Biorremediación de suelos contaminados por hidrocarburos pesados y caracterización de comunidades microbianas implicadas

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BIORREMEDIACIÓN DE SUELOS CONTAMINADOS POR HIDROCARBUROS PESADOS Y CARACTERIZACIÓN DE COMUNIDADES MICROBIANAS IMPLICADAS

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CONTENIDO

PRESENTACIÓN DE LA TESIS

La presente tesis doctoral tiene como principal objetivo aportar conocimiento en el área de la biorremediación de suelos contaminados por hidrocarburos, mediante el estudio a escala real y de laboratorio de tratamientos enfocados a mejorar la degradación de los contaminantes y el uso de técnicas moleculares para determinar la biodiversidad microbiana presente en el suelo, así como su evolución a lo largo de dichos tratamientos.

Este reto no se podría haber abordado sin pertenecer a un grupo de investigación con amplia experiencia en el campo de la biodegradación microbiana aeróbica de hidrocarburos del petróleo. Durante los años ochenta y principios de los noventa, los estudios del grupo se centraron en el aislamiento de cepas degradadoras de crudo de petróleo y su capacidad de biodegradación (Solanas, 1981; Solanas et al., 1984; Grimalt et al., 1991), junto con trabajos donde se concluyó la genotoxicidad ambiental presente en matrices contaminadas por hidrocarburos aromáticos policíclicos (HAPs) (Grifoll et al., 1990 y 1992a; Casellas et al., 1995). Como consecuencia de los resultados obtenidos, posteriormente, se abrió una línea de investigación en metabolismo bacteriano de HAPs específicos, consiguiendo aislar varias cepas degradadoras (Grifoll et al., 1992b; Sabaté et al., 1999 y 2003) y describir vías metabólicas de degradación, tanto de HAPs, como de HAPs alquilados, más recalcitrantes y genotóxicos que sus representantes no substituidos. Los hidrocarburos elegidos fueron el fluoreno (Casellas et al., 1997) y el 2-metilfenantreno (Sabaté et al., 1999). A partir del año 1999 se amplía el campo de investigación a la biorremediación de suelos contaminados por hidrocarburos. Esta línea se ha mantenido hasta el día de hoy, demostrando la ralentización, a lo largo del tiempo, de la biodegradación bacteriana de HAPs, debido a un progresivo enriquecimiento de la fracción más recalcitrante y de la baja biodisponibilidad de ciertos compuestos (Sabaté et al., 2006), junto con la importancia de los estudios de biodiversidad microbiana para aumentar el conocimiento de las poblaciones asociadas a degradación de hidrocarburos y sus dinámicas a lo largo de procesos de biorremediación (Viñas et al., 2005). Además, siempre ha sido un objetivo del grupo insistir en la necesidad de realizar estudios de ecotoxicidad para mejorar el análisis de riesgos de suelos contaminados.

Por lo tanto, es gracias al trabajo realizado previamente en el grupo que el presente trabajo de tesis doctoral ha sido posible y nace con el objetivo de dar respuesta a las nuevas preguntas que surgieron durante los anteriores estudios de biorremediación de suelos contaminados por hidrocarburos.

Los estudios llevados a cabo pretenden abordar aspectos todavía no resueltos y por tanto necesitados de investigación. En primer lugar la presencia de hidrocarburos de elevado peso molecular como las fracciones alifáticas pesadas o los HAPs de 4 y 5 anillos, en concentraciones excesivamente elevadas después de aplicar la biotecnología de la biorremediación con técnicas convencionales como la aireación y la adición de nutrientes. Otro aspecto a resolver es la falta de accesibilidad de estas moléculas de excesivo tamaño molecular a aquellos microrganismos capaces de metabolizarlas. Cabe resaltar que mientras el grupo de investigación logró muy buenos resultados en la utilización de biosurfactantes para mejorar la biodisponibilidad de hidrocarburos de elevado peso molecular en medio líquido, los intentos llevados a cabo en distintos suelos, no condujeron a ninguna mejora de la biodegradación. Finalmente el reto más importante que actualmente tiene la comunidad científica, es aumentar el conocimiento de las poblaciones microbianas implicadas en los procesos de biorremediación. Deberíamos dejar la pura descripción para alcanzar el objetivo último que sería conocer qué función está llevando a cabo cada microorganismo identificado. Por suerte, las metodologías moleculares que están evolucionando vertiginosamente nos están ofreciendo el camino. Relacionado con este aspecto, y que quizás no ha sido abordado suficientemente, estaría el conocimiento de las interacciones entre bacterias y hongos o entre poblaciones autóctonas y aquellas introducidas como inóculos en procesos debioaumentación.

El presente trabajo de tesis doctoral aborda todos estos aspectos que se exponen en seis capítulos: (I) a multi-approach assessment to evaluate biostimulation and bioaugmentation strategies for heavily oil-contaminated soil, (II) ensayo piloto de biorremediación por tecnología de la biopila dinámica para la descontaminación de suelos contaminados por creosotas provenientes de las actividades dedicadas a la preparación de la madera, (III) microbial populations related to PAH biodegradation in an aged biostimulated creosote-contaminated soil, (IV) Fungal/bacterial interactions throughout bioremediation assays in an aged creosote polluted soil, (V) Comparative assessment of bioremediation approaches to highly recalcitrant PAH degradation in a

real industrial polluted soil y (VI) Combining DGGE and Bar-Coded Pyrosequencing for microbial community characterization throughout different soil bioremediation strategies in an aged creosote-polluted soil. Cada uno de los seis capítulos contenidos en la tesis ha dado como resultado artículos científicos publicados o en vía de publicación.

Siguiendo los requerimientos para la mención europea, las lenguas escogidas para el desarrollo de la memoria han sido el castellano y el inglés. Tanto los artículos publicados como los enviados constituyen la base de la presente tesis doctoral.

LISTA DE ABREVIACIONES

ADN: Ácido Desoxiribonucleico
ADNc: ADN Complementario
ADNr 16S: ADN Ribosómico 16S
ARN: Ácido Ribonucleico
ARNm: ARN Mensajero
ARNr 16S: ARN Ribosómico 16S
ARNr 18S: ARN Ribosómico 18S
BLAST: Basic Local Alignment
BOE: Boletín Oficial del Estado
BS: Biostimulation
BTEX: Benceno, Tolueno, Etilbenceno y Xileno
CHB: Cultivable Heterotrophic Bacteria
CHDB: Cultivable Hydrocabon-Degrading Bacteria
CERCLA: Comprehensive Environmental Response, Compensation and Liability Act
CMC: Concentración Crítica Miceliar
DGGE: Electroforesis en Geles de Gradiente Desnaturalizante
FISH: Hibridación *In-Situ* Fluorescente
GC-FID: Gas Chromatography with Flame Ionization Detector
GC-MS: Gas Chromatography with Mass Detector
HAPs: Hidrocarburos Aromáticos Policíclicos
HMW: High Molecular Weight
ITS: Internal Transcribed Spacer
LiP: Lignina Peroxidasa
LMEs: Lignin-Modifying Enzymes
LS: Lignocellulosic Substrate
LT: *Lentinus tigrinus*
MAs: Mobilizing Agents
MnP: Manganeso Peroxidasa
MPN: Most Probable Number
NAPLs: Non-Aqueous Phase Liquids
NGR: Nivel Genérico de Referencia
OECD: Organisation for Economic Cooperation and Development

OTU: Unidad Taxonómica Operativa

PAH: Polycyclic Aromatic Hydrocarbon

PCA: Principal Component Analysis

PCBs: Bifeniles Policlorados

PCR: Polymerase Chain Reaction

PLFA: Análisis de los Ácidos Grasos de Fosfolípidos

PM: Peso Molecular

qPCR: Quantitative Real-Time Polymerase Chain Reaction

RDP: Ribosomal Data Project

SIP: Stable Isotope Probing

SO: Soybean Oil

TEFAP: Tag-Encoded FLX Amplicon Pyrosequencing

TGGE: Electroforesis en Geles con Gradiente Térmico

TOE: Total Organic Extracts

TPH: Total Petroleum Hydrocarbons

T-RFLP: Polimorfismo del Fragmento Terminal de Restricción

TV: *Trametes versicolor*

UCM: Unresolved Complex Mixture

UE: Unión Europea

US EPA: Agencia de Protección Medioambiental de los Estados Unidos

WHC: Water Holding Capacity

WRF: White Rot Fungi

LISTA DE PUBLICACIONES

El presente trabajo de tesis se basa en las siguientes publicaciones:

1. **S. Lladó, A.M. Solanas, J. de Lapuente, M. Borras and M. Viñas**. 2012. A diversified approach to evaluate biostimulation and bioaugmentation strategies for heavy-oil contaminated soil. *Science of the Total Environment*, 2012, Vol. 435-436: 262-269.
2. **E. Realp, J.A. Doménech, R. Martínez-García, C. Restrepo, S. Lladó, M. Viñas y A.M. Solanas.** 2008. Ensayo piloto de biorremediación por la tecnología de la biopila dinámica para la descontaminación de suelos contaminados por creosotas provenientes de las actividades dedicadas a la preparación de la madera. *Revista Técnica Residuos*. Año 2008. Año nº 18. Número 103: 38-49.
3. **S. Lladó, N. Jiménez, M. Viñas and A.M. Solanas.** 2009. Microbial populations related to PAH biodegradation in an aged biostimulated creosote-contaminated soil. *Biodegradation*, 2009, Vol. 20(5): 593-601.
4. **S. Lladó, E. Gràcia, A.M. Solanas and M. Viñas.** 2012. Fungal/bacterial interactions throughout bioremediation assays in an aged creosote polluted soil. *Submitted to Soil Biology and Biochemistry.*
5. **S. Lladó, S. Covino, A.M. Solanas, M. Viñas, M. Petruccioli and A. D'Annibale.** 2012. Comparative assessment of bioremediation approaches to highly recalcitrant PAH degradation in a real industrial polluted soil. *Submitted to Science of the Total Environment.*
6. **S. Lladó, S. Covino, A.M. Solanas, M. Petruccioli, A. D'Annibale and M. Viñas.** 2012. Combining DGGE and Bar-Coded Pyrosequencing for microbial community characterization throughout different soil bioremediation strategies in an aged creosote-polluted soil. *Submitted to Soil Biology and Biochemistry.*

Introducción general

1.1 Suelos contaminados por hidrocarburos

1.1.1 El suelo

El suelo constituye la capa superficial del manto terrestre y su profundidad es variable. Está formado por partículas minerales, organismos vivos, materia orgánica, agua y sales. La mayoría de los componentes provienen de la meteorización de rocas, descomposición de restos vegetales y acción de microrganismos, formando uno de los recursos naturales más importantes del planeta.

El suelo es un medio altamente complejo, formado, prevalentemente por tres fases: sólida (50%), líquida y gaseosa. Estas tres fases se pueden organizar de muy diferentes formas, adquiriendo diversas proporciones para dar lugar a centenares de tipos de suelos.

Una de las formas de clasificación de suelos es mediante el tamaño de las partículas que forman su fase sólida, tal y como se puede observar en la tabla adjunta 1.1.

Tabla 1.1 Clasificación de suelos según el tamaño de las partículas

División	Tamaño de partícula (mm)
Grava	Mayor de 2
Arena	2,0 a 0,2
Arena fina	0,2 a 0,02
Limo	0.02 a 0.002
Arcilla	Menor de 0.002

La materia orgánica y los compuestos minerales se organizan en el espacio generando una estructura porosa, donde puede haber agua o aire. En el agua contenida en estos poros hay sales minerales y nutrientes y, por lo tanto, es el medio donde se puede desarrollar la actividad metabólica de los microorganismos que habitan el suelo.

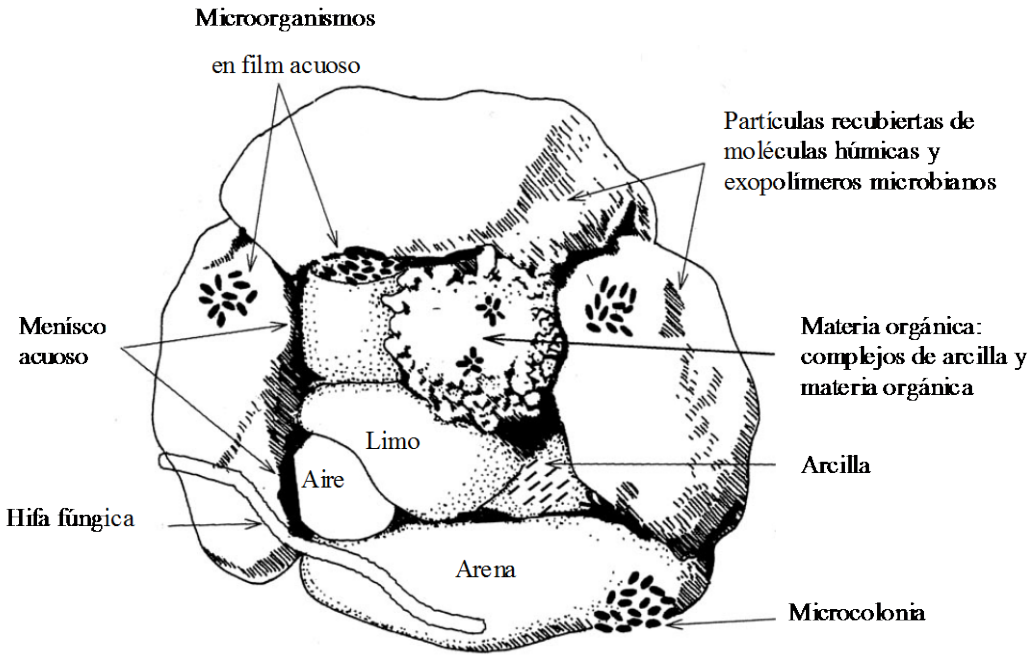

Fig. 1.1. Esquema de la disposición en el espacio de los agregados constituyentes de un suelo

1.1.2 Destino ambiental de los contaminantes orgánicos del suelo

La entrada de contaminantes al medio ambiente va ligada tanto a procesos naturales como antropogénicos. Como resultado de estos últimos (vertidos accidentales, incineración de residuos…), cada año se liberan al medio, grandes cantidades de compuestos químicos orgánicos e inorgánicos. La contaminación de suelos es una consecuencia común de la actividad industrial (Ulrici, 2000).

