadmium did not decrease Lac or MnP activity. The bioavailability of Cd during the assay was lower than 150 μmol kg^{-1}, and as was shown in the Lac-Cd interaction in liquid medium, low Cd concentration did not inhibit Lac activity. Lead produced significant differences during the assay. On the 7th day, MnP activity of PAH + Pb treatment was lower than treatments without Pb. No significant differences were obtained in Lac activity on the 7th day, but on the 21th day, PAH + Pb proved to be the treatment with highest Lac activity. These results contrast with those obtained in the Lac-Pb interaction in liquid medium, where Pb inhibited Lac activity at all concentrations tested (Table 2). The different behavior can be explained because, although Pb inhibited Lac activity, Pb could promote the enzyme production by *A. bisporus*. Baldrian et al. (2005) reported a similar pattern for *P. ostreatus* during lignocellulose degradation in the presence of Pb. At the end of the assay (28 days), the treatments that use xenobiotics, PAH with and without Cd or Pb, showed higher Lac activity than control (without both PAH and metals).

Biodegradation of PAH by spent A. bisporus substrate in the presence of Cd and Pb

Flu, Phe, Ant, and Py biodegradation with SAS in the absence of, and with 500 μmol kg^{-1} of Cd or Pb, was comparatively determined (values of the remaining PAH are shown in Fig. 4). No biodegradation nor PAH abiotic losses in sterilized SAS throughout the 28 days of the assay were detected. SAS polluted with PAH, PAH + Cd, and PAH + Pb was able to biodegrade the four PAHs tested (Flu, Phe, Ant, and Py). Biodegradation was more intense on the first 7 days. This higher biodegradation at the beginning of the assay agrees with the highest Lac and MnP activities (Fig. 3) and with a probable higher PAH bioavailability due to PAH aged decrease their bioavailability (Smith et al. 2011). On day 7, biodegradation of Flu was lower in the presence of Pb (51 %) than Cd (59 %) or no heavy metal treatment (61 %). Biodegradation of Phe in the presence of Cd was 15 % higher than in the Pb treatment, and no differences were shown between Cd and no heavy metal treatment. No significant differences were found between treatments for Ant and Py, although their biodegradation in the presence of Cd showed a tendency to be increased. On the 21st day, Phe, Ant, and Py biodegradation in the presence of Pb was lower than in the treatment without heavy metals, but on the other hand, the addition of Cd to SAS did not change PAH biodegradation. Flu biodegradation did not show differences in this sampling period.

At the final sampling time (28 days), treatment without heavy metals showed the highest biodegradation rates for Flu (75 %), Phe (41 %), Ant (51 %), and Py (37 %). Flu biodegradation was lower in the presence of Pb than with Cd. No differences were found between Cd and Pb treatments for Phe, Ant, and Py biodegradation, in contrast with the other sampling times where Pb inhibited PAH biodegradation. Therefore, the inhibition effect of Cd was only presented at the final sampling time. According to Baldrian (2003), the mechanism for Cd^{2+} uptake by white rot fungi is linked to the transportation of Ca^{2+}; due to the

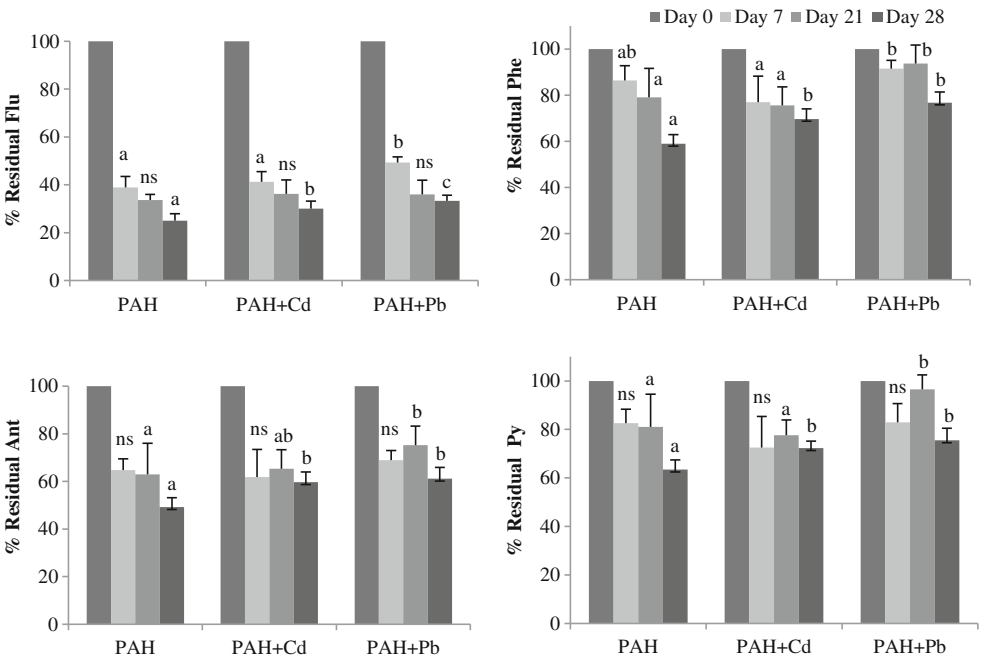

Fig. 4 Percentage of residual PAH (initial Flu, Phe, Ant, Py 100 mg kg^{-1}) during the 28 days of biodegradation process by spent *A. bisporus* substrate with and without Cd or Pb 500 μmol kg^{-1}. Significant differences were provided at $p<0.1$. *Different letters* indicate significant differences among treatments in the same sampling time. *Bars* indicate standard deviation. $n=4$. *ns* indicates no significant differences

high Ca^{2+} contents in SAS (García-Delgado et al. 2012), a competition between both cations can occur and the negative effects of Cd on *A. bisporus* were delayed. In the case of Flu, lower biodegradation occurred in the presence of Pb than with Cd. These results mean that Cd and Pb tend to decrease biodegradation potential of *A. bisporus* and other microorganisms present in SAS. A similar pattern was reported in the case of lignocellulose biodegradation in the presence of heavy metals by *P. ostreatus* (Baldrian et al. 2005). Nevertheless, taking into account that the presence of heavy metals in soil inhibited the mycelium penetration, and therefore PAH biodegradation (Baldrian et al. 2000), and that SAS adsorbs heavy metals as is demonstrated in this work, the use of SAS as a fungi carrier to multi-polluted soil could decrease the heavy metal bioavailability and increase mycelium development.

As a general pattern, the number of aromatic rings of each PAH is an indicator of biodegradation difficulty for microorganisms (Haritash and Kaushik 2009). According to this, Flu with two aromatic rings was the most biodegradable PAH (67–75 %). Both Phe and Ant are formed by three aromatic rings, although Ant was more biodegradable than Phe, which presents similar behavior to Py, with four aromatic rings. Leonardi et al. (2008) reported similar Phe biodegradation behavior for *P. ostreatus* and *I. lacteus* in soil, due to Phe higher ionization potential (IP=8.03 eV) than Ant (IP=7.43 eV) and its higher resistance to oxidation. Mayolo-Deloisa et al. (2011) reported that Lac from SAS of *A. bisporus* was not able to biodegrade Phe (IP=8.03 eV) and Flu (IP=7.89 eV) in liquid medium due to their high IP. Farnet et al. (2009) reported that Lac partially purified from *Marasmius quercophilus* strain19 were not able to biodegrade PAH with IP>7.55 eV (e.g., naphthalene or Phe). Majcherczyk et al. (1998) obtained similar results for Flu and Phe (IP>7.55) using Lac extract from *T. versicolor* and suggest that PAH with IP below 7.45 eV revealed very good oxidation by Lac without a mediator redox compound. Instead, Li et al. (2010) got Flu biodegradation rates between 25 and 79 %, and very low Phe biodegradation using crude extract from SAS and others spent mushroom substrates. The mechanisms of PAH biodegradation by Lac and the production of the oxidative enzymes are not directly correlated with the PAH metabolism (Haritash and Kaushik 2009). This can be confirmed in our results with the PAH + Pb treatment, which showed higher Lac activity on the 21st day and equal Lac activity as the other treatments on the 28th day; however, their biodegradation percentages were the same as, or less than, PAH + Cd and PAH without heavy metal treatment.

In this work, *A. bisporus* was not the only PAH degrader agent. The PAH biodegradation process was carried out in SAS where a consortium of *A. bisporus* and other microorganisms was present. Some of them were probably able to biodegrade PAH and their metabolites and increase their ability to oxidize PAH with high IP. PAH metabolites detected by HPLC-PDA were fluoren-9-one; 9,10-anthracenedione and pyrene hydroxides for Flu, Ant, and Py, respectively. These compounds have been described as final biodegradation metabolites of white rot fungi (Aranda et al. 2010).

Consequently, SAS behaves adequately to be reused and revaluated to bioremediate heavy metals and PAH simultaneously. The main environmental effects of SAS input in soil will be the adsorption of heavy metals, which causes their immobilization, and PAH degradation by *A. bisporus* and other microorganisms present in the material. In addition, the well-known advantages of adding organic matter to soil, such as the improvement of soil texture, water retention capacity, nutrients content, and microbial activity, have to be taken into account in soil remediation. On the other hand, the reuse of SAS will prevent or decrease the environmental impact in mushroom production areas, mainly soil and groundwater pollution, caused by leaching of salts and soluble organic carbon during SAS storage.

Conclusions

SAS showed a greater tendency to immobilize Cd than SPS, probably because SAS has a higher degree of humification. Nevertheless, SPS presented higher capacity to adsorb Pb than SAS because of the higher cellulose–hemicellulose content. Different Lac activity behaviors in the presence of Cd and Pb were found for *T. versicolor*, *A. bisporus*, and *P. ostreatus*, although all Lac were inhibited by Pb. Lac extracted from SAS was more resistant to Cd and Pb than Lac extracted from SPS. SAS was able to biodegrade PAH in the presence of Cd and Pb. However, both Cd and Pb decreased PAH biodegradation rate. This study shows the potential of SAS to biodegrade PAH and to immobilize Cd and Pb simultaneously.

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Implications of polluted soil biostimulation and bioaugmentation with spent mushroom substrate (*Agaricus bisporus*) on the microbial community and polycyclic aromatic hydrocarbons biodegradation

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HIGHLIGHTS

- Augmentation with *A. bisporus* led to the degradation of high molecular weight PAH.
- Sterile SAS stimulated the bacterial population with ensuing 3-ring PAH degradation.
- Richness of soil bacterial and fungal biota increased in the SAS-amended microcosm.
- The results support the adequacy of SAS for environmental remediation purposes.

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ABSTRACT

Different applications of spent *Agaricus bisporus* substrate (SAS), a widespread agro-industrial waste, were investigated with respect to the remediation of a historically polluted soil with Polycyclic Aromatic Hydrocarbons (PAH). In one treatment, the waste was sterilized (SSAS) prior to its application in order to assess its ability to biostimulate, as an organic amendment, the resident soil microbiota and ensuing contaminant degradation. For the other treatments, two bioaugmentation approaches were investigated; the first involved the use of the waste itself and thus implied the application of *A. bisporus* and the inherent microbiota of the waste. In the second treatment, SAS was sterilized and inoculated again with the fungus to assess its ability to act as a fungal carrier. All these treatments were compared with natural attenuation in terms of their impact on soil heterotrophic and PAH-degrading bacteria, fungal growth, biodiversity of soil microbiota and ability to affect PAH bioavailability and ensuing degradation and detoxification. Results clearly showed that historically PAH contaminated soil was not amenable to natural attenuation. Conversely, the addition of sterilized spent *A. bisporus* substrate to the soil stimulated resident soil bacteria with ensuing high removals of 3-ring PAH. Both augmentation treatments were more effective in removing highly condensed PAH, some of which known to possess a significant carcinogenic activity. Regardless of the mode of application, the present results strongly support the adequacy of SAS for environmental remediation purposes and open the way to an attractive recycling option of this waste.

1. Introduction

Polycyclic aromatic hydrocarbons (PAH) are ubiquitous organic contaminants which comprise two or more condensed benzene rings with toxic, mutagenic and carcinogenic properties (IARC, 2010). As a consequence of their physico-chemical properties, these compounds are accumulated in soil via their sorption onto organic matter (Krauss et al., 2000; Liang et al., 2006) and/or clay fractions (Hundal et al., 2001). The selection of a particular remediation technique for PAH-polluted soils is not an easy choice. There are many alternatives including in situ or ex situ techniques which rely on either physico-chemical or biological approaches (Gan et al., 2009; Pelaez et al., 2013). Among them, bioremediation is increasingly deemed to be the most environmentally friendly technique to clean-up polluted soils. Bioremediation of PAH-contaminated soils is based either on the biostimulation of the indigenous microbiota (Sayara et al., 2010) or on the addition of

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exogenous microorganisms, the latter approach being referred to as bioaugmentation (Haritash and Kaushik, 2009). The biostimulation of resident microbial communities of PAH-impacted soil can be achieved in a variety of ways including the addition of organic matter, which has been found to be successful (Covino et al., 2010a; Sayara et al., 2010).

Bioaugmentation with lignin-degrading fungi (LDF) to perform the clean-up of PAH-contaminated soils has received increasing attention in recent years due to its reported efficacy (Covino et al., 2010b; Federici et al., 2012a; Li et al., 2012). LDF are known to produce extracellular lignin-modifying enzymes (LME) with low substrate specificity which enable them to degrade a wide range of organic pollutants, including PAH (Covino et al., 2010c; Majcherczyk et al., 1998). The main LME enzymes include multi-copper oxidases, such as laccase, and heme-peroxidases (Mn-peroxidase (MnP), versatile peroxidase and lignin peroxidase).

Soil colonization and the ensuing contaminant degradation by LDF in soil requires the addition of lignocellulosic materials either as amendments, or inoculum carriers (Covino et al., 2010a; Lestan and Lamar, 1996). The use of these additives has been found to have a favorable impact on the resident microbiota, including specialized populations (Federici et al., 2012a). PAH degradation via bioaugmentation with LDF has been shown to involve either synergistic or antagonist interactions between the fungi added and the autochthonous microbiota. In one study a cooperative effect on the degradation of highly condensed PAH was reported between resident bacteria and *Bjerkandera* sp. (Kotterman et al., 1998) or *Trametes versicolor* (Borràs et al., 2010). However, in another study *Pleurotus ostreatus* enhanced PAH degradation in non-sterile, artificially spiked soils but also inhibited the growth of the indigenous bacteria and changed the composition of the bacterial community (Andersson et al., 2000). Appropriate inocula formulations of LDF, relying on lignocellulosic materials as the carriers have been shown to improve the competitive ability of the fungi added to the resident microbiota (Covino et al., 2010a, 2010b; Federici et al., 2012a). The use of pent mushroom substrate of some LDF as a source of viable inocula for soil clean up applications has been proposed (Li et al., 2012). However spent *Agaricus bisporus* substrate (SAS), without previous treatment, has never been used for bioremediation of PAH polluted soil. Spent *Agaricus bisporus* substrate (SAS), has been shown to enhance the ability of the fungi to endure the toxic effects of both cadmium and lead in a PAH-contaminated substrate (García-Delgado et al., 2013a). Marín-Benito et al. (2012a, 2012b, 2014) reported the ability of pesticide degradation by SAS composted with spent *P. ostreatus* substrate (75:25) and its adsorption capacity for fungicides with low polarity that reduce their mobility in the environment. In addition, SAS has been reported to be an excellent source of LME, mainly laccase, that were able to biodegrade PAH in aqueous solutions (Mayolo-Deloisa et al., 2011). The annual production of this organic waste in Europe has been estimated at 3.5×10^6 t (Pardo-Giménez and Pardo-González, 2008) consequently its potential use in bioaugmentation applications would certainly help to reduce this figure.

Therefore, in view of the profitable and environmentally sound use of SAS, this study has thoroughly investigated its feasibility in PAH remediation applications. This necessarily implies a variety of manipulations of the waste prior to its application, to gain more insight into its ability to act as an organic amendment, fungal carrier or a supplier of exogenous complex microbiota.