La distribución de los contaminantes orgánicos en el suelo está muy condicionada por la existencia de tres fases bien diferenciadas. En relación a los contaminantes, estos pueden estar adsorbidos sobre las partículas, la materia orgánica o incluso en disolución o en forma de vapor. Los más hidrofóbicos, como los HAPs y los aceites minerales, suelen estar adsorbidos, mientras que los que lo son menos, suelen estar disueltos en el agua de los poros (Mackay & Betts, 1991; Weissenfels et al., 1992).

En el conjunto del suelo, los contaminantes pueden verse transformados, a causa de la acción de agentes físicos o químicos, o pueden sufrir biodegradación (Bossert & Bartha, 1984), por parte de los microrganismos presentes en el suelo en forma de

microcolonias (Harms & Bosma, 1996). Este potencial degradador de las poblaciones microbianas naturales es explotado por las tecnologías de biorremediación.

1.1.3 Suelos contaminados por hidrocarburos

Los procesos de combustión dan lugar a un tipo de contaminación difusa que afecta mayoritariamente a la atmósfera, pero que a causa de procesos de precipitación y lixiviado se puede acumular en suelos y sedimentos (Casellas et al., 1995). En cambio, las actividades de producción, transporte y utilización de combustibles fósiles dan lugar a emplazamientos definidos, con la posibilidad de la existencia de un alto grado de contaminación. Los hidrocarburos se encuentran en el suelo en forma de mezclas complejas que suelen constituir fases líquidas no acuosas (NAPLs, en inglés).

Los crudos de petróleo son mezclas formadas por diversos miles de compuestos, mayoritariamente hidrocarburos, formado a partir de la fosilización o diagénesis sufrida por restos orgánicos sometidos a condiciones de elevada presión y temperatura durante millones de años (Rosini, 1960). Así mismo, contienen derivados heteroatómicos sulfurados, nitrogeneados y oxigenados, en menor medida, y también metales como hierro, vanadi, níquel, en forma de complejos organometálicos. La composición química de crudos procedentes de diversas regiones puede presentar gran variabilidad, aunque siempre son las mismas familias de hidrocarburos, en mayor o menor medida, las presentes en la mezcla de compuestos (Figura 1.2):

- Hidrocarburos saturados, básicamente n-alcanos e isoprenoides pero también cicloalcanos. Contienen el número máximo de hidrógenos posibles.
- Hidrocarburos aromáticos, mono (con un anillo bencénico) o policíclicos (con más de uno) y/o con sustituyentes alquilados, representan entre un 1 y un 20% de los hidrocarburos totales en la mayoría de crudos.
- Compuestos polares (resinas y asfaltenos), más pesantes, con azufre, nitrógeno y/o oxígeno y pueden contener también metales.

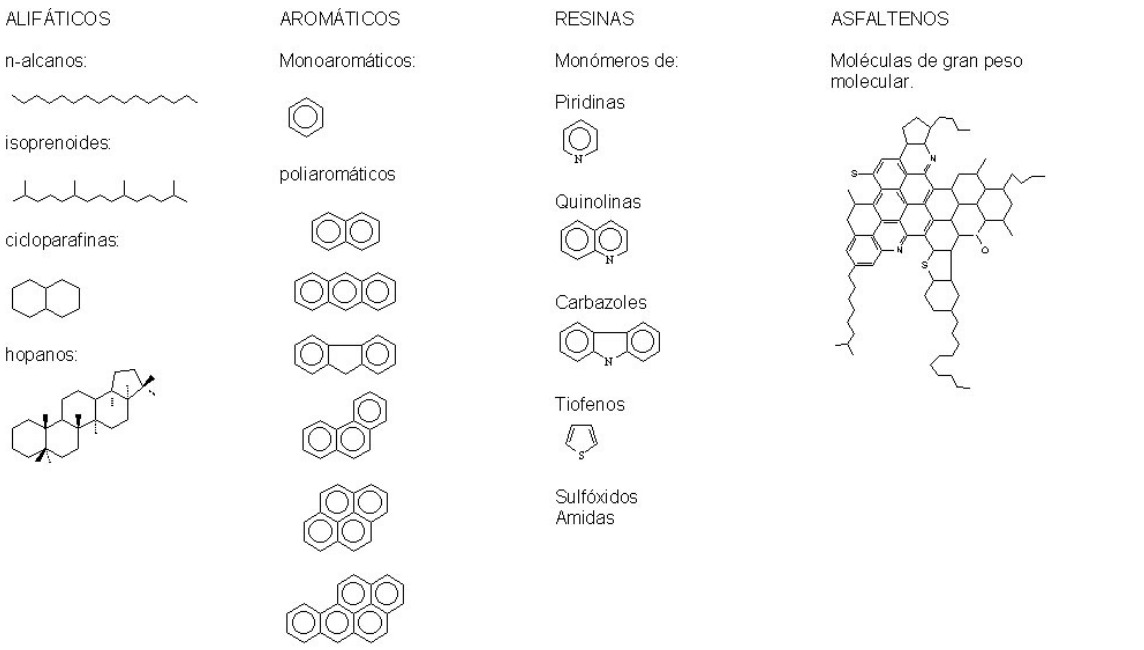

Fig. 1.2. Estructuras químicas de diferentes componentes mayoritarios de un crudo de petróleo.

A partir de la destilación del crudo de petróleo, se producen diferentes fracciones, como se puede observar en la tabla 1.2. Conocer las diferencias entre los hidrocarburos que forman estas fracciones es imprescindible para entender su destino ambiental y para prever la dificultad que implicaría en un proceso de biorremediación. Estas diferencias, a nivel estructural, conducen a diferentes comportamientos en relación a la adsorción a las partículas del suelo y solubilidad en el agua, entre otros procesos que harán de la descontaminación, un proceso más o menos complicado y/o costoso.

El crudo se destila a temperaturas crecientes y como más elevada es la temperatura, más carbonos tienen los hidrocarburos que se obtienen y diferentes sus usos así como su peligrosidad y recalcitrancia como contaminantes.

1.1.3.1 Suelos contaminados por gasolina

La gasolina forma parte de la fracción ligera del crudo de petróleo, ya que se obtiene a entre 20 y 180ºC (Tabla 1.2), aunque su producción es menos directa que la del diesel y los fueles. Este hecho implica que los suelos contaminados por gasolina son

ricos en hidrocarburos de cadena corta (C_6-C_{11}) y no en otros de cadena más larga, como en el caso de suelos contaminados por otros derivados del petróleo.

Tabla 1.2. Fracciones que se pueden obtener en la destilación fraccionada de un crudo de petróleo.

Fracciones	Tª ebullición (ºC)	Composición	Uso
Gasolina ligera	20-100	C_5H_{12}-C_7H_{16}	Disolvente
Benzina	70-90	C_6-C_7	Lavado en seco
Ligroína	80-120	C_6-C_8	Disolvente
Gasolina	20-180	C_6-C_{11}	Carburante
Queroseno, Jet fuel	200-300	C_{12}-C_{16}	Iluminación y carburante
Gasoil. Diesel	200-350	C_{13}-C_{18}	Carburante
Aceite lubricante	200-350	C_{16}-C_{20}	Lubricantes
Grasas , vaselinas	250-400	C_{18}-C_{22}	Farmacéutica
Cera de parafina	245-540	C_{20}-C_{45}	Velas
Betún asfáltico (35% peso)	> 540	C_{30}-C_{45}	Alquitrán asfáltico Coque de petróleo

Los componentes más importantes de la gasolina y, por lo tanto, los contaminantes más abundantes en suelos con presencia de esta substancia son: n-butano, isopentano, pentano, mono y dimetilpentanos, hexano, BTEX, mono y dimetilhexanos, trimetilbenzenos, metiletilbenzenos, naftalenos y heptano, estos dos últimos en menor grado. Entre ellos, los BTEX generan una gran preocupación medioambiental debido a su toxicidad y relativamente elevada solubilidad en agua.

1.1.3.2 Suelos contaminados por diesel

El diesel forma parte de la fracción mediana del crudo de petróleo, esto implica un rango de puntos de ebullición entre 200 y 350ºC (Tabla 1.2). Dentro del diesel se encuentran compuestos de entre 10 y 25 átomos de carbono, aunque los más abundantes son los que en tienen entre 15 y 17 (Figura 1.3). En el cas de suelos contaminados por diesel, encontraremos, por lo tanto, compuestos más pesados que en los suelos contaminados por gasolina. Estos compuestos son: n-alcanos e isoprenoides (parafinas), cicloalcanos (naftenos) y aromáticos. Los más abundantes son los cicloalcanos,

representando un 45% del total de hidrocarburos del diesel. En general, los alcanos no se considera tóxicos, sin embargo, los compuestos de menor peso molecular pueden causar daños por inhalación o por su acción como disolventes.

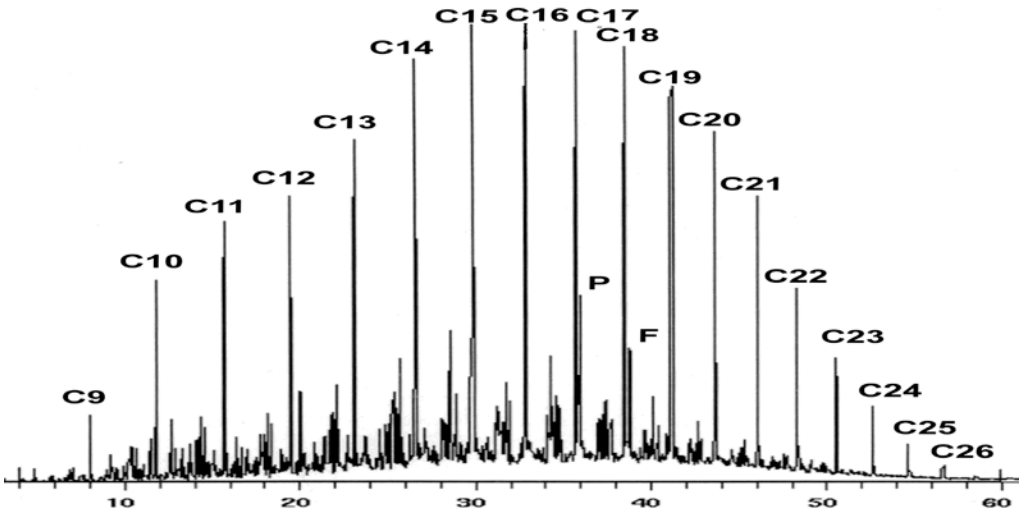

Fig. 1.3. Perfil cromatográfico de GC-MS, de diesel. P, pristano; F, fitano.

1.1.3.3 Suelos contaminados por aceites minerales

Los aceites minerales son productos muy utilizados en motores, luces e, incluso, como disolventes. Provienen del crudo de petróleo y se obtienen gracias al proceso de refinado. Su composición es una mezcla de hidrocarburos alifáticos y aromáticos. Los aceites minerales se diferencian entre ellos por los rangos de ebullición de los compuestos presentes en su estructura. Los más pesados son ricos en compuestos de entre 25 y 40 carbonos y, por lo tanto, tienen unos rangos de temperatura de ebullición más elevados que los aceites minerales ligeros, más ricos en compuestos de entre 15 y 25 carbonos. Por otro lado, los suelos contaminados con aceites minerales presentan un alto contenido de alcanos alifáticos y cicloalcanos, que son los hidrocarburos que tienen un papel principal en la composición de los aceites minerales, ya que forman parte en un 80-90%, mientras que los compuestos aromáticos solo representan entre un 10 y un 20% del total de hidrocarburos presentes en estos suelos, como se hablará más adelante en el capítulo 3 de la presente tesis, donde se presenta un ensayo de tratabilidad realizado a escala de laboratorio en un suelo contaminado por aceites minerales.

1.1.3.4 Suelos contaminados por creosota

La creosota es un producto líquido y viscoso que se utiliza para la conservación de la madera, de la cual se hablará en más profundidad durante el capítulo 4, donde se realizó un ensayo piloto en campo, con un suelo industrial contaminado por este producto derivado del carbón.

El producto final, se obtiene por procesos de destilación a entre 200 y 400ºC de alquitranes procedentes de la combustión de carbones minerales grasosos, como la hulla. Está formada por, entre, 150 y 200 compuestos diferentes, aunque un 85% de estos compuestos son hidrocarburos aromáticos policíclicos (PAHs), un 10% son compuestos fenólicos y el 5% restante son compuestos heterocíclicos (Nestler, 1974). El 50% de los PAHs de la creosota son de entre 2 y 3 anillos, es decir, de bajo peso molecular (Mueller et al., 1989). Aunque, como se verá con mayor amplitud entre los capítulos 5 y 8, donde se trabaja con un suelo contaminado por creosota después de haber sufrido el ensayo descrito en el capítulo 4, son los PAHs de mayor peso molecular, entre 4 y 6 anillos, los que suponen un mayor reto en un posible proceso de biorremediación, debido a sus propiedades físico-químicas. Actualmente, la fabricación y utilización de creosota en Europa y Estados Unidos está prohibida, aunque los emplazamientos contaminados son numerosos.

En relación a los suelos contaminados por creosota, los diferentes tipos de compuestos que la forman se comportan de forma diferente. Los compuestos fenólicos y algunos hidrocarburos heterocíclicos presentan una gran solubilidad. Este hecho causa que los contaminantes puedan ser movilizados por el agua del suelo y puedan afectar a sistemas acuosos colindantes con el suelo contaminado, eso incluye tanto aguas superficiales como subterráneas. Por otro lado, los compuestos contaminantes más volátiles pueden disminuir su concentración porque pasan a la atmosfera. Dentro de este grupo destacan los HAPs de 2 anillos y los compuestos fenólicos y heterocíclicos de bajo pes molecular.

1.1.4 HAPs

Los hidrocarburos aromáticos policíclicos son compuestos químicos formados per dos o más bencenos fusionados. Se conocen unos 100 tipos diferentes de HAPs. La estructura atómica del anillo bencénico les confiere una gran estabilidad (Dagley, 1981).

Los HAPs pueden tener diferentes orígenes, pero los dos más importantes son la pirolisis, que es la exposición de moléculas orgánicas a altas temperaturas, y la petrogénesis, que consiste en una exposición a menor temperatura pero a presiones mucho más elevadas, durante millones de años (Blumer, 1976).

Tabla 1.3. Peso molecular (PM) y solubilidad de los 16 HAPs incluidos en la lista de contaminantes de estudio prioritario de la EPA.

Compuesto	PM	Hidrosolubilidad (mg/L)	Factor carcinogénico (Nisbet & La Goy, 1992)
Naftaleno	128	31,7	0,001
Acenaftileno	152	16,1	0,001
Acenafteno	154	3,9	0,001
Fluoreno	166	1,8	0,001
Fenantreno	178	1,3	0,001
Antraceno	178	0,07	0,01
Fluoranteno	202	0,26	0,001
Pireno	202	0,14	0,001
Benz(a)antraceno	228	0,002	0,1
Criseno	228	0,0006	0,01
Benzo(b)fluoranteno	252	0,0012	0,1
Benzo(k)fluoranteno	252	0,00055	0,1
Benzo(a)pireno	252	0,003	1
Dibenzo(a,h)antraceno	278	0,0005	5
Benzo(g,h,i)perileno	276	0,00026	0,01
Indeno(1,2,3-cd)pireno	276	0,062	0,1

Las características fisicoquímicas de los HAPs son las que condicionan su comportamiento, en el medio ambiente. Las dos más importantes son la hidrofobicidad y la recalcitrancia. Ambas aumentan como más anillos bencénicos presente la estructura

del hidrocarburo. Debido a sus propiedades hidrofóbicas, los HAPs tienden a adsorberse a las superficies, hecho que dificulta su degradación (Clements et al., 1994). Además de su elevada persistencia ambiental, los HAPs suponen un riesgo para la salud pública y en 1979 la Agencia de Protección Ambiental de los Estados Unidos (US EPA, en inglés) incluyó los 16 HAPs de 2 a 6 anillos más frecuentes en su lista de contaminantes de investigación prioritaria (Keith & Telliard, 1979).

Los HAPs pueden acumularse en la cadena trófica y presentan toxicidad por inhalación, contacto o ingestión (Eisler, 1987). Su baja hidrosolubilidad (Tabla 1.3) los convierte en altamente liposolubles, favoreciendo su absorción a través del tracto intestinal de mamíferos y su rápida distribución en los tejidos, preferentemente en el adiposo (Twiss et al., 1999). Especialmente, los de elevado peso molecular como el benzo(a)antraceno, criseno, benzo(b)fluoranteno, benzo(k)fluoranteno, benzo(a)pireno, dibenzo(a,h)antraceno e indeno(1,2,3-c,d)pireno, poseen potencial carcinogénico y mutagénico, ya que forman aductos con el ADN tras su activación biológica (Goldman, 2001). De entre los 16 HAPs prioritarios de la EPA, el benzo(a)pireno está descrito como uno de los más potentes carcinógenos (Juhasz & Naidu, 2000); el potencial carcinogénico de otros HAPs se representa en relación al del benzo(a)pireno, como se puede observar en la tabla 1.3.

En el capítulo 5 el trabajo se centra en el benzo(a)antraceno y el criseno (Fig 1.4) ejemplos de HAPs de cuatro anillos aromáticos, durante la realización de una estrategia diseñada para llegar a conocer mejor las poblaciones bacterianas presentes en el suelo contaminado por creosota asociadas a su degradación. Estos dos HAPs, al ser de elevado peso molecular, presentan elevadas tasas de hidrofobicidad y de recalcitrancia.

Finalmente, a partir del capítulo 6 el estudio se cierne sobre todos los HAPs de entre 4 y 5 anillos aromáticos, incluidos en la lista de los 16 más importantes para la EPA.

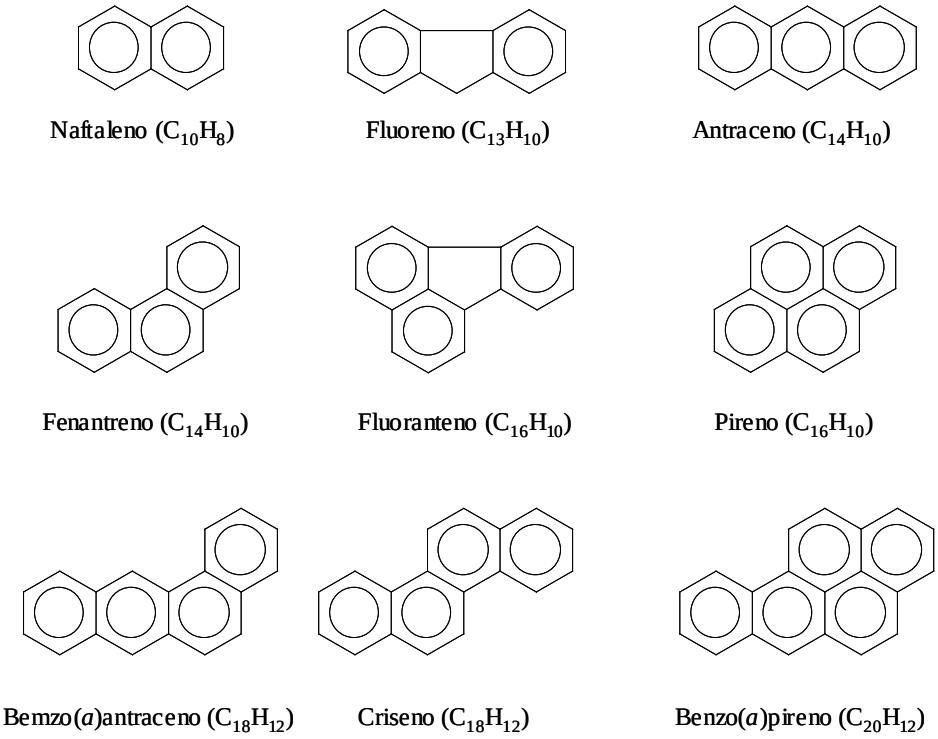

Fig. 1.4. Estructura química de hidrocarburos aromáticos policíclicos representativos

1.1.5 Marco legal

El programa CERCLA (Comprehensive Environmental Response, Compensation and Liability Act), gestionado por la US EPA y aprobado por parte del congreso de los Estados Unidos en 1980, fue el primero en responder al despertar de las preocupaciones de las sociedades sobre el riesgo que suponen los emplazamientos contaminados para la salud humana. Este programa determina las prohibiciones y requerimientos en relación a los sitios contaminados y establece, por primera vez, la responsabilidad de la contaminación.

Casi dos décadas después, en 1996, la Unión Europea estableció la directiva 96/61, con aspectos generales relacionados con la prevención y el control de la contaminación. Esto obligó a los distintos países a realizar tareas de identificación de los emplazamientos contaminados, con especial interés en aquellos dedicados a actividades industriales, militares y vertederos de residuos.

En España, siguiendo las directrices europeas, se creó el Plan Nacional de Recuperación de Suelos Contaminados, que obligó a cada una de las comunidades autónomas a realizar un inventario de los posibles emplazamientos contaminados. A este respecto, se dictó la ley de Residuos (Ley 10/1998, del 21 de abril), apoyada en el Real Decreto 9/2005, del 14 de enero. Factores clave, como la definición del concepto de suelo contaminado y de riesgo inaceptable, la realización de un inventario de suelos contaminados, el listado de las actividades potencialmente contaminantes o el régimen de responsabilidades, se establecen por primera vez en España, junto con los niveles de referencia de los contaminantes (NGR), a partir de los cuales se pudieran llevar a cabo los estudios de riesgo previo a la declaración de un suelo como contaminado.

Sin embargo tal, en la Unión Europea (UE) existen diferentes NGR dependiendo del país legislador, con incluso marcadas diferencias en los valores de ciertos hidrocarburos considerados de alto riesgo por la EPA. Estas marcadas diferencias, así como la ausencia de algunos compuestos, implican que todavía será necesario un tiempo para completar e incluso corregir algunos valores ya definidos. Además no se contempla el hecho de que los contaminantes, aun estando a una misma concentración, pueden tener efectos muy diferentes, dependiendo del suelo (tanto por la textura como por el contenido en materia orgánica) y de su biodisponibilidad real.

Posteriormente, con la ley 22/2011, del 28 de julio, se matizaron en España ciertos conceptos como la determinación de los sujetos responsables de la contaminación, así como las obligaciones de información a la que quedan sujetos tanto los titulares de las actividades potencialmente contaminantes como los titulares de los suelos contaminados.

Por otro lado, el marco normativo vigente en Catalunya, está configurado, además, por el decreto legislativo 1/2009, de 21 de julio, que modificó la ley reguladora de residuos 6/1993, de 15 de julio.

Una vez llevado a cabo el análisis de riesgos, si se aconseja la descontaminación del suelo, hay que tener en cuenta tanto el tiempo del proceso, como el coste económico de las diferentes metodologías potenciales.

Desafortunadamente, con la llegada de la crisis económica, se produjo una grave ralentización de muchos trabajos de descontaminación, básicamente, por razones presupuestarias. De forma un tanto contradictoria, el gran golpe recibido por el sector inmobiliario en nuestro país, se traduce en menos limpieza de suelos contaminados, al existir menos proyectos urbanísticos. Este hecho se constata con la reducción, durante el periodo 2009-2010, de la facturación de las empresas del sector en más del 60% para los trabajos de recuperación, con respecto a años anteriores en el estado español.

1.2. Metabolismo microbiano de hidrocarburos

La transformación microbiana de los compuestos orgánicos va ligada a dos procesos principales, el crecimiento y el cometabolismo. La utilización de un sustrato para el crecimiento por parte de catabolismo microbiano siempre implica el mismo principio básico: una degradación gradual de la molécula para formar al final uno o más fragmentos capaces de pasar a metabolismo central. Durante el proceso conocido como mineralización, una parte de los elementos que constituyen la materia orgánica son convertidos en productos inorgánicos como CO_2 o H_2O. En algunos casos, sólo una parte del sustrato es degradado, mientras que el resto del compuesto persiste en forma parcialmente oxidada. Por su parte, el cometabolismo se basa en la transformación o metabolización de un compuesto orgánico por un microrganismo que no es capaz de utilizarlo como fuente de carbono y energía. De hecho, la mayoría de transformaciones cometabólicas y las de degradación parcial son producto de la baja especificidad de algunos enzimas presentes en las rutas metabólicas de degradación.