To elucidate the isolated and/or combined effects exerted by SAS, several application options were compared using a historically PAH-contaminated soil. All were assessed for their ability to (i) affect the densities of heterotrophic and PAH-degrading bacteria (ii) enable fungal growth (iii) modify the biodiversity of the bacterial and fungal communities (iv) remove PAH (v) modify the bioavailability of PAH and finally to (vi) detoxify the soil. A non-amended contaminated soil microcosm was incubated in parallel and was used as a natural attenuation control.

2. Materials and methods

2.1. Materials

The polluted soil was collected from a creosote wood treatment plant (42° 10′ 31″N 1° 41′ 36″ W Navarra, Spain). The total soil sample (40 kg) was obtained by mixing 20 sub-samples taken from an area close to the treated wood stock zone at a depth of 0–20 cm. The soil sample was homogenized, air-dried at room temperature and finally passed through a 2 mm-sieve. The main properties of the soil are shown in Table 1. According to the US textural classification, the soil was a clay loamy soil (sand 39%, silt 39% and clay 22%) with a water holding capacity of 37%. Thirteen out of the 16 PAH US EPA were present in the polluted soil. The PAH concentrations are shown in Table 2.

SAS was collected from a composting plant of agricultural waste located at 39° 22′ 16″N 1° 59′ 43″ W (Cuenca, Spain). The main characteristics of the SAS are shown in Table 1. Total carbon, nitrogen, hydrogen and sulfur were determined by elemental analysis (LECO CHNS-932 analyzer, St. Joseph, MI).

2.2. Preparation of the remediation microcosms

Irrespective of the type of microcosm, the experiments were carried out in 1 l glass reactors using unsterilized contaminated soil (50 g dry mass). The microcosms were designed in order to simulate:

- Natural attenuation: the preparation of this microcosm simply involved the adjustment of the soil moisture content to 70% of its water-holding capacity prior to the beginning of the incubation. This microcosm will be referred to as soil microcosm (SM)
- Biostimulation: the moisture content of the polluted soil was adjusted to 70% as above and amended with sterilized SAS (121 °C, 30 min) at a 4:1 mass ratio to yield the SSAS microcosm. This approach was designed to assess the stimulatory effect of a sterilized organic waste on resident microbiota in the soil.
- Bioaugmentation I: the moisture content of the polluted soil was adjusted to 70% as above and amended with SAS (4:1 mass ratio) to yield the SAS microcosm. This approach was designed to assess the combined effect of both *A. bisporus* and the indigenous SAS microbiota.
- Bioaugmentation II: to prepare this microcosm, sterilized SAS was inoculated with 3 agar plugs (1 cm diameter) from a 14 day old culture of *A. bisporus* grown on malt extract agar and incubated for 10 d at 20 °C. The colonized matrix was mixed with the contaminated soil (1:4 mass ratio) and the moisture content adjusted to 70% as described above. This bioaugmentation approach, called the Abisp microcosm, was designed to eliminate the contribution of the SAS microbiota.

Each microcosm was carried out in triplicate and incubated at 20 °C for 0, 7, 21, 42 and 63 days under static conditions in the dark.

Table 1
Characteristics of the PAH-polluted soil and spent *Agaricus bisporus* substrate (SAS). $n = 3$.

Parameter	Soil	SAS
pH	8.20 ± 0.03	6.7 ± 0.3
Electronic conductivity (dS m^{-1})	0.58 ± 0.02	7.1 ± 0.8
Organic matter (%)	1.2 ± 0.1	61.9 ± 1.7
Carbonates (%)	30 ± 1	n.d.†
C/N ratio	n.p.§	7.96 ± 0.13
% C	n.p.	32.4 ± 0.02
% N	n.p.	4.07 ± 0.07
% H	n.p.	1.95 ± 0.06
% S	n.p.	0.92 ± 0.12

†n.d.: not detected; § n.p.: not performed.

Table 2
Initial (t_o) and residual PAH concentrations after 63 d incubation (t_f) in the non amended soil microcosm (SM), amended with sterilized spent *Agaricus* substrate (SSAS), spent *Agaricus* substrate (SAS) and sterilized spent *Agaricus* substrate reinoculated with the fungus (Abisp). Data are mean ± standard deviation of 3 replicated microcosms; the asterisk denotes significant differences between the initial and residual concentrations for each microcosm (ANOVA, $p < 0.05$).

Contaminant	Toxic equivalency factor†	Contaminant concentration (mg kg^{-1}) in							
		SM		SSAS		SAS		Abisp	
		t_o	t_f	t_o	t_f	t_o	t_f	t_o	t_f
FLU	0.001	6.11 ± 3.78	2.96 ± 1.77	3.47 ± 0.64	0.859 ± 0.159*	3.86 ± 1.13	1.27 ± 0.29*	2.54 ± 0.57	1.38 ± 0.16*
PHE	0.001	16.8 ± 5.9	6.30 ± 3.80	13.3 ± 2.1	1.39 ± 0.43*	12.1 ± 3.9	2.39 ± 0.52*	10.2 ± 1.6	4.62 ± 0.43*
ANT	0.01	23.7 ± 10.3	13.8 ± 7.6	20.7 ± 7.1	3.69 ± 1.19*	20.1 ± 3.1	8.95 ± 3.9*	14.8 ± 2.2	16.6 ± 1.2
FLT	0.001	21.7 ± 2.6	7.59 ± 1.39*	16.7 ± 0.5	8.05 ± 4.08*	16.5 ± 1.5	5.11 ± 0.35*	1.60 ± 1.9	8.73 ± 4.08*
PYR	0.001	10.1 ± 1.1	4.11 ± 0.25*	8.02 ± 0.04	4.84 ± 2.68	8.46 ± 0.96	2.60 ± 0.15*	7.42 ± 1.0	3.70 ± 2.68*
BaA	0.1	4.35 ± 0.77	2.61 ± 0.82	3.21 ± 0.04	2.72 ± 1.16	3.68 ± 0.35	2.16 ± 0.31*	3.50 ± 0.39	1.81 ± 1.16*
CHR	0.01	11.8 ± 5.0	9.55 ± 4.61	8.01 ± 1.22	8.99 ± 3.84	8.61 ± 0.75	5.97 ± 1.1*	8.25 ± 0.46	5.49 ± 3.84*
BbF	0.1	5.94 ± 0.59	5.45 ± 0.18	4.81 ± 0.09	5.40 ± 1.04	5.35 ± 0.23	4.42 ± 0.21*	5.09 ± 0.37	4.26 ± 1.04*
BkF	0.1	2.42 ± 0.22	2.09 ± 0.08	1.94 ± 0.02	2.03 ± 0.42	2.19 ± 0.10	1.73 ± 0.13*	2.05 ± 0.16	1.52 ± 0.42*
BaP	1	2.91 ± 0.27	2.91 ± 0.05	2.13 ± 0.03	3.02 ± .059	2.78 ± 0.08	2.29 ± 0.13*	2.59 ± 0.22	1.26 ± 0.59*
DBhaA	5	0.755 ± 0.071	0.853 ± 0.047	0.576 ± 0.022	0.782 ± 1.16	0.676 ± 0.062	0.643 ± 0.066	0.683 ± 0.022	0.466 ± 0.162*
BghiP	0.01	2.51 ± 0.17	2.54 ± 0.03	2.03 ± 0.06	2.31 ± 0.41	2.33 ± 0.06	1.99 ± 0.05*	2.23 ± 0.11	1.79 ± 0.41*
IcdP	0.1	2.18 ± 0.18	2.15 ± 0.04	1.75 ± 0.02	2.03 ± 0.29	1.98 ± 0.04	1.68 ± 0.02*	1.87 ± 0.10	1.47 ± 0.29*
Σ3rings		43.6 ± 19.8	23.1 ± 13.1	37.5 ± 9.8	5.94 ± 1.75*	36.1 ± 8.0	12.6 ± 4.5*	46.2 ± 9.8	22.6 ± 1.7
Σ4rings		48.0 ± 8.3	23.9 ± 7.0*	36.0 ± 1.2	24.6 ± 10.1	37.3 ± 3.3	15.8 ± 1.8*	35.1 ± 1.2	19.7 ± 10.1*
Σ5-6rings		16.7 ± 1.5	16.0 ± 0.1	13.2 ± 0.2	15.6 ± 2.9	15.3 ± 0.6	12.8 ± 0.55*	14.5 ± 0.18	10.8 ± 2.9*
ΣPAH		111 ± 29	63.0 ± 15.4	86.7 ± 10.2	46.1 ± 13.7*	88.6 ± 4.7	41.2 ± 6.6*	82.4 ± 10.2	53.1 ± 13.7*

† Nisbet and LaGoy (1992).

2.3. Extraction and analysis of ergosterol and PAH

Total ergosterol was extracted and analyzed as described in the method by Covino et al. (2010b). Samples (0.5 g) were sonicated at 70 °C for 90 min with 3 ml methanolic solution KOH (10%, w/v). Distilled water (1 ml) was added to each sample and the sample extracted three times with 2 ml of n-hexane. The solvent was evaporated under a nitrogen stream and the solid residue dissolved in methanol (1 ml). The samples were analyzed using high performance liquid chromatography (Waters 2695 Separation Module) coupled with a Waters 996 photodiode array detector equipped with Phenomenex Luna C18 column (250 mm × 4.60 mm; particle size 5 μm; pore size 100 A) equilibrated with methanol:water (95:5) at a flow rate of 1 ml min^{-1}. The sample injection volume was 20 μl. The elution profile was monitored at 282 nm.

The extraction of PAH was performed by pressurized liquid extraction (PLE) (ASE350, Dionex). Soil samples (10 g) were loaded into the extraction cell (32 ml) and subsequently extracted with a dichloromethane–acetone mixture (DAM, 1:1, v/v). Static heating was applied to the vessel (100 °C, 5 min) and the extraction performed for 7 min at the same temperature under 1500 psi. The cell was then flushed with 7 ml DAM and finally the solvent purged from the cell with argon for 60 s. This extraction cycle was repeated twice for each sample. The resultant organic extract was dried under gentle N_2 flow at room temperature and finally dissolved in acetonitrile. HPLC analyses were performed using a system consisting of a 2695 Separations Module (Waters, Milford, MA) equipped with a Superguard™ LC-18 guard column (20 × 3 mm) prior to the separation Supelcosil™ LC-PAH column (250 × 3.0 mm; particle size 5 μm) and a 2996 diode-array detector (Waters). Separation of the PAH was achieved using a gradient elution program, using (A) acetonitrile and (B) Milli-Q water. The elution program was: isocratic elution with 60% (A) for 5 min, gradient to 100% (A) for 15 min, isocratic elution at 100% A for 20 min. The column temperature was fixed at 28 °C. The sample injection volume was 20 μl. The chromatograms were monitored at 254 nm. PAH were identified on the basis of both UV spectra and matching the retention times with commercially available standards (Sigma-Aldrich). The PAH detected and quantified were: fluorene (FLU); phenanthrene, (PHE), anthracene (ANT), fluoranthene (FLT), pyrene (PYR), benzo[a]anthracene (BaA), chrysene (CHR), benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF), benzo[a]pyrene (BaP), dibenzo[a,h]anthracene (DBahA), benzo[g,h,i]perylene (BghiP), indeno[c,d]pyrene (IcdP). Naphthalene, acenaphthylene, and acenaphthene were below detection limits probably due the volatilization of these compounds (García-Delgado et al., 2013b).

2.4. Estimation of PAH bioavailability

The bioavailable fraction of each PAH was determined in all microcosms by hydroxypropyl-β-cyclodextrin (HPCD) extraction according to the method described by Stokes et al. (2005). The extraction was carried out by mixing the soil (1.5 g) with a 25 ml solution of 50 mM HPCD in deionized water and incubating the mixture for 20 h on an orbital shaker (250 rpm). The mixture was then centrifuged (2500 rpm, 15 min) and the supernatant discarded. The resulting soil pellet was washed with deionized water (25 ml) and manually shaken for 10 s, centrifuged and the supernatant discarded again to remove residual HPCD. Finally, the soil pellet underwent exhaustive PLE extraction (as described above) to determine residual PAH concentration after HPCD extraction.

2.5. Biochemical determinations and toxicity tests

Lignin-modifying enzymes (LME) were extracted from the remediation microcosms (3 g) at 5 °C for 1 h using the buffered solution described by D'Annibale et al. (2006). The aqueous suspension was centrifuged (6000 *g*, 30 min) and the supernatant assayed for LME activities.

Laccase activity was spectrophotometrically determined by following the oxidation of 0.2 mM 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid in 100 mM sodium acetate buffer (pH 4.5) at 420 nm ($\varepsilon = 36{,}000\ M^{-1}\ cm^{-1}$). MnP activity was assayed by the oxidation of 1 mM $MnSO_4$ in 50 mM sodium malonate buffer (pH 4.5) in the presence of 0.1 mM H_2O_2. Manganic ions, Mn^{3+} form a complex with malonate which absorbs at 270 nm ($\varepsilon = 11{,}590\ M^{-1}\ cm^{-1}$) (Wariishi et al., 1992). One unit of enzyme activity (IU) is defined as the amount of enzyme which produced 1 μmol of product per minute under the assay conditions.

Ecotoxicological assessment of remediation microcosms was carried out using two independent methods. The first was an acute toxicity test on the springtail *Folsomia candida* Willem based on the percentage mortality of adults, as previously reported by Leonardi et al. (2008). The second test was based on the determination of dehydrogenase activity which has been shown to be a sensitive ecological index in soils

contaminated with hydrocarbons (Dawson et al., 2007). Remediation microcosms were assayed for dehydrogenase activity as follows: 1 ml 1.5% 2,3,5-triphenyltetrazolium chloride dissolved in 0.1 M Tris–HCl buffer at pH 7.5 and added to 1 g fresh samples. The reaction mixture was incubated at 30 °C for 24 h in the dark. At the end of incubation, the triphenylformazan was extracted with 8 ml acetone, the extract was centrifuged (3500 rpm, 15 min) and the absorbance of the supernatant measured at 546 nm. ($\varepsilon = 15.4\ mM^{-1}\ cm^{-1}$).

The percentage reduction of carcinogenic risk assessment (RCRA) of the PAH present in the soil was based on the Nisbet and LaGoy (1992) toxic equivalency factors (TEF) of PAH for environmental exposure and was determined as follows:

$$RCRA(\%) = \frac{\sum_{i=1}^{13}[PAH_i]_{to} \cdot DR \cdot TEF_i}{\sum_{i=1}^{13}[PAH_i]_{to} \cdot TEF_i} \cdot 100$$

where $[PAH_i]_{to}$ is the initial concentration of PAH, DR and TEF the degradation rate and toxic equivalency factor (Table 2).

2.6. Enumeration of cultivable heterotrophic and PAH-degrading bacteria

Soil bacterial counts were performed using a miniaturized most probable number (MPN) method using 96-well microtiter plates, with eight replicate wells per dilution according to the method of Lladó et al. (2009). The total number of heterotrophs were counted in tryptone soy broth and the PAH-degrading bacteria were counted in a mineral medium containing a PAH mixture composed of PHE 0.5 g l^{-1} and FLU, ANT, PYR, at a final concentration of 0.05 g l^{-1} as the sole carbon sources. To avoid fungal contamination, cycloheximide at a final concentration of 100 mg l^{-1} was added to both growth media (i.e., tryptone soy broth and mineral medium). The MPN plates were incubated at room temperature for 30 days. Positive wells were detected by turbidity for heterotrophs, and the presence of a brownish/yellow coloration for PAH degrading bacteria. The MPN calculation was carried out using US EPA MPN Calculator v1.1 software.