La biodegradación de hidrocarburos ha sido descrita tanto en condiciones aeróbicas como anaeróbicas, aunque los procesos anaeróbicos de degradación son más lentos y los mecanismos bioquímicos todavía no están descritos, en su mayoría (Meckenstock & Mouttaki, 2011). Recientemente, nuevas rutas de biodegradación anaeróbica, por bacterias sulfato reductoras, han sido propuestas para el fluoreno y el fenantreno (Tsai et al., 2009). En cambio, las rutas aeróbicas para hidrocarburos alifáticos y HAPs de hasta tres anillos están bien caracterizadas. En presencia de oxígeno, las reacciones clave para la biodegradación de hidrocarburos están catalizadas por oxigenasas tanto en hongos como en bacterias, que actúan incorporando átomos de

oxígeno, procedentes de oxígeno molecular (O_2), al sustrato. Las monooxigenasas incorporan un solo átomo de oxígeno y el otro es reducido a agua, mientras que las dioxigenasas incorporan ambos átomos de oxígeno. Como consecuencia, se entiende que los microrganismos degradadores de hidrocarburos, en condiciones aeróbicas, requieren la presencia de oxígeno tanto para realizar la oxidación inicial del sustrato como al final de la cadena respiratoria, donde su papel es de aceptor final de electrones.

1.2.1 Degradación de hidrocarburos alifáticos

Los n-alcanos son los compuestos alifáticos que se degradan más rápidamente en una mezcla de hidrocarburos (Atlas, 1981). Tanto en hongos como en bacterias, el paso clave para la biodegradación de este tipo de hidrocarburos es una monooxigenación inicial de la cadena hidrocarbonada. En función de la posición en que se produce esta oxidación, se han descrito 3 rutas metabólicas distintas (Figura 1.5). En la ruta mayoritaria, la oxidación terminal de la molécula, para formar un grupo alcohol, va seguida de una segunda a aldehído y una tercera al ácido graso correspondiente. Estos ácidos grasos pueden incorporarse a metabolismo central vía β-oxidación, donde se generan otros ácidos grasos de cadena más corta. Los n-alcanos de entre C_{10}-C_{20} son los más biodegradables, ya que al aumentar su peso molecular disminuye su solubilidad en el agua (Kremer & Anke, 1997; Watkinson & Morgan, 1990).

Para un mismo número de carbonos, los alcanos ramificados, como los isoprenoides, presentan una biodegradabilidad inferior (Alexander, 1999). De hecho, la oxidación de isoprenoides puede verse inhibida por la presencia de n-alcanos (Pirnik et al., 1974). También se ha descrito la degradación de cicloalcanos o compuestos que contienen un anillo alifático en su estructura (Trower et al., 1985).

Por otro lado, poco se conoce de la degradación de este tipo de hidrocarburos mediante enzimas extracelulares de hongos de podredumbre blanca o ligninolíticos, ampliamente descritos, en cambio, como capaces de romper el anillo aromático de los HAPs. Finalmente, enzimas como el citocromo P450 pueden jugar un papel importante en la degradación de hidrocarburos alifáticos, tanto en microrganismos eucariotas como procariotas (van Beilen & Funhoff, 2005). Un ejemplo, muy bien estudiado, de asimilación de alcanos relacionado con el citocromo P450 se realizó con especies de

Candida. El sistema monooxigenasa requerido comprende diferentes enzimas citocromo P450 inducidos por alcanos (Scheller et al., 1996; Seghezzi et al., 1991).

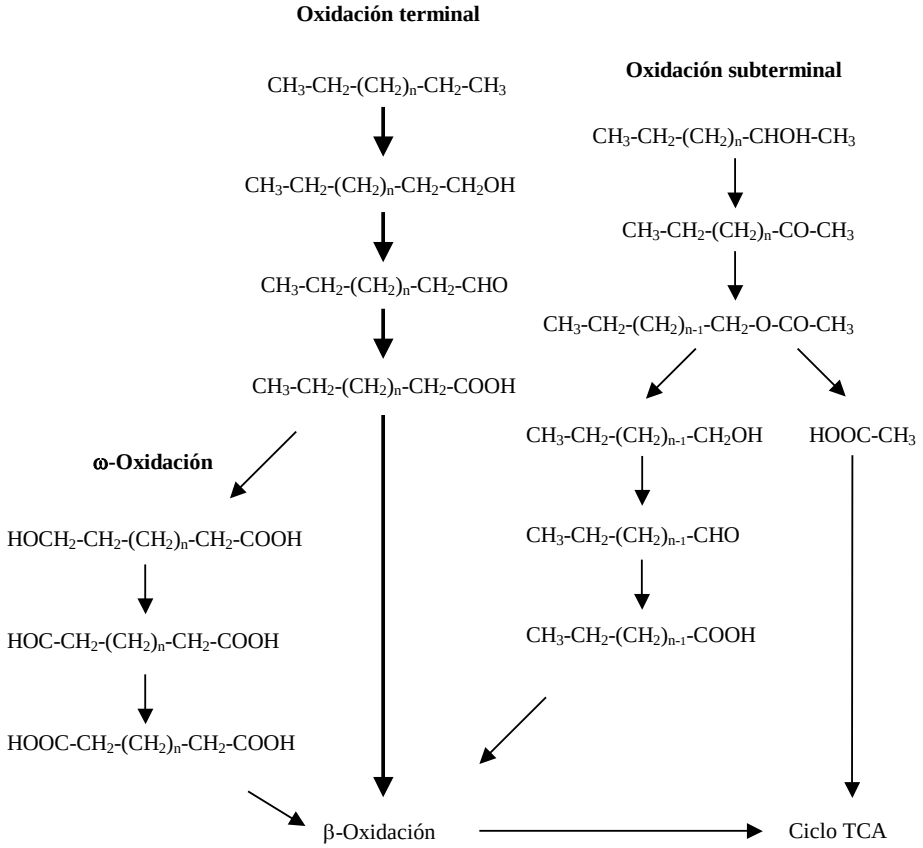

Fig. 1.5. Rutas de degradación microbiana de n-alcanos (Adaptado de (van Beilen & Witholt, 2003))

1.2.2 Degradación de HAPs

En general, los compuestos aromáticos se degradan con más dificultad, respecto a los hidrocarburos alifáticos, debido a la mayor estabilidad de los enlaces entre carbonos presentes en su estructura. Como en los alcanos, la degradabilidad disminuye al aumentar el número de carbonos o anillos aromáticos (Prince et al., 2003). A pesar de su elevada estabilidad, la capacidad de degradar compuestos aromáticos se ha descrito en una gran variedad de microrganismos, entre ellos, bacterias y hongos capaces de degradar compuestos de entre 1 y 5 anillos aromáticos (Cerniglia, 1992; Kanaly & Harayama, 2000), debido a que es una de las estructuras químicas más ampliamente distribuidas en la naturaleza, formando parte de compuestos mono y poliaromáticos, así como de compuestos más complejos como la lignina (Dagley, 1981).

Para desestabilizar el anillo aromático, los microrganismos han desarrollado una estrategia común que consiste en la activación mediante la introducción de uno o dos grupos hidroxilo, mediante reacciones catalizadas por mono- o dioxigenasas, respectivamente (Harayama & Rekik, 1989). Las diferentes estrategias existentes en la naturaleza pueden observarse en la figura 1.6.

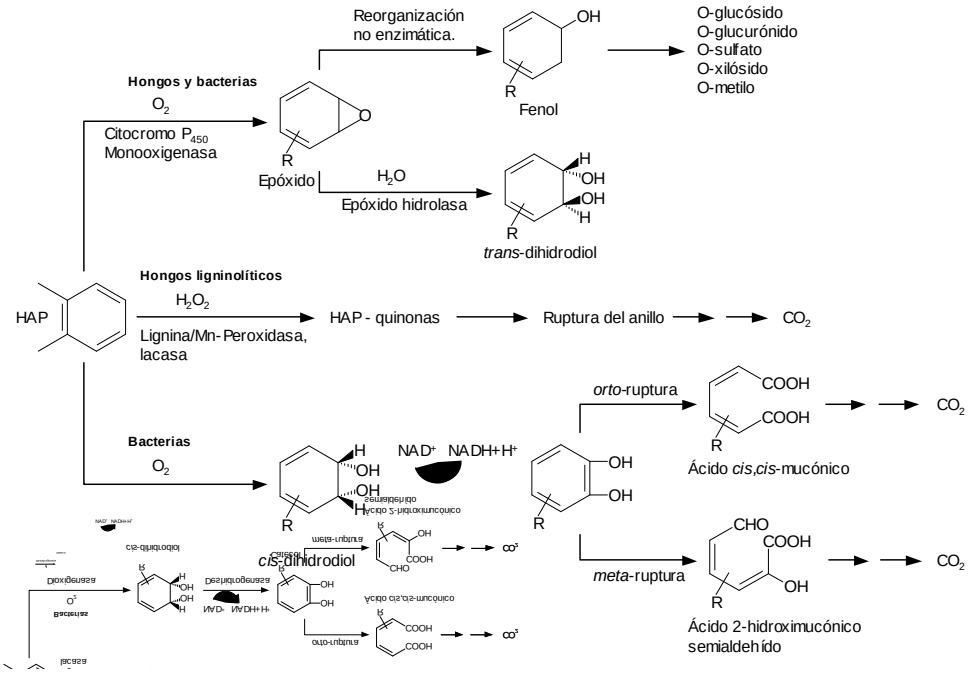

Fig. 1.6. Primeras reacciones de la degradación y/o transformación de los hidrocarburos aromáticos por bacterias y hongos (Adaptado de (Kästner, 2000)).

El metabolismo de los HAPs por parte de microrganismos ha sido un tema de gran interés para varios investigadores (Bamforth & Singleton, 2005; Cerniglia, 1992; Haritash & Kaushik, 2009; Juhasz & Naidu, 2000; Sutherland et al., 1995).

1.2.2.1 Biodegradación bacteriana de HAPs

Actualmente, numerosos géneros bacterianos han sido descritos como degradadores de HAPs. Una gran diversidad bacteriana es capaz de degradar los HAPs de bajo peso molecular y unos cuantos géneros (*i.e. Rhodococcus*, *Mycobacterium*, *Alcaligenes*, *Pseudomonas*, *Arthrobacter* o *Nocardia*, entre otros) han sido catalogados como degradadores de HAPs de elevado peso molecular.

El principal mecanismo, que presentan la mayoría de bacterias, para degradar HAPs de forma aeróbica consiste en la oxidación del anillo bencénico por parte de dioxigenasas para formar *cis*-dihidrodiol, como se puede observar en la figura 1.6. Estos dihidrodioles son dehidrogenados, formando intermediarios dihidroxilados, que pueden ser metabolizados via catecoles, después de entrar a metabolismo central, hasta CO_2 y agua. Por su parte, algunas bacterias pueden catalizar la degradación de HAPs a *trans*-dihidrodioles, mediante la acción del enzima citocromo P450 monooxigenasa, como *Mycobacterium* sp. (Kelley et al., 1990).

Durante las dos últimas décadas, se han descrito géneros bacterianos (*Pseudomonas, Agrobacterium, Bacillus, Burkholderia, Sphingomonas, Rhodococcus, Mycobacterium* o *Flavobacterium*) capaces incluso de degradar benzo(a)pireno (Aitken et al., 1998; Schneider et al., 1996) y se han propuesto rutas metabólicas donde las dioxigenasas tienen un papel principal (Schneider et al., 1996). Finalmente, se ha descrito también que ciertas cepas bacterianas pueden degradar benzo(a)pireno, de forma cometabólica, cuando crecen a partir de otra fuente de carbono (Ye et al., 1995).

1.2.2.2 Biodegradación fúngica de HAPs

Aunque se ha avanzado mucho durante los últimos veinte años, el conocimiento actual sobre el metabolismo fúngico de HAPs es mucho menor que el referente al metabolismo bacteriano. Sin embargo, los hongos son tan importantes como las bacterias en la biorremediación de HAPs en ambientes acuáticos y en suelos. En los capítulos 6 y 8 de la presente tesis se presentan dos estudios llevados a cabo con el objetivo de aportar conocimiento sobre las comunidades tanto bacterianas como fúngicas en suelos contaminados por HAPs de elevado peso molecular.

Al contrario de las bacterias, los hongos no asimilan los HAPs como fuente de carbono y energía, así que requieren de cometabolismo para detoxificarlos (Casillas et al., 1996). En general, los hongos son más lentos y menos eficientes que las bacterias en la degradación de HAPs. Por el contrario, la mayoría de cepas bacterianas no son capaces de degradar de forma eficiente HAPs de 4 o más anillos bencénicos, mientras que los hongos pueden incluso mineralizar HAPs de más de 4 anillos aromáticos. La oxidación de HAPs es un preludio para su asimilación, mientras que en hongos es un

primer paso hacia la detoxificación. El papel de los hongos en ecología es muy importante, ya que los metabolitos formados, más polares y más reactivos que los productos parentales, pueden ser mineralizados o detoxificados por la población autóctona bacteriana del suelo.

Se ha demostrado que varias especies fúngicas pueden metabolizar HAPs, como el zigomiceto *Cunninghamella elegans*, ascomicetos *Aspergillus niger* y *Penicillium* sp., y los basidiomicetos de podredumbre blanca *Phanerochaete chrysosporium*, *Trametes versicolor*, *Pleurotus ostreatus* y *Lentinus tigrinus.* Tanto *Trametes versicolor* como *Lentinus tigrinus* han sido utilizados en distintos capítulos de la presente tesis, con el objetivo de mejorar la biodegradación de HAPs de elevado peso molecular, teniendo en cuenta sus capacidades metabólicas.