2.7. DNA extraction

Whole genomic DNA from each soil was extracted using the Power Soil DNA extraction Kit (Mo Bio Laboratories Inc., Carlsbad, CA) according to the manufacturer's instructions. The bacterial V3 region of the 16S rRNA gene was amplified using the universal bacterial 341-f (5′-CGCC CGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGC AGCAG-3′) and 534-r (5′-ATT ACC GCG GCT GCT GG-3′) primers with a length of approximately 200 bp (Muyzer et al., 1993). Fungal 18S ribosomal DNA was amplified using the universal fungal primers FUN_NS1 (5′-GTAGTCATATGCTTGTCTC-3′) and GC fung (5′-CGCCCGCCGCGCCC CGCGCCCGGCCCGCCGCCCCCGCCCCATTCCCCGTTACCCGTTG-3) (Das et al., 2007). The reaction mixture consisted of 2 μL of template DNA (ca. 20 ng), Tris–HCl (20 mM, pH 8.3), KCl (100 mM), $MgCl_2$ (3 mM), Taq-polymerase (0.1 IU) (NzyTech, Lisbon, Portugal), primers (0.2 mM of each) and double deionized water to bring the final volume up to 50 μL. For Bacterial amplification, the touchdown PCR program was performed in a *Primus* PCR thermo cycler (MWG biotech, Ebersberg, Germany) using the same procedure (Muyzer et al., 1993). For fungal amplification the PCR program previously reported by Das et al. (2007) was used. All amplicons (5 μL) were analyzed on agarose gel before being used for Denaturing Gradient Gel Electrophoresis (DGGE).

2.8. Denaturing gradient gel electrophoresis (DGGE) analysis

The INGENY phorU DGGE system (Ingeny, Goes, NL) was used for sequence-specific separation of PCR amplified fragments. For PCR amplicons obtained with the 341f-GC and 534r primers, electrophoresis was performed in a polyacrylamide gel (8% (w/v) acrylamide/bis-acrylamide gel 37.5:1), containing 40–60% urea-formamide denaturing gradient (100% corresponds to 7 M urea and 40% (w/v) formamide). For fungal amplicons the urea-formamide denaturating gradient ranged from 20 to 35%. After DGGE electrophoresis the gels were stained with Gel star solution (Lonza, Ltd group, USA) at room temperature for 45 min and photographed using a UV-transillumination table with a GelDoc XR digital camera (Bio-Rad, Carlsbad, CA). The fingerprinting profile obtained from DGGE was investigated using the Quantity one software (Bio-Rad). To characterize the community composition obtained by DGGE analyses, the Shannon diversity index H′ was calculated as follows:

$$H' = -\Sigma(P_i \times \ln P_i)$$

where P_i represents the ratio of the single intensity band to the sum of the bands intensity of each lane, Richness (S) is the number of bands revealed, and Evenness (E) was calculated as H′/ln S.

3. Results and discussion

3.1. Time- and microcosm-dependent evolution of fungal biomass and cultivable bacteria

In the SM microcosm control the ergosterol content, a specific indicator of fungal biomass was invariably lower than the detection limits (Fig. 1A). Irrespective of the sampling time, no fungal growth was observed in the SM throughout the incubation period.

There were no significant ($p \leq 0.05$) time dependent changes in ergosterol in the SSAS microcosm which indicates that despite the presence of the sterilized *Agaricus* substrate, fungal growth was not stimulated at all. This result was unexpected because several studies have shown that the application of sterilized lignocellulose waste exerts a positive effect on the resident fungi in contaminated soils (Federici et al., 2011, 2012a; Lladó et al., 2013). Conversely, in the SAS microcosm an approximate three-fold increase in ergosterol content was observed in the first week of incubation. This was followed by a decline in the values which remained constant in subsequent harvests (Fig. 1A).

No changes in the ergosterol content were observed throughout the incubation in the Abisp microcosm which suggests that the sterilized SAS was neither an adequate carrier for the subsequent growth of *A. bisporus* in the soil, nor a valuable trophic supplement for the resident fungi. This could be explained by the fact that most of the available organic components in the sterilized SAS had been consumed by the reinoculated *A. bisporus* prior to its addition to the soil, resulting in a microcosm with a low nutrient status. Therefore, the potential trophic contribution of the carrier to the competitive ability of the added inoculum was impaired (Covino et al., 2010b; Lestan and Lamar, 1996).

The density of the cultivable heterotrophic and PAH-degrading bacteria over time is shown in Fig. 1B and C′ respectively. Heterotrophic microbial counts in the SAS and SSAS microcosms increased by approximately two orders of magnitude and were significantly higher than those in the SM microcosm over the whole incubation period (Fig. 1B). The same result was also found in the bioaugmented Abisp microcosm where heterotrophic bacterial counts increased by more than one order of magnitude (Fig. 1B). In the amended microcosms, the lowest densities of heterotrophic bacteria throughout the incubation period were found in the Abisp microcosm. This may be explained by the low nutrient input associated with this inoculant. Its preparation had involved the reinoculation of *A. bisporus* into spent Agaricus substrate and subsequent incubation for 14 d prior to its application onto soil with ensuing consumption of organic nutrients by the growing fungus. This is also corroborated by the observed increase in heterotrophs in the other bioaugmented SAS microcosm where the addition of the inoculant ensured a higher nutrient input than in Abisp. Similar results were also found for the cultivable PAH-degrading bacteria. The highest densities were observed in the SSAS and SAS microcosms (Fig. 1C). However,

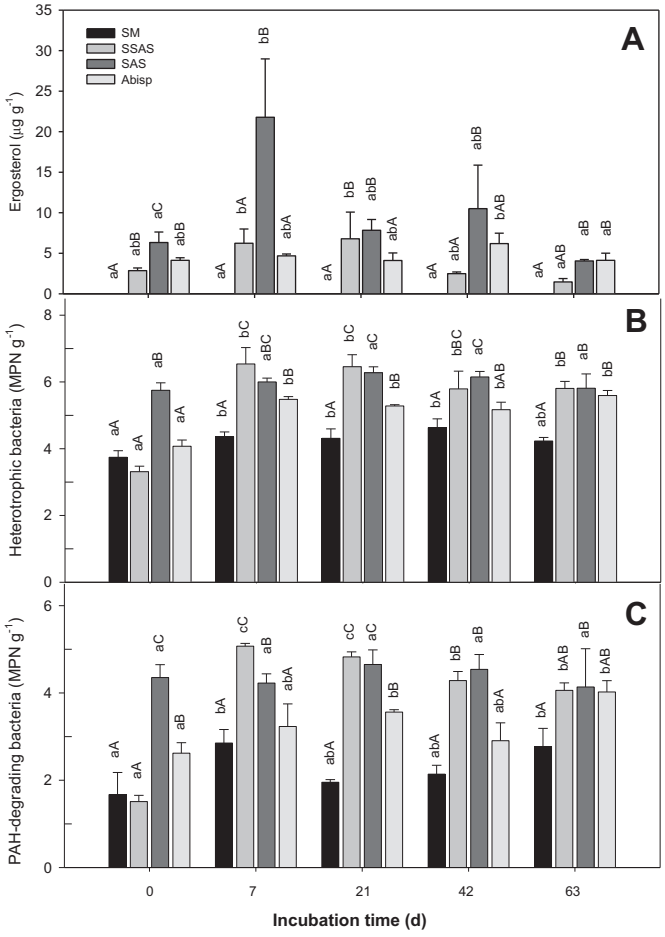

Fig. 1. Time course of ergosterol concentrations (A), total heterotrophic (B) and PAH degrading (C) bacteria in non amended soil microcosm (SM), amended with sterilized spent Agaricus substrate (SSAS), spent Agaricus substrate (SAS), and sterilized spent Agaricus substrate reinoculated with the fungus (Abisp). Data are the mean ± standard deviation of three replicated microcosms. Different uppercase and lowercase letters indicate significant differences between microcosms at the same incubation time and between incubation times within the same microcosm (Tukey post-hoc test; $p \leq 0.05$), respectively.