En general, los hongos filamentosos y las levaduras forman *trans*-dihidrodioles, dihidrodiol epóxidos, quinonas y fenoles, a la vez que productos conjugativos que se forman a partir del fenol (Figura 1.6). Estos productos conjugativos no son mutagénicos, mientras que los productos oxidativos son tóxicos y bioactivos. Las rutas metabólicas de degradación de HAPs por hongos involucran diversos enzimas, como el citocromo intracelular P450 y los extracelulares lignina peroxidasa, manganeso peroxidasa y lacasa, de las cuales se hablará con más detalle en la sección 1.3.3.3.2 de esta introducción. La formación de metabolitos hidroxilados es muy importante para la biorremediación, ya que incrementa la mineralización de dichos compuestos.

1.3. Biorremediación de suelos

La biorremediación es una tecnología basada en la utilización de los microorganismos y su potencial metabólico degradador para eliminar o transformar los contaminantes del medio en productos inocuos (Alexander, 1999). Tiene ciertas ventajas respecto a los métodos fisico-químicos (excavación, extracción química e incineración), ya sea por su bajo coste económico (Ulrici, 2000), como por la no afectación de otros compartimentos ambientales y la optimización de los recursos (Exner, 1994; Klein, 2000).

La capacidad metabólica de las poblaciones microbianas, frente a los contaminantes presentes en un suelo, es el fundamento sobre el que se sustenta la tecnología de la biorremediació (Alexander, 1999). Generalmente, en un suelo con contaminación recurrente o con episodios previos de contaminación, las poblaciones microbianas autóctonas se habrán seleccionado en favor de la metabolización del contaminante, el cual puede ser transformado con mayor rapidez que la materia orgánica húmica del suelo (Kästner, 2000). Por este motivo, en emplazamientos previamente contaminados, la bioestimulación de la población microbiana indígena, tanto hongos como bacterias, puede acelerar el proceso de biodegradación de los contaminantes (Alexander, 1999). Únicamente en aquellos casos de contaminación puntual o de compuestos de gran recalcitrancia, puede ser necesaria la inoculación de poblaciones alóctonas, con capacidades degradativas especializadas, para posibilitar la degradación de los contaminantes existentes, técnica conocida como bioaumento. Sin embargo, a menudo la falta de adaptación de las poblaciones alóctonas puede poner en peligro su supervivencia (Atlas, 1993; Dejonghe et al., 2001; Kästner, 2000). En los capítulos 6 y 7 de la presente tesis se entrará a valorar la conveniencia de aplicar un bioaumento fúngico, con dos cepas alóctonas, en un suelo donde los contaminantes remanentes tienen elevados niveles de recalcitrancia.

1.3.1 Tecnologías de biorremediación: ventajas y desventajas de su uso

La biorremediación tiene como objetivo degradar o transformar compuestos peligrosos, utilizando microrganismos (normalmente, bacterias heterótrofas y hongos), en otros menos dañinos como: CO_2, agua, sales inorgánicas y/o biomasa. Los tratamientos biológicos llevan muchos años siendo importantes en el tratamiento de aguas residuales, tanto industriales como urbanas, pero es en los últimos tiempos cuando empiezan a implementarse de forma habitual en el tratamiento de aguas subterráneas y suelos (US-EPA, 2000; US-EPA, 1998).

Así como en los tratamientos físico-químicos, las técnicas de biorremediación se pueden clasificar en *in situ* o *ex situ*. En los tratamientos *ex situ* el suelo es excavado y transportado hasta la localización donde se implementará la tecnología, mientras que en los *in situ* el suelo es tratado en su emplazamiento natural. Esto conlleva una serie de ventajas como un mayor abanico de tecnologías disponibles, mejor control sobre el

proceso y su evolución junto con una mayor velocidad y homogeneidad, que hacen preferibles los tratamientos *in situ* en muchos casos. En cambio, el proceso de excavación y transporte conlleva mayores costes pero las tecnologías asociadas pueden ser más efectivas en relación a contaminantes de elevada persistencia en el ambiente.

Los tratamientos *in situ* más comunes son:

- *Atenuación natural*: proceso natural de descontaminación sin alterar las condiciones del suelo.
- *Bioventing aeróbico*: Suelos con bajos niveles de oxígeno son tratados insuflando aire u oxígeno, para facilitar la biodegradación aeróbica de los contaminantes. Se podrían añadir otros aditivos, como nutrientes o surfactantes, en caso que se considerara necesario.
- *Bioventing cometabólico*: Muy similar al bioventing aeróbico, pero añadiendo un sustrato orgánico apropiado.
- *Bioventing anaeróbico*: Se inyecta nitrógeno y un donador de electrones en lugar de aire, generando así condiciones anaeróbicas en el suelo.

Por otro lado, los tratamientos *ex situ* se pueden clasificar según si se implementan en la zona contaminada (*on site*) o en un emplazamiento adyacente (*off site*). Los más comunes son:

- *Landfarming*: Se genera una capa de entre 30 y 40 cm de suelo contaminado para promocionar la degradación aeróbica de contaminantes. Los suelos se airean periódicamente y se añade agua para controlar la humedad cuando sea necesario. En algunos casos, se puede suplementar nutrientes o incluso moderar el pH del suelo.
- *Compostaje*: Proceso biológicamente controlado que trata contaminantes orgánicos usando microrganismos en condiciones termófilas (40-50ºC). El suelo se excava y se mezcla con compuestos para aumentar su porosidad. La degradación de estos compuestos añadidos, genera las condiciones termofílicas.
- *Biopila*: Tecnología similar al landfarming pero la capa de suelo puede llegar a tener entre 2 y 3 metros de alto. Humedad, nutrientes, temperatura, pH y concentración de oxígeno son controladas durante el

proceso. En el capítulo 4 se presentará el trabajo realizado para construir una biopila con un suelo contaminado por creosota, así como los resultados de biodegradación de los diferentes HAPs.

- *Biorreactores en "slurry"*: Es una tecnología costosa y se suele aplicar para casos de contaminantes persistentes y altamente recalcitrantes. El suelo se suele tamizar y se suspende en un tanque de agua. La suspensión y la agitación del suelo deberían promover la transferencia de masas y aumentar el contacto entre los compuestos contaminantes y la microbiota del suelo. En los capítulos 5 y 6 se presentan dos diseños experimentales en "slurry" a escala de laboratorio.

Resumiendo, los costes de las técnicas biológicas son más rentables económicamente que las técnicas físicoquímicas (Tabla 1.4) y no afectan otros compartimentos ambientales. Una de las limitaciones que presentan las técnicas biológicas respecto a las técnicas físico-químicas es el tiempo necesario para alcanzar una biodegradación aceptable. Durante un proceso de biorremediación se produce una ralentización del proceso de biodegradación ya sea por un enriquecimiento de componentes más recalcitrantes o por una disminución de la biodisponibilidad de los contaminantes (Alexander, 2000; Alexander, 1999).

Tabla 1.4. Costes económicos de la remediación de suelos contaminados, según diferentes técnicas (datos obtenidos de la empresa Geotecnia 2000 en el año 2011).

Tipo de tratamiento	Coste medio (€/m^3)
Bioventing/Biosparging	26-28
Biopila	42-46
Landfarming	40-43
Lavado del suelo	68
Excavar + vertedero	70

Sin embargo, las técnicas físico-químicas, aun pudiendo ser más rápidas y efectivas en la disminución de la concentración de contaminantes, alteran o eliminan por completo la microbiota autóctona del suelo, modifican las características físico-químicas del suelo, y además, no eliminan los contaminantes, si no que los trasladan a

otro compartimento ambiental. Por lo tanto no cumplen con los criterios de sostenibilidad en los que se debería basar la ley de protección de suelos.

1.3.2 Factores que limitan la biorremediación

Un buen número de factores que afectan la biodegradación de hidrocarburos derivados del petróleo han sido ampliamente descritos (Bamforth & Singleton, 2005; Das & Chandran, 2011). La estructura de la matriz contaminante puede ser uno de los factores más importantes y por eso debe estar muy bien caracterizada, durante los ensayos de tratabilidad de suelos contaminados. Por otro lado, factores físicos como la temperatura, el pH y la humedad siempre se deben tener en cuenta, ya que afectan directamente a las características químicas de los compuestos contaminantes, como su solubilidad en agua, así como a la fisiología y diversidad de la microbiota autóctona del suelo (Das & Chandran, 2011). Además, la presencia de tanto nutrientes inorgánicos (nitrógeno, fósforo, potasio…) como aceptores de electrones es básica para que las tecnologías implementadas tengan resultados aceptables. En suelos contaminados, donde la presencia de carbono orgánico suele ser alta, debido a la naturaleza del contaminante, los nutrientes disponibles pueden agotarse rápidamente (Breedveld & Sparrevik, 2001). Las proporciones molares de C:N:P, descritas en la bibliografía, respecto al contenido de carbono a degradar son muy variadas. La EPA recomienda utilizar proporciones C:N de 100:10 a 1000:10 para la biodegradación de suelos contaminados por hidrocarburos (US-EPA, 1995). Además, La mayor parte de hidrocarburos presentes en los productos petrolíferos son degradados con mayor extensión y rapidez de forma aeróbica (O_2 como aceptor de final de electrones), ya que en ausencia de O_2, y en presencia de aceptores de electrones alternativos (NO_3^-, SO_4^{2-}, CO_2, Mn^{4+} y Fe^{3+}) los hidrocarburos pueden ser degradados, pero con unas tasas de biodegradación muy inferiores a las aeróbicas (Boopathy, 2002; Grishchenkov et al., 2000; Holliger & Zehnder, 1996; Leutwein & Heider, 1999; Massias et al., 2003).

Finalmente, en suelos industriales como los utilizados en el presente trabajo, la biodisponibilidad del compuesto contaminante puede ser el factor más importante para limitar el éxito de un proceso de biorremediación (Bamforth & Singleton, 2005). Tanto los hidrocarburos alifáticos de cadena larga como los HAPs, especialmente los de elevado peso molecular, tienen valores bajos de biodisponibilidad y están clasificados

como compuestos orgánicos hidrofóbicos, es decir, moléculas con niveles bajos de solubilidad en agua y, por lo tanto, resistentes a la degradación (Semple et al., 2003). Además, los hidrocarburos tienen tendencia a adsorberse rápidamente a superficies minerales, como las arcillas del suelo, y a la materia orgánica, como los ácidos húmicos. Como más tiempo pase en contacto el hidrocarburo con el suelo, mayor será su adsorción y menor su extractabilidad, ya sea química o biológica. Este proceso es conocido como envejecimiento o "*ageing*", en inglés, y es uno de los principales problemas a los que se enfrenta la tecnología de la biorremediación en suelos históricamente contaminados, ya que está íntimamente relacionado con la persistencia de los contaminantes en el medio ambiente.

1.3.3 Estrategias para aumentar la biodegradación de hidrocarburos en suelos

Como ya se ha comentado en el apartado anterior, los suelos industriales contaminados con hidrocarburos pueden presentar fuertes limitaciones que se tienen que tener en cuenta al implementar tecnologías de biorremediación y que deben activar la investigación y el desarrollo en este campo, siempre buscando una mayor aplicabilidad y una contención en el coste. Dependiendo de tipo de suelo y de matriz contaminante, así como de la microbiota presente, las estrategias habituales para favorecer la bioestimulación de las poblaciones (humedad óptima, nutrientes en proporciones adecuadas etc.) autóctonas pueden no obtener los resultados que se esperarían, si las condiciones fuesen otras. Como consecuencia de ello, el uso de surfactantes (biológicos y/o químicos) para aumentar la biodisponibilidad de los contaminantes, de co-sustratos para añadir una fuente de carbono fácilmente asimilable, para la microbiota capaz de degradar hidrocarburos, o el bioaumento con hongos de podredumbre blanca para aprovechar su potencial metabólico, pueden ser estrategias a tener en cuenta si las condiciones son las adecuadas y todas ellas se presentarán con más profundidad a lo largo de la tesis, evaluando los resultados de su implementación a escala de laboratorio.

1.3.3.1 Surfactantes

Una posible forma de aumentar la biodisponibilidad de compuestos orgánicos hidrofóbicos, como los hidrocarburos, en un suelo contaminado es mediante el uso de surfactantes, que se caracterizan por tener una cabeza hidrofílica y una cola hidrofóbica.

Gracias a esta estructura amfifílica, agregados de entre 10 y 200 moléculas, llamados micelas (Figura 1.7), se forman, en solución, cuando la concentración del surfactante está por encima de la concentración crítica miceliar (CMC), siendo capaces de disolver moléculas hidrofóbicas en su interior.