it should be noted that the spent *Agaricus* substrate contained viable PAH-degrading bacteria which would have resulted in a higher initial density in the SAS microcosm than in the SM (2.3×10^4 vs. 0.5 × x 10^2 MPN g^{-1}) (Fig. 1C). In the SSAS microcosm, the addition of the sterilized organic waste also exerted a high stimulation on the PAH-degrading bacteria which were increased by more than three orders of magnitude after one and three weeks of incubation, although after this their densities declined. As previously observed for heterotrophic bacterial counts in the amended remediation microcosms, the Abisp exhibited the lowest densities of PAH-degrading bacteria for most of the incubation period. Regardless of the different application options in addition to leading to a higher density of heterotrophs, when compared with the SM, it also enhanced specialized ones. As described above, the non-sterile spent *Agaricus* substrate in the SAS microcosm contained allochthonous PAH-degrading bacteria. This is not surprising because a molecular characterization of the bacterial community in this substrate has revealed the presence of several genera *Paenibacillus*, *Arthrobacter*, *Comamonas* and *Sphingobacterium*, (Ntougias et al., 2004; Watabe et al., 2004) which included several species with reported PAH-degrading capacity (Haritash and Kaushik, 2009). However, the addition of the sterilized SAS, irrespective of whether it had been reinoculated with *A. bisporus* or not, also appeared to stimulate PAH-degrading bacteria. Previous studies have also found that the addition of sterilized organic wastes such as wheat straw and maize stalks activated the specialized resident bacterial populations in soils contaminated with PAH (Lladó et al., 2013), and polychlorobiphenyls (Federici et al., 2012a, 2012b). It has been suggested that the stimulatory effect exerted by organic waste is due to an enhancement of the oxygen transfer via an increase in soil porosity without necessarily involving either trophic factors or modifications in contaminant bioavailability (Federici et al., 2012b).

3.2. Microcosm-dependent impact on bacterial and fungal community structure

It is widely known that the cultivable microbiota in soil represent a minor fraction of the whole microbial community (Daniel, 2005). Therefore, an investigation into the structure of both the bacterial and fungal communities as a function of the remediation treatment necessitated a cultivation-independent approach which relied on DGGE. Table 3 shows the Shannon Weaver Index, which gives the richness and evenness values of bacterial and fungal communities in the microcosms at the start, and at the end of incubation. These values have been calculated by a numerical analysis of the DGGE profiles of PCR-amplified 16S and 18S rDNA fragments respectively. Both the richness and the diversity of the bacterial and fungal communities in the SM did not significantly change during incubation. This result is not surprising because both communities presumably had a well established adaptation to the conditions exerted by the historical contamination of the soil.

As a result of the fungi and bacteria already present in the SAS microcosm which contained the unsterilized spent *Agaricus* substrate, higher initial richness values for bacterial and fungal biota were found than in those in the SM microcosm (18 vs. 11, respectively, and 23 vs. 17, respectively). In addition, the 63 day old SAS microcosms produced a significantly higher H′ value for the bacterial community than that from the coeval SM (2.95 ± 0.08 vs. 2.23 ± 0.08, respectively), as well as a higher richness in the fungal biota (27 vs. 17, respectively) but without substantial differences in their respective evenness.

Conversely, the presence of the sterilized spent *Agaricus* substrate in the SSAS microcosm did not substantially modify the test parameters with respect to those found in the SM. In the 63 day old SSAS microcosm, significantly higher S and H′ values for the bacterial community than those in the coeval SM were found, although no substantial differences were observed in their fungal biota. Regardless of the sampling time, similar results to those described for SSAS were found in the Abisp microcosm. Interestingly, the microcosms to which sterilized spent *Agaricus* substrate had been added, irrespective of whether they had been reinoculated with Agaricus or not, i.e. the SSAS and Abisp microcosms had the same impact on the fungi. They both failed to promote fungal growth throughout incubation (Fig. 1A) and/or to substantially change the richness and diversity of the fungal community when compared to the SM microcosm (Table 3).

3.3. PAH removal in the remediation microcosms

Table 2 shows the initial and residual PAH concentrations in the remediation microcosms after 63 days incubation. In the SM, no significant reduction in the total PAH concentration was observed ($p = 0.07$). Significant differences between the initial and final concentrations were only found for FLT and PYR, with a percentage removal of 65%, and 59% respectively. In the biostimulation SSAS microcosm a statistically significant decrease ($p < 0.05$) in 3-ring PAH content, FLU (75%), PHE (90%), ANT (82%), and FLT (52%), was observed and the overall PAH residual content significantly differed from that at the start. Therefore, the biostimulation treatment was effective at degrading low molecular weight PAH which were the most abundant contaminants in the soil. In the bioaugmented microcosms, namely SAS and Abisp, significant differences between the initial and the residual concentrations were found for the majority of the individual contaminants as well as the overall

Table 3
Shannon Weaver Index (H′), richness (S) and evenness (E) values of bacterial and fungal communities at start (t_0) and at the end (t_{63}) of the incubation in the non amended soil microcosm (SM), amended with sterilized spent *Agaricus* substrate (SSAS), spent *Agaricus* substrate (SAS) and sterilized spent *Agaricus* substrate reinoculated with the fungus (Abisp).

Microcosms	Bacteria			Fungi		
	H′	S	E	H′	S	E
SM t_o	2.27 ± 0.08^{Aa}	11 ± 0	0.95 ± 0.03^{Aa}	2.61 ± 0.02^{Ba}	17 ± 1	0.91 ± 0.00^{Ca}
SM t_{63}	2.23 ± 0.03^{Aa}	10 ± 0	0.97 ± 0.01^{Ba}	2.59 ± 0.04^{Ba}	19 ± 1	0.96 ± 0.12^{Aa}
SAS t_0	2.64 ± 0.08^{Ba}	18 ± 1	0.90 ± 0.04^{Aa}	2.55 ± 0.13^{ABa}	23 ± 3	0.82 ± 0.01^{Ba}
SAS t_{63}	2.95 ± 0.08^{Ca}	27 ± 1	0.89 ± 0.01^{Aa}	2.74 ± 0.03^{Ba}	27 ± 0	0.83 ± 0.01^{Aa}
SSAS t_0	2.42 ± 0.03^{Aa}	12 ± 1	0.97 ± 0.02^{Aa}	2.25 ± 0.10^{Aa}	15 ± 1	0.83 ± 0.01^{Ba}
SSAS t_{63}	2.86 ± 0.04^{BCb}	21 ± 1	0.95 ± 0.01^{Ba}	2.66 ± 0.07^{Bb}	25 ± 2	0.83 ± 0.00^{Aa}
Abisp t_0	2.41 ± 0.02^{Aa}	12 ± 1	0.96 ± 0.00^{Aa}	2.09 ± 0.01^{Aa}	15 ± 0	0.77 ± 0.00^{Aa}
Abisp t_{63}	2.63 ± 0.11^{Bb}	16 ± 2	0.94 ± 0.01^{Ba}	2.41 ± 0.02^{Ab}	20 ± 0	0.81 ± 0.03^{Aa}

Data are the mean ± standard deviation of 3 independent microcosms. Multiple pair-wise comparisons were performed by the Fisher LSD test ($p \leq 0.05$): same uppercase and lowercase letters denote the absence of statistically significant differences between different microcosms at same time of treatment and between the same microcosm at different time, respectively.

PAH concentrations (Table 2). These findings indicate that the bioaugmentation treatments with *A. bisporus* were efficient at degrading both low and high molecular weight PAH.

A comparison of the degradation performances of SSAS, SAS and Abisp microcosms, revealed that SSAS and SAS, which exhibited the highest density of PAH-degrading bacteria, were very efficient at degrading low molecular weight PAH. However, Abisp was superior to SSAS and SAS in the removal of highly condensed PAH (Table 2). Therefore, for PAH which are potentially carcinogenic to humans or animals (IARC, 2010), the Abisp microcosm was more efficient than the SAS in degrading compounds such as BaP and DBahA (52 vs. 18% respectively, and 32 vs. 6% respectively). In this respect, a very limited number of bacteria able to grow in pure cultures on either 5- or 6-ring PAH have been identified (Haritash and Kaushik, 2009).