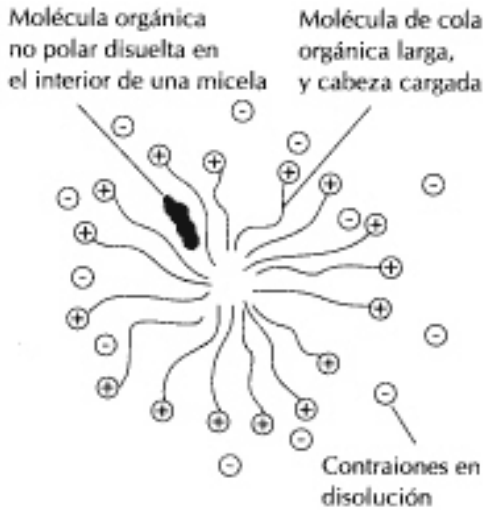

Fig. 1.7. Estructura de una micela en disolución a concentración superior a la CMC.

Dos mecanismos explican el aumento de la biodisponibilidad de compuestos hidrofóbicos en presencia de surfactantes. El primero es que aumenta su solubilidad como consecuencia de la fracción lipofílica de las micelas (Edwards et al., 1991) y el segundo es que el transporte del contaminante desde la fase sólida hasta la fase líquida está favorecido, debido a la disminución de la tensión superficial del agua de los poros presentes en el suelo (Volkering et al., 1997).

En función de los grupos hidrofílicos que presenten, los surfactantes pueden clasificarse en iónicos y no iónicos. Por otro lado, según su origen se pueden clasificar en químicos (producidos por síntesis química) y biosurfactantes (producidos por microrganismos). Generalmente, los surfactantes no iónicos son menos tóxicos para los microrganismos ya que la carga negativa de la superficie de las células las hace más sensibles a la introducción de surfactantes cargados (Li & Chen, 2009).

Existen muchos trabajos anteriores donde se describe que los surfactantes no-iónicos estimulan la biodegradación de HAPs (Kotterman et al., 1998; Marquez-Rocha et al., 2000; Zheng & Obbard, 2001). Sin embargo, también se pueden encontrar

literatura donde los surfactantes inhiben la degradación (Avramova et al., 2008). Este hecho puede ser debido a que los microrganismos no puedan acceder a los hidrocarburos disueltos en las micelas, que los surfactantes puedan resultar tóxicos para la microbiota o incluso que puedan ser utilizados como fuente de carbono y energía. Estos aspectos se discutirán en mayor profundidad en el capítulo 7 del presente trabajo, donde dos surfactantes fueron añadidos a un suelo contaminado por creosota.

Finalmente, también existe la posibilidad de usar biosurfactantes, aunque la tecnología se encuentra todavía en fases iniciales y los resultados descritos son muy contradictorios (Lu et al., 2011). De todas formas, el interés en su uso es creciente ya que se consideran menos tóxicos que los surfactantes químicos. En el capítulo 3 de la tesis se describe el uso de un biosurfactante producido por *Pseudomonas aeruginosa* en un suelo contaminado con aceites minerales.

1.3.3.2 Co-sustratos

Un número considerable de compuestos (glucosa, extracto de levadura, etanol, metanol, piruvato, acetato, etc.) han sido usados en la literatura como co-sustratos en la degradación de hidrocarburos en suelos y sedimentos (Chang et al., 2008; Liang et al., 2007).

Generalmente, la presencia de estos sustratos podría incrementar la presencia de bacterias heterótrofas en el suelo, así como hongos autóctonos, y como consecuencia aumentar la biodegradación de los hidrocarburos contaminantes. Además, el co-sustrato puede hacer variar la relación C:N en el suelo, favoreciendo el crecimiento microbiano e incrementando la producción de enzimas involucrados en la degradación de hidrocarburos y sus intermediarios (Lu et al., 2011). Sin embargo, el efecto de los co-sustratos también puede ser adverso, al competir como fuente de carbono y energía con el contaminante presente en el suelo (Quantin et al., 2005). Estas consideraciones se tratarán en más profundidad en los capítulos 3, 6 y 7, donde paja de arroz, glucosa y paja de trigo son utilizados como fuente de carbono para el bioaumento fúngico llevado a cabo en los capítulos mencionados, aunque al mismo tiempo, su efecto como co-sustratos asimilables por la microbiota autóctona del suelo, debe ser considerado.

1.3.3.3 Micorremediación

Aunque la mayoría de los estudios de biorremediación se han centrado en las bacterias, la capacidad metabólica de los hongos para transformar una gran variedad de compuestos orgánicos, e incluso mineralizarlos, ya sea a través de enzimas intracelulares o excretadas al medio, ofrece un gran potencial para su uso en descontaminación de suelos y una ventaja sobre el metabolismo bacteriano, obligado a la internalización de los compuestos, antes de ser degradados.

Los hongos filamentosos, entre los cuales se encuentran los hongos de podredumbre blanca, representan alrededor del 75% de la biomasa microbiana en suelos, con redes que se pueden extender a lo largo de cientos de hectáreas (Harms et al., 2011), ya que crecen por extensión de sus hifas, suponiendo este hecho otra ventaja sobre las bacterias para acceder a los contaminantes del suelo. Por el contrario, investigaciones recientes han demostrado que, en ciertas ocasiones, las bacterias autóctonas del suelo pueden aprovechar este crecimiento fúngico para difundirse a través del suelo y acceder a nuevos hábitats donde crecer a expensas de fuentes de carbono como posibles contaminantes (Banitz et al., 2011). Estas llamadas “autopistas fúngicas” pueden conectar poros llenos de agua a través de espacios del suelo llenos de aire e inaccesibles para las bacterias, que de otra forma restarían inmovilizadas. Como se verá más adelante, dentro de esta misma introducción, ésta no es la única forma de interacción entre hongos y bacterias en el suelo, pero pone de manifiesto la importancia de estudiar las relaciones que se establecen entre ellos con respecto a procesos como la biodegradación de contaminantes.

1.3.3.3.1 Hongos de podredumbre blanca

Este tipo de basidiomicetos son considerados los degradadores de lignina más eficientes de la naturaleza. Catalizan su degradación mediante una maquinaría enzimática basada en radicales libres y con baja especificidad de sustrato. La ligninolisis *per se* no puede soportar el crecimiento fúngico, pero abre las puertas de los materiales leñosos para que los hongos puedan acceder a los sustratos polisacáridos

(Hammel, 1995). De hecho, los materiales lignocelulósicos, son los sustratos ideales para la aplicación de los hongos de podredumbre blanca en suelos contaminados.

Su sistema ligninolítico es extracelular, y eso les permite degradar sustratos sin tener que internalizarlos, permitiendo la oxidación de compuestos poco solubles en agua y aumentado su tolerancia a concentraciones de contaminantes relativamente altas. Esta maquinaría extracelular es inducida por la limitación de nutrientes (Reddy & Mathew, 2001). Además del sistema enzimático extracelular, los hongos de podredumbre blanca poseen otro intracelular donde se involucra el citocromo P450 monooxigenasa-epoxido hidrolasa. Esta ruta intracelular está presente en todos los organismos eucariotas, donde regula la bioconversión de hormonas y la detoxificación de drogas y xenobióticos (Bernhardt, 2006).

A pesar de esto, en los estudios realizados en suelos, los niveles de transformación y mineralización suelen ser bajos, comparados con los estudios a escala de laboratorio y en cultivo líquido (Pointing, 2001). Este hecho puede estar directamente relacionado con la baja biodisponibilidad de ciertos contaminantes en el suelo por procesos de adsorción y a la poca solubilidad en agua e invita a la investigación sobre la combinación de hongos y surfactantes en suelos contaminados. De hecho, un diseño experimental similar se explica en el capítulo 7 del presente trabajo.

Finalmente, los hongos pueden ver su crecimiento limitado al interactuar con las poblaciones microbianas autóctonas. Se ha descrito tanto que los hongos de podredumbre blanca pueden inhibir a las poblaciones autóctonas como el caso contrario (Gramss et al., 1999). Por este motivo y siempre con la finalidad de mejorar la aplicabilidad de la biorremediación, los estudios poblacionales, adentrándose en las interacciones entre hongos y bacterias son muy necesarios. Tanto en el capítulo 6 como en el 6 se muestran los resultados de estudios moleculares llevados a cabo para conocer con más detalle las poblaciones microbianas presentes en el suelo contaminado por creosota, así como sus dinámicas de cambio durante diferentes estrategias de biorremediación.

1.3.3.3.2 El sistema ligninolítico de los hongos de podredumbre blanca

La degradación de la lignina se ha descrito en otros microrganismos que no son los hongos de podredumbre blanca (eubacterias y hongos ascomicetos), aunque su capacidad parece limitada (Zimmermann, 1990). La lignina se encuentra en la pared celular de la mayoría de los vegetales, formando una matriz compleja sobre las fibras de celulosa y hemicelulosa, confiriendo rigidez a la pared, protegiendo las células del ambiente externo y aportando hidrofobicidad. Su polimerización es al azar y su estructura es heterogénea e irregular, produciendo una macromolécula altamente compleja, amorfa e insoluble en agua (Figura 1.8) (Boominathan & Reddy, 1992).

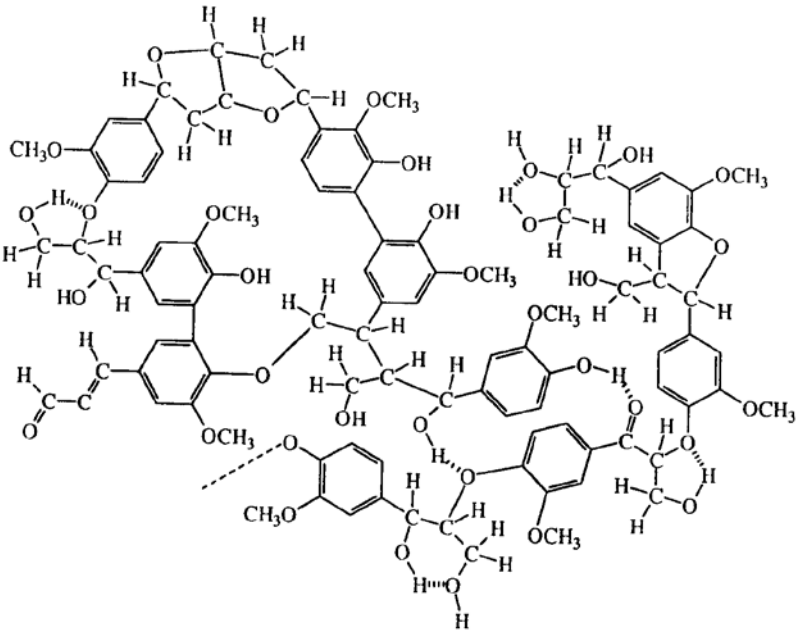

Fig. 1.8. Estructura de la lignina (Haider et al., 1964)

Los hongos de podredumbre blanca producen, principalmente, dos tipos de enzimas ligninolíticos, las lacasas y las peroxidasas, de entre las cuales la lignina peroxidasa (LiP) y la manganeso peroxidasa (MnP) son las más importantes. La principal diferencia entre las lacasas y las peroxidasas recae en la naturaleza del aceptor electrónico, ya que las lacasas utilizan O_2, mientras que las peroxidasas necesitan H_2O_2. Otras enzimas producidas por los mismos hongos se encargan de producir el H_2O_2 requerido por, tanto la LiP, como la MnP.

La producción de enzimas ligninolíticos ocurre durante metabolismo secundario y se ha descrito que puede estar afectada tanto por compuestos mediadores, otros compuestos químicos y/o metales requeridos como el Mn^{2+} o Cu^{2+}. (Dittmer et al., 1997; Galhaup et al., 2002; Scheel et al., 2000). Cada especie de hongo tiene un patrón determinado de producción enzimática y este puede servir para clasificarles (Hatakka, 1994).

La lacasa (bencenodiol:oxígeno oxidorreductasa, EC 1.10.3.2) es el enzima ligninolítico más descrito y conocido y que se encuentra más ampliamente distribuido entre los hongos. En general, se expresa en forma de diferentes isoenzimas, que pueden ser constitutivos y/o inducibles, con una masa molecular típica de entre 60 y 80 kDa. La lacasa contiene cuatro átomos de cobre por molécula y éstos se encuentran directamente implicados en el ciclo catalítico del enzima (Figura 1.9)

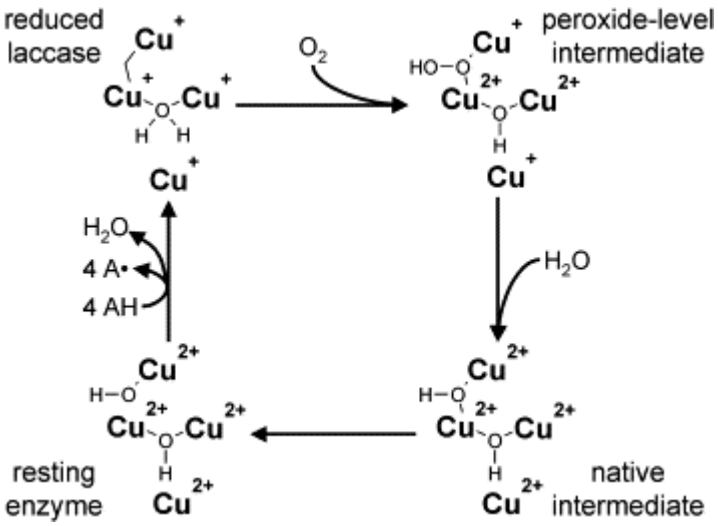

Fig. 1.9. Ciclo catalítico de la lacasa (Wesenberg et al., 2003).

Las lacasas son, característicamente, muy poco específicas y mediante su ciclo catalítico pueden oxidar diferentes compuestos como sustrato reductor, como por ejemplo, fenoles, polifenoles, aminas aromáticas así como compuestos orgánicos no fenólicos, generando radicales altamente reactivos que pueden conducir a nuevas oxidaciones, ya sean espontáneas o por vía enzimática (Thurston, 1994).

Las lacasas, a parte de la degradación de la lignina, están involucradas en múltiples procesos del ciclo vital del hongo como el desarrollo de los cuerpos fructíferos, pigmentación, patogenicidad y diferenciación sexual (Leonowicz et al., 2001) y pueden ser intracelulares o extracelulares. Además, su expresión se puede inducir con la adición de Cu^{2+}, que regula su expresión a nivel transcripcional (Saparrat et al., 2002), o incluso con otros compuestos como el veratril alcohol o el guaiacol (Quaratino et al., 2007).

La lignina peroxidasa (diarilpropano:peróxido de hidrogeno oxidorreductasa, EC 1.11.1.14) fue la primera peroxidasa descubierta implicada en la degradación de la lignina (Tien & Kirk, 1988) y su masa molecular es de entre 40 i 45 kDa. Aunque, posteriormente se describió en otros hongos, la LiP no es uno de los componentes habituales de los sistemas enzimáticos ligninolíticos.

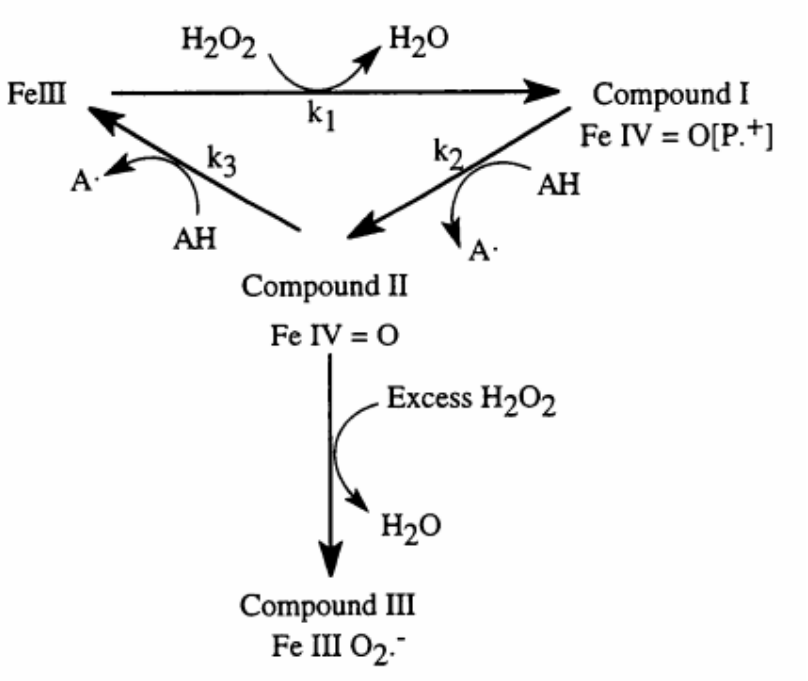

Fig. 1.10. Ciclo catalítico de la lignina peroxidasa (Castillo, 1997).

Durante su ciclo catalítico (Figura 1.10), el peróxido de hidrogeno oxida por dos electrones la LiP a un compuesto intermediario referido como compuesto I. Este compuesto puede oxidar sustratos por un electrón, produciendo un intermediario todavía más reducido conocido como compuesto II. Finalmente, este compuesto puede catalizar el mismo tipo de reacciones que el compuesto I, devolviendo la LiP a su estado inicial,

pero con un exceso de H_2O_2 en el medio, el compuesto II reacciona dando lugar a un compuesto III inactivo (Waarishi & Gold, 1989).

La LiP es secretada de forma extracelular, mayoritariamente durante metabolismo secundario, y en condiciones donde el nitrógeno es limitante (Tien & Kirk, 1988), aunque también se ha descrito su producción con altas concentraciones de nitrógeno en el medio (Collins & Dobson, 1995). Además, se observó que la producción del enzima está regulada por manganeso, y esta regulación por manganeso es independiente del nitrógeno, tanto para la producción de LiP como de MnP (Reddy & Dsouza, 1994). Altos niveles de Mn pueden inhibir la producción de LiP mientras que deficiencias en este elemento pueden reponer los elevados niveles de oxigeno requeridos para la formación de LiP (Rothschild et al., 1999).

Finalmente, la manganeso peroxidasa (Mn(II):peróxido de hidrogeno oxidorreductasa, EC 1.11.1.13) es una peroxidasa extracelular, normalmente producida en varias isoformas diferentes con un peso molecular de entre 45 i 55 kDa. Estas isoformas se diferencian en el punto isoeléctrico, normalmente en rango acido (pH 3-4) (Ha et al., 2001). La expresión de la MnP en hongos está regulada a nivel transcripcional por peróxido de hidrógeno así como otros compuestos químicos y por las concentraciones de Mn^{2+} (Li et al., 1995; Scheel et al., 2000).

Su ciclo catalítico es muy similar al de la LiP (Figura 1.11) pero difiere en dos aspectos. Por un lado, el compuesto I oxida Mn^{2+} a Mn^{3+} y este ión, estabilizado con ciertos ácidos orgánicos (oxalato, malonato, malato, tartrato o lactato), causa oxidaciones de un electrón sobre varios sustratos, generando radicales libres. Por otro, el compuesto II solo puede ser reducido a su estado original por Mn^{2+} i depende estrictamente de este ion para cerrar su ciclo catalítico (Hofrichter, 2002). La MnP, al igual que la LiP, es muy sensible a elevadas concentraciones de H_2O_2, que pueden causar la inactivación del enzima, formando el MnP-compuesto III.

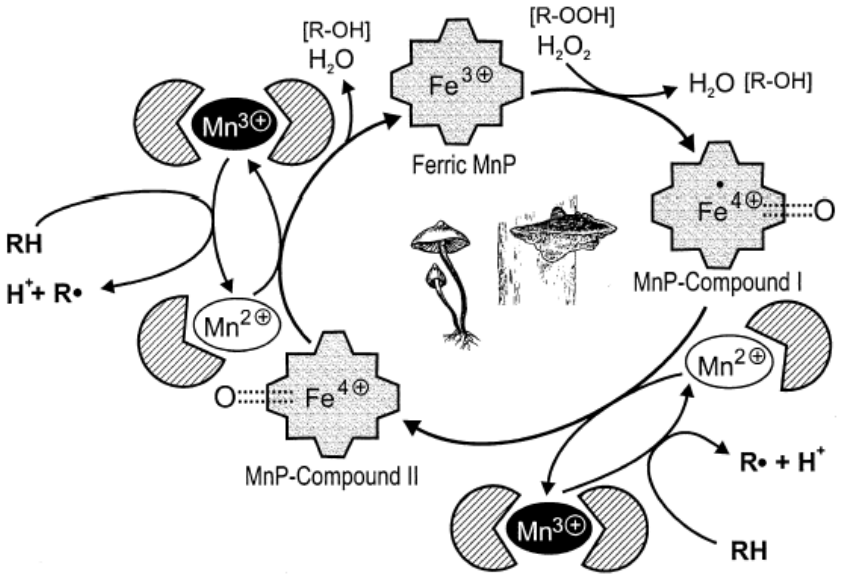

Fig. 1.11. Ciclo catalítico de la manganeso peroxidasa (Hofrichter, 2002).

1.3.3.3.3 Correlación entre biomasa fúngica, expresión enzimática y biodegradación de contaminantes

La importancia de una fuerte colonización del suelo y de una elevada producción y secreción de enzimas extracelulares debe ser tenida en cuenta al evaluar procesos de biorremediación en suelos que involucren hongos ligninolíticos. Desafortunadamente, no siempre es fácil poder cuantificar de forma correcta la biomasa fúngica presente en un suelo ni poder relacionar una mayor actividad enzimática con una mayor degradación de los contaminantes presentes en el suelo.

El lípido de membrana ergosterol se encuentra casi exclusivamente en hongos y se usa frecuentemente como indicador de biomasa fúngica viva. Sin embargo, ciertas limitaciones como una significativa degradación fotoquímica (Mille-Lindblom et al., 2004) o la elevada variabilidad del contenido de ergosterol en suelos (Zhao et al., 2005) y entre diferentes especies fúngicas (Barajas-Aceves et al., 2002) aconsejan interpretar los resultados con precaución y emplear el método combinado con otras técnicas, como por ejemplo la estimación del contenido de quitina (Roche et al., 1993) o la cuantificación de fosfolípidos (PLFA) (Zhao et al., 2005). En el presente trabajo, y como se tratará en más profundidad en los capítulos 6 y 7, se usó la PCR cuantitativa (qPCR) para comparar con los resultados obtenidos de la cuantificación de ergosterol en

el suelo contaminado. Esta técnica se usa de forma habitual a partir de ADN extraído de suelos (van Elsas & Boersma, 2011), ya que permite cuantificar genes de interés como los genes codificantes para el ARNr 16S, cuando se trata de cuantificar presencia de bacterias en el suelo, o el ARNr 18S o la región ITS para hongos o incluso genes funcionales de interés, como los de ciertas rutas degradativas. Las limitaciones de las técnicas dependientes de PCR serán evaluadas más adelante, dentro de la misma introducción, en la sección 1.4.

Por otro lado, existen controversia y resultados contradictorios en la literatura sobre los niveles de actividad enzimática extracelular producidos por hongos en suelos, sobretodo de podredumbre blanca, y su correlación con resultados óptimos de biodegradación de contaminantes como PCBs o HAPs (Novotný et al., 2004). Este hecho puede deberse, *in vivo*, a la adsorción de los enzimas a las partículas de suelo y a la más que probable perdida de actividad una vez externalizados (Burns, 1982). Por lo tanto, la falta de correlación e incluso de conocimiento, en este campo, se deben a la elevada complejidad de los mecanismos de degradación implicados, ya que otros sistemas bioquímicos, a parte de los enzimas ligninolíticos, pueden estar interviniendo en el proceso de biodegradación, como el citocromo P450 de hongos, radicales libres, niveles de H_2O_2 o toda la batería bacteriana, presente en los suelos no estériles.

1.3.3.3.4 Descripción de los hongos ligninolíticos *Trametes versicolor* y *Lentinus tigrinus*

Desde el descubrimiento de las aplicaciones de los enzimas ligninolíticos durante los años 80 del siglo pasado, se ha avanzado mucho en el estudio de los hongos de podredumbre blanca y su sistema enzimático.

Trametes versicolor (Figura 1.12.A) es, juntamente con *Phanerochaete chysosporium* y *Pleurotus ostreatus*, uno de los hongos ligninolíticos más utilizados en aplicaciones biotecnológicas a escala de laboratorio. Este hongo es también conocido como cola de pavo debido a los colores que recubren sus cuerpos fructíferos. Se encuentra, sobretodo, en arboles planifolios y caducifolios, especialmente en encinares y robledas (Llimona, 1991) y coloniza la madera, siendo competitivo en contacto con otras especies de hongos, aunque como discutiremos en más profundidad en los

capítulos 6 y 7, puede no serlo tanto al inocularlo como hongo alóctono en un suelo con una comunidad autóctona fuertemente aclimatada.

En tanto a su sistema enzimático, muestra una fuerte estimulación de su sistema ligninolítico en medios donde el nitrógeno es limitante (Leatham & Kirk, 1983) y puede producir lacasa tanto de forma constitutiva como inducida (Sariaslani, 1989). Aunque la lacasa es la enzima que produce *Trametes versicolor* de forma mayoritaria, también se ha descrito, en determinadas condiciones, la producción de LiP y MnP (Hatakka, 1994). Se ha demostrado, ampliamente, su capacidad para degradar un amplio espectro de xenobióticos (Hundt et al., 1999; Vyas et al., 1994).

Por su parte, *Lentinus tigrinus* es una especie de hongo ligninolítico poco frecuente en nuestro entorno cercano y suele fructificar en árboles de ribera como el chopo o el sauce (Figura 1.12.B). Es un hongo mucho menos utilizado, hasta el momento, que *Trametes versicolor* en biotecnología pero su sistema enzimático ligninolítico está suficientemente descrito, sobretodo en la cepa 8/18 (Quaratino et al., 2006). Se ha podido observar que *Lentinus tigrinus* puede producir cantidades importantes de lacasa y MnP, siendo este último el enzima más característico e importante de su sistema ligninolítico (Pozdnyakova et al., 1999). En cambio, la LiP no forma parte de su metabolismo (Maltseva et al., 1991).