However, in the SAS microcosm, the augmented fungus appeared to exert a lower inhibitory action on the resident bacterial biota as deduced by comparing its better degradation performance on low molecular weight PAH and its higher density of PAH-degrading bacteria than those found in the Abisp microcosm. One of the possible synergistic mechanisms which might occur between bacteria and fungi, relies on the ability of the latter to convert PAH into more polar degradation intermediates, such as PAH diones and hydroxylated derivatives (Covino et al., 2010a).

Linear regression analyses were performed to relate the percentage removal of each PAH in the microcosm with respect to the chemical characteristics of these contaminants (Table 4). Therefore, the degradation results were related to those parameters which have been suggested to significantly affect PAH degradation, such as molecular weight (MW), organic carbon sorption coefficient (log K_{oc}), hydrophobicity (log *P*), water solubility (WS) and ionization potential (IP), (Table 4). These analyses showed that the degradation results were positively and significantly ($p < 0.05$) correlated with WS in all the microcosms tested. Conversely, with the exception of the Abisp microcosm, PAH degradation results were negatively correlated ($p < 0.01$) with their respective MW, log K_{oc} and log *P* values (Table 4). These results clearly indicate that the bioavailability of PAH contaminants strongly affect their ability to be degraded in the SM, SSAS and SAS microcosms, where the bacteria make an important contribution to PAH depletion. Bacterial uptake of PAH and their subsequent metabolism have been shown to be governed by the mass transfer rates of contaminants from the solid to the liquid phase of soil, which are in turn affected by the aforementioned physico-chemical properties (Haritash and Kaushik, 2009). The distinctive behavior of the Abisp microcosm, namely the lack of correlation between PAH degradation and MW, log K_{oc} and log *P*, might indicate that different PAH degradation mechanisms are involved here. In particular, the direct involvement of PAH oxidation by LME which was high during the initial incubation phases (See subsection 3.5) might be as result of their ability to diffuse into the soil matrix and potentially oxidize PAH with low bioavailability (Haritash and Kaushik, 2009). This hypothesis may explain why the Abisp microcosm was able to partially degrade 5- and 6-ring PAH.

The IP has been shown to affect in vitro PAH oxidation by either laccase or fungal heme-peroxidases, whose activities were found in the bioaugmented microcosms (see subsection 3.4). The susceptibility of PAH to oxidation by fungal LME has been shown to increase as the IP decreases (Majcherczyk et al., 1998). In the present study the opposite trend was found when relating PAH depletions and respective IP values in all the microcosms. However, the Pearson Coefficients related to these regressions, which were significant with the exception of Abisp, were the lowest among the physico-chemical properties tested (Table 4). Similar findings were obtained with two historically PAH-contaminated soils augmented with either *Irpex lacteus* or *Lentinus tigrinus* (Covino et al., 2010b). This clearly suggests that even in augmented microcosms, LME-triggered PAH oxidation is not the sole mechanism involved in PAH degradation. This was made particularly evident in the bioaugmented microcosms by the high depletion extents of PHE and FLT, their high IP values (8.03 and 7.91 eV respectively) make them poorly susceptible to mono-electronic oxidation by LMEs (Majcherczyk et al., 1998; Mayolo-Deloisa et al., 2011). In addition to the action of the PAH-degrading bacteria, it should be taken into account that PAH degradation in fungi also relies on the intracellular cytochrome P-450/epoxide hydrolase complex whose activity is not dependent on IP (Haritash and Kaushik, 2009).

Table 4
Pearson product moment correlation coefficients between percent PAH removal in non amended soil microcosm (SM) or amended with either sterilized spent Agaricus substrate (SSAS) or spent Agaricus substrate (SAS) or sterilized spent Agaricus substrate reinoculated with the fungus (Abisp) and respective PAH physico-chemical properties including molecular weight (MW), organic carbon adsorption coefficient (log K_{oc}), hydrophobicity (log *P*), water solubility (WS) and ionization potential (IP).

Microcosm	MW	log $K_{oc}^{\S}$	log $P^{\S}$	WS§	IP†
SM	−0.720**	−0.705**	−0.688**	0.402*	0.339*
SSAS	−0.896**	−0.895**	−0.872**	0.582*	0.490**
SAS	−0.848**	−0.839**	−0.813**	0.546**	0.468**
Abisp	n.s.	n.s.	n.s.	0.327*	n.s.

§These parameters were calculated using the Advanced Chemistry Development v. 11.02 software package (ACD/Labs, Toronto, Canada) available from the on-line SciFinder chemical database (American Chemical Society, Columbus, OH); †From Covino et al., 2010a; n.s., no significant correlation; * significant correlation at $p < 0.05$: ** significant correlation at $p < 0.01$.

3.4. Time- and microcosm-dependent modifications in the PAH bioavailable fraction

The bioavailable fraction of each PAH was determined in all the microcosms at the start and after 63 days incubation using HPCD extraction (Stokes et al., 2005). At the start the different soil application options, i.e. SSAS, SAS and Abisp microcosms did not significantly affect the percentage bioavailable fraction of the large majority of contaminants in each microcosm (Table 5). The only exceptions were observed

in some highly condensed PAH such as DBahA. The bioavailable fractions were lowest in the SAS and Abisp microcosms. BghiP showed the highest bioavailability in the SSAS and Abisp microcosms.

After 63 days incubation a microcosm-dependent modification of the bioavailable fraction of individual PAH was observed when compared to the beginning of the experiment. In particular, in the SM an increase in the bioavailable fraction was observed for high molecular weight PAH (i.e., DBahA, BghiP and IcdP). In the SSAS microcosm, the bioavailable fractions of BbF and DBahA increased while those of PHE and ANT decreased (Table 5). For the latter two compounds, it could be envisaged that their high degradation extents in this microcosm (90 and 82%, respectively) left only the most recalcitrant fraction. In the bioaugmented microcosms (SAS and Abisp) a different scenario was found. In particular, in the SAS a decrease in the bioavailable fraction of PHE, FLT, PYR, CHR and BaP was observed. Conversely, in the Abisp microcosm, decreased bioavailabilities were found for FLU, PHE, BbF, BkF, BaP, BghiP and IcdP. The reason underlying the decrease in the bioavailable fraction of the majority of individual PAH was not solely due to their respective depletions since these parameters were not found to be correlated in all the microcosms. Therefore, the changes observed might have derived from a combination of PAH sorption onto organic matter and partial degradation. In this respect, the organic matter of spent *Agaricus* substrate analyzed by cross-polarization and magic angle spinning nuclear magnetic resonance, was found to contain a high percentage of aliphatic carbon (about 21%) that could contribute significantly to PAH sorption via hydrophobic interactions (García-Delgado et al., 2013a).

3.5. Lignin-modifying enzyme activity and residual toxicity in remediation microcosms

The remediation microcosms were assayed for their ligninolytic activity due to the involvement of these enzymes in the early oxidation steps of PAH (Li et al., 2010; Majcherczyk et al., 1998). Among them, laccase and to a much lower extent Mn-peroxidase activity (Fig. 2A and B, respectively) were only detected in the bioaugmented microcosms (SAS and Abisp). The highest laccase and MnP activity were found in the Abisp microcosms where the activity of the former enzyme markedly declined with incubation time (Fig. 2A). Noteworthy, in this microcosm the best depletions were observed for BaP and DBahA

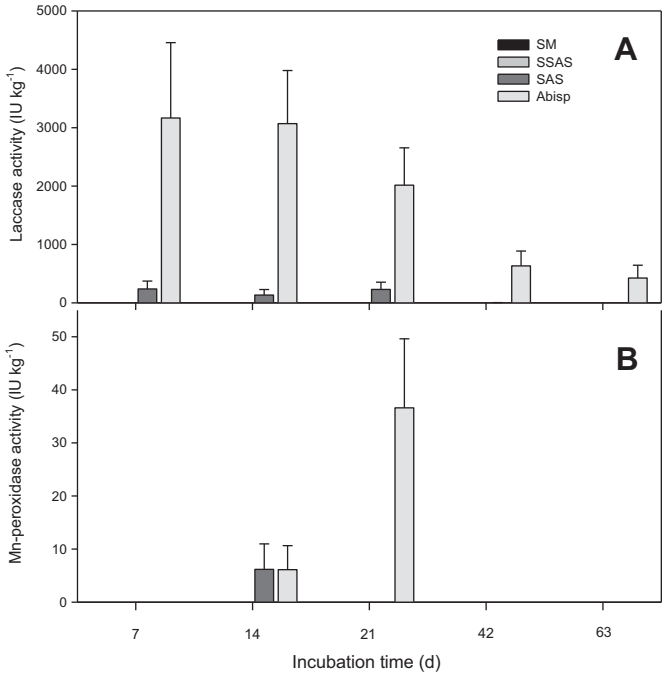

Fig. 2. Time courses of laccase (A) and Mn-peroxidase (B) activities in non amended soil microcosm (SM), amended with sterilized spent Agaricus substrate (SSAS), spent Agaricus substrate (SAS) and sterilized spent Agaricus substrate reinoculated with the fungus (Abisp). Data are the mean ± standard deviation of three replicated microcosms.

which despite being high molecular weight PAH, are characterized by low IP values (i.e., 7.12 and 7.38 respectively) and therefore highly susceptible to oxidation by both laccase and MnP (Bogan and Lamar, 1995). In this regard the results of the present study are in agreement with those of Li et al. (2010) who showed high removal rates of BaP and DBahA by crude laccase extracts from spent *Agaricus* substrate.

Dehydrogenase activity was used as a possible index of detoxification in the remediation microcosms as previously suggested for hydrocarbon-impacted soils (Dawson et al., 2007) in addition to the *F. candida* mortality test. In the present study, the adoption of this parameter did not provide an unequivocal clue to the detoxification by augmented microcosms. This was the result of a low dehydrogenase activity in the contaminated soil at the start (Table 6). The level of dehydrogenase activity was markedly and predictably boosted by the addition of viable fungal inocula in the SAS and Abisp microcosms (Table 6). The retention of high levels of activity in the 63 day old SAS and Abisp microcosms, albeit being lower in the SAS and equal in the Abisp, indicate that the soil microbiota retained a high functional activity despite a prolonged incubation time.

Table 6 also shows that the initial toxicity of the microcosms towards *F. candida* was high, leading to mortalities that ranged from 77.5 to 89%. Although a partial detoxification was observed in all microcosms, the best results were observed with SAS and Abisp where mortality was reduced by 45.1 and 41.4%, respectively. In these microcosms, a generalized decrease in the bioavailable fractions of individual contaminants were observed together with the highest percentage removal of both 4-ring and highly condensed PAH. The lowest mortality reduction (26.8%) was observed in SSAS where the lowest depletion of 4-ring PAH, and no depletion of highly condensed PAH were found. In this respect, the higher detoxification observed in Abisp than SSAS suggest that 3-ring PAH were less toxic to *F. candida* than 4-ring and 5,6-ring PAH. This might be explained by the fact that in the SSAS the former contaminant group was degraded at a significantly higher extent and, in the same microcosm, with the exception of PHE, their bioavailable fractions were comparatively lower than in Abisp.

Table 5
Percent bioavailable PAH fraction at start (t_0) and at the end (t_{63}) of the incubation in non amended soil microcosm (SM) or the same soil added with sterilized spent Agaricus substrate (SSAS) or spent Agaricus substrate (SAS) or sterilized spent Agaricus substrate reinoculated with the fungus (Abisp).

PAH	Percent bioavailable PAH fraction† in							
	SM		SSAS		SAS		Abisp	
	t_0	t_{63}	t_0	t_{63}	t_0	t_{63}	t_0	t_{63}
FLU	92	98^{A}	94	99^{A}	99	96^{A*}	97	86^{B*}
PHE	75	83^{A}	87	0^{D*}	89	57^{C*}	88	68^{B*}
ANT	86	93^{A}	82	29^{B*}	90	86^{A}	79	90^{A}
FLT	71	69^{A}	71	59^{AB}	74	39^{B*}	76	58^{AB}
PYR	17	0	7	4	14	0^{*}	3	0
BaA	0	2	8	4	0	0	5	3
CHR	43	61^{A}	64	68^{A}	58	30^{B*}	56	44^{AB}
BbF	5	19^{B}	7	33^{A*}	0	0^{C}	13	0^{C*}
BkF	23	30^{B}	46	44^{A}	10	1^{C}	14	1^{C*}
BaP	10	25^{B}	45	52^{A}	16	0^{C*}	20	0^{C*}
DBahA	28^{a}	54^{B*}	33^{a}	64^{A*}	0^{b}	34^{C}	2^{b}	16^{D}
BghiP	0^{b}	21^{A*}	24^{a}	20^{A}	0^{b}	0^{B}	12^{a}	0^{B*}
IcdP	0	14^{A*}	30	24^{A}	0	0^{B}	8	0^{B*}

† Data are the mean of three independent microcosms. Different lowercase letters indicate that differences between microcosms at start (t_0) were significant (Tukey post-hoc test, $p < 0.05$). Different uppercase letters indicate that differences between 63-d-old microcosms (t_{63}) sampling time were significant (Tukey post-hoc test, $p < 0.05$). The asterisk * denotes significant differences between sampling times within the same microcosm (ANOVA, $p < 0.05$).

Table 6
Reduction of carcinogenic risk assessment (RCRA) and dehydrogenase activity and *F. candida* mortality at start (T_0) and after 63 d (T_f) incubation in non amended soil microcosm (SM) or the same soil added with sterilized spent Agaricus substrate (SSAS), spent Agaricus substrate (SAS) or sterilized spent Agaricus substrate reinoculated with the fungus (Abisp).

Microcosm	RCRA[a] (%)	Dehydrogenase activity[b] (IU kg^{-1})		*F. candida* mortality[b] (%)	
	T_f	T_0	T_f	T_0	T_f
SM	5.3 ± 2.2AB	0.011 ± 0.004Aa	0.041 ± 0.003Ab	89.5 ± 2.5Bb	60.6 ± 3.2Ca
SSAS	4.5 ± 0.6A	0.106 ± 0.006Aa	0.223 ± 0.075Ab	77.5 ± 4.1Ab	58.7 ± 2.1BCa
SAS	15.3 ± 6.3B	0.598 ± 0.140Cb	0.195 ± 0.006Aa	89.6 ± 3.8Bb	49.2 ± 3.1Aa
Abisp	37.6 ± 8.6C	0.336 ± 0.060Ba	0.499 ± 0.162Ba	85.5 ± 4.1Bb	50.1 ± 2.6Aa

[a] Reduction of carcinogenic risk assessment expressed as percentage was based on toxic equivalency factors proposed by Nisbet and LaGoy (1992).
[b] Data are the mean ± standard deviation of 3 independent microcosms; same lowercase and uppercase letters denote the absence of statistically significant differences between column and row means, respectively, as assessed by the Tukey post-hoc test ($P < 0.05$).

A comparison of the 63 day old microcosms showed that the best reduction in the carcinogenic risk assessment (RCRA) was observed in the bioaugmented microcosms (SAS and Abisp). This is a consequence of the higher reduction in the amount of 4- and 5,6-ring PAH which have the highest carcinogenic properties (IARC, 2010). Significant differences were also found when comparing the bioaugmented microcosms. The Abisp microcosm showed higher RCRA than the SAS because of its better efficacy in the removal of BaP and DBahA (Table 2) which exhibited the highest TEF values.

4. Conclusions

The addition of the sterilized spent *A. bisporus* substrate to the PAH-polluted soil was effective in stimulating the resident soil bacteria which resulted in higher levels of 3-ring PAH being removed. Bioaugmentation treatments with *A. bisporus*, in the SAS and Abisp microcosms, were more effective in removing 5, 6-ring PAH in particular BaP. The best detoxification results were obtained in the Absip microcosm where a high retention of microbiological functional activity, a significant decrease in *F. candida* mortality, and a reduction in carcinogenic risk assessment were observed. The wide spatio-temporal availability of this agro-waste combined with its proved efficacy in PAH biodegradation make its use technically feasible for environmental remediation purposes.

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