Fig. 1.12. Hongos de podredumbre blanca *Trametes versicolor* (A) y *Lentinus tigrinus* (B)

1.3.4. Estudios de ecotoxicidad en biorremediación de suelos contaminados

El objetivo principal en la recuperación de suelos contaminados es la disminución de la concentración de contaminantes, hasta unos niveles establecidos por la legislación vigente que impliquen la ausencia de un riesgo inaceptable para la salud humana. Se define riesgo, como la probabilidad de que un contaminante entre en contacto con algún receptor con consecuencias adversas para la salud de las personas o para el medio ambiente. Por lo tanto, el riesgo de un suelo contaminado se relaciona habitualmente con la concentración de ciertos componentes específicos, para los que se ha establecido el efecto dosis-respuesta en ensayos con organismos vivos. No obstante, la mayor parte de los suelos contaminados difieren de estas condiciones al menos en dos factores importantes:

- Los contaminantes, extrañamente existen de forma individual, ya que se presentan en forma de mezclas orgánicas complejas, que pueden ser tóxicas o alterar, positivamente o negativamente, el comportamiento de los constituyentes legislados o de interés (Efroymson & Alexander, 1995; Rutherford et al., 1998).

- Los contaminantes habitualmente están presentes en el suelo durante años o décadas, pudiéndose encontrar secuestrados en los microporos del suelo e incluso reaccionando con la materia orgánica o incorporándose en las substancias húmicas del suelo (Alexander, 1995; Guthrie & Pfaender, 1998).

En consecuencia, es poco probable que la biodisponibilidad de un contaminante presente en una matriz orgánica compleja y que además puede estar envejecida *(“ageing”*), coincida con la que resulte de ensayar el contaminante de forma individual y en un suelo recientemente dopado. Además, las interacciones sinérgicas o antagónicas que pueden producirse tanto entre los diferentes contaminantes, como con la matriz del suelo, así como también la dificultad de detectar y cuantificar todos los componentes y los metabolitos de degradación de una matriz contaminante, justifican la necesidad de complementar el análisis químico con estudios ecotoxicológicos (Hund & Traunspurger, 1994). En realidad, en los últimos años se ha observado un creciente

interés en incorporar los ensayos ecotoxicológicos para complementar la toma de decisiones en proyectos de recuperación (Salanitro et al., 1997; Saterbak et al., 1999) y para redefinir las concentraciones límite a nivel legal (Dorn & Salanitro, 2000; Dorn et al., 1998).

Durante los últimos años, se han estado utilizando ensayos de ecotoxicidad, tanto en el laboratorio como en estudios de campo, para el seguimiento de procesos de biorremediación en suelos contaminados con hidrocarburos (Gandolfi et al., 2010; Garcia Frutos et al., 2010). Los diferentes ensayos de ecotoxicidad descritos en suelos utilizan diferentes niveles tróficos, como bacterias, invertebrados, protozoos, anfibios, algas y plantas (Dorn et al., 1998; Dumont et al., 1983; Greene et al., 1988; Hund & Traunspurger, 1994; Juvonen et al., 2000; Keddy et al., 1995). A continuación se describen los ensayos de ecotoxicidad más utilizados en suelos, que corresponden a ensayos de toxicidad aguda y a ensayos de genotoxicidad:

Los ensayos de toxicidad aguda más comunes son:

- Microtox® (*Vibrio fischeri*): bioensayo de toxicidad aguda, basado en la bioluminiscencia natural de la bacteria marina *Vibrio fischeri*. Se ha demostrado que la luminiscencia natural de la cepa se ve afectada de forma directamente proporcional a la presencia de tóxicos.
- Lumbrícidos (*Eisenia foetida*): ensayo de toxicidad aguda (letalidad) llevado a cabo con el invertebrado *Eisenia foetida* (oligoqueto), en el que se determina como afecta un suelo o muestra problema a la letalidad (14 días) y reproducción (28 días) de una población de *Eisenia foetida* adulta.
- Plantas superiores: se han utilizado diferentes especies de plantas superiores para ensayos de toxicidad aguda en suelos, en estudios de crecimiento (incluye el estudio de la germinación de semillas y del crecimiento de cotiledones y planta adulta) y de supervivencia como *Lactuca sativa* o *Lemma minor*.

Los ensayos de genotoxicidad más comunes son:

- Ensayo de teratogénesis en embriones de *Xenopus laevis* (FETAX): el FETAX es un ensayo en el que se evalúa el efecto teratogénico y tóxico (inhibición de crecimiento y letalidad) de una muestra ambiental en embriones del anuro *Xenopus laevis*.
- Comet test: este ensayo de toxicidad es capaz de detectar lesiones en el ADN causadas por agentes alquilantes, intercalantes y por daño oxidativo.
- Ensayos de mutagénesis en bacterias: los ensayos de mutagenicidad se basan en la detección, en organismos diana, de fenotipos específicos después de un contacto con agentes fisicoquímicos.
- Test de Ames: el test de Ames utiliza cepas de *Salmonella typhimurium* como organismo indicador de mutagénesis. Se utilizan cepas modificadas genéticamente para que sean auxótrofas para la histidina, tengan una permeabilidad más elevada de la membrana externa y que las mutaciones que tengan lugar no sean reparadas de forma eficiente.

Tanto el ensayo de toxicidad aguda en lumbricidos como el ensayo de genotoxicidad del "comet test" se presentarán en el capítulo 3, ya que fueron utilizados para determinar la evolución de la ecotoxicidad durante un ensayo de tratabilidad de un suelo contaminado con aceites minerales.

1.4 Estudio de comunidades microbianas

El conocimiento de las poblaciones microbianas presentes en suelos contaminados, así como su evolución durante procesos de biorremediación, es todavía muy limitado. Aumentar el conocimiento en este campo debería ser prioritario para futuras investigaciones, ya que sería útil para disponer de más información sobre qué microrganismos son capaces de adaptarse a estos hábitats y poder explotar así sus capacidades. Además, es necesario estudiar las comunidades de microrganismos porque el metabolismo de sustratos orgánicos en sistemas naturales suele producirse mediante interacciones metabólicas entre distintos organismos. Por ello, es prioritario aumentar nuestro conocimiento sobre las distintas comunidades microbianas existentes en

diferentes sitios contaminados, así como de las interacciones que se establecen entre ellas.

Durante los últimos 20 años han aumentado, de forma significativa, las metodologías para estudiar la diversidad microbiana presente en la biosfera. Pero la realidad es que todavía queda mucho camino por recorrer. De hecho, existe mucha controversia y el orden de magnitud todavía es desconocido, aunque algunos estudios apuntan que en un único gramo de suelo podrían estar presentes entre 10^2 i 10^7 especies distintas de microrganismos (Gans et al., 2005; Schloss & Handelsman, 2006). Por otro lado, hay que tener en cuenta que solo entre un 0,001 y un 1% de los microrganismos viables son cultivables (Torsvik et al., 2003). Por este motivo, los estudios clásicos de diversidad microbiana, basados únicamente en el aislamiento de microrganismos, representan una parte minoritaria de la diversidad real existente (Amann et al., 1995), aunque siguen siendo muy útiles para proporcionar cierta información, como se verá en la sección 1.4.1. En cambio, las nuevas técnicas de biología molecular, aplicadas a estudios de biorremediación, nos ofrecen nuevas y atractivas posibilidades para analizar la estructura, composición y cambio poblacional de las comunidades microbianas del suelo, durante procesos de biodegradación.

Desafortunadamente, existen todavía pocos trabajos que hayan estudiado de forma exhaustiva las comunidades bacterianas en suelos contaminados (Federici et al., 2007; Singleton et al., 2011; Viñas et al., 2005). Además, los pocos estudios llevados a cabo con suelos reales, no realizaron ningún seguimiento de la dinámica de las poblaciones fúngicas autóctonas durante el proceso de biorremediación de los mismos.

1.4.1 Técnicas dependientes de cultivo

Las estimaciones de la diversidad bacteriana se han basado, de forma tradicional, en métodos dependientes de cultivo. En este grupo se incluyen el aislamiento y el cultivo en medio sólido o en medio líquido, como el número más probable (NMP) u otros que incluyen baterías de pruebas miniaturizadas de utilización de sustratos como el método Biolog®, que permite estudiar las comunidades microbianas en base a su perfil fisiológico (El Fantroussi et al., 1999). Como se ha comentado anteriormente en la sección 1.4., se ha comprobado que estos métodos

tienden a subestimar la biodiversidad en comparación con los métodos no dependientes de cultivo.

A pesar de las limitaciones, las técnicas dependientes de cultivo presentan ciertas ventajas y todavía son de uso habitual en los laboratorios de microbiología ambiental. La principal de sus ventajas es poder obtener cultivos puros, útiles para posteriores estudios, y para caracterizar actividades metabólicas de interés. Por último, si se es consciente de las limitaciones que presentan, pueden ser útiles en estudios comparativos.

1.4.2 Técnicas independientes de cultivo

Como consecuencia de las restricciones que presentan las técnicas dependientes de cultivo, en el campo de la ecología microbiana cada vez es más frecuente el uso de metodologías que no requieren cultivar los microrganismos (Riesenfeld et al., 2004).

Los primeros métodos se basaban en la observación directa de los microrganismos metabólicamente activos, utilizando distintas tinciones específicas. Posteriormente, se introdujeron nuevas técnicas como el análisis de los perfiles de ácidos grasos de los fosfolípidos de membrana (PLFA). Esta técnica es útil para detectar cambios rápidos en la estructura de las poblaciones pero no permite asignar afiliaciones filogenéticas. Por este motivo, se suele utilizar combinada con otras metodologías (Ibekwe et al., 2002).

Los siguientes avances en el campo de la biología molecular permitieron implementar metodologías basadas en el análisis de los ácidos nucleicos existentes en los emplazamientos de interés. Generalmente, estos métodos se fundamentan en el estudio de los genes codificantes para el ARNr 16S cuando son las poblaciones procariotas las que suscitan interés, mientras que el ARNr 18S y la región ITS cada vez son más usados para el estudio de comunidades fúngicas, aunque las bases de datos disponibles no son tan extensas como las que disponemos para procariotas.

Las técnicas basadas en la amplificación por PCR son diversas: librerías de clones, análisis mediante electroforesis en geles de gradiente desnaturalizante, tanto

químico como térmico (DGGE y TGGE, respectivamente) o PCR acoplada al análisis de polimorfismos en la longitud del fragmento terminal de restricción (T-RFLP). También se desarrollaron técnicas de hibridación *in situ* con sondas fluorescentes (FISH).

Hasta hace unos pocos años, el abordaje más común para caracterizar una comunidad de microrganismos en un suelo o emplazamiento contaminado consistía en la amplificación por PCR del gen codificante para el ARNr 16S, en caso de bacterias, o la región ITS para hongos y el posterior análisis por DGGE. De hecho, este tipo de diseño se usa en los capítulos 3, 6 y 7 del presente trabajo, con la finalidad de estudiar la dinámica de las poblaciones presentes en los suelos contaminados de interés. Esta técnica permite separar fragmentos de ADN de la misma longitud (o similar) pero con secuencias diferentes (Fischer & Lerman, 1979), mediante un gradiente desnaturalizante optimizado. Se asume que cada banda presente en un gel de DGGE, corresponde a una unidad taxonómica operativa (OTU). Lamentablemente, y como se comentará en más profundidad en los capítulos 6 y 8, puede darse el caso que más de una banda corresponda al mismo OTU o que una banda sola corresponda a varios OTU (Green, 2009). Sólo después de cortar, reamplificar y secuenciar las bandas se puede ver el resultado definitivo de un DGGE, aunque no siempre es posible obtener toda la biodiversidad presente en el gel, por las limitaciones intrínsecas de la técnica.

Sin embargo, en estos últimos años, se han desarrollado técnicas que han supuesto una auténtica revolución en el estudio de la biodiversidad presente en suelos como son las metodologías metagenómicas. La unión del desarrollo de la PCR en emulsión junto a los nuevos métodos de secuenciación, como la pirosecuenciación, basada en acoplar la síntesis de ADN a una reacción quimioluminiscente, proporciona una resolución incomparable con las técnicas anteriormente descritas, ya que de 10 o 20 bandas secuenciadas, con suerte, de un carril de DGGE o de 50 a 100 de una librería de clones clásica, podemos pasar a obtener miles de secuencias útiles, de una sola muestra de suelo, de una forma mucho más rápida y eficaz y, por lo tanto, realizar estudios taxonómicos mucho más profundos (Sogin et al., 2006). Esta cantidad ingente de datos, ha requerido un fuerte desarrollo de herramientas bioinformáticas para poder procesar, lo más correctamente posible, la diversidad taxonómica presente en las muestras secuenciadas (Simon & Daniel, 2011). Se puede decir que la metagenómica junto con

las nuevas técnicas de secuenciación han revolucionado como describimos y comparamos comunidades microbianas complejas (Amend et al., 2010). En el presente trabajo de tesis doctoral, en el capítulo 8, se comparará con más profundidad los resultados de biodiversidad, tanto de hongos como de bacterias, obtenidos mediante DGGE y pirosecuenciación, en diferentes tratamientos de biorremediación para un suelo contaminado por creosota. Siendo una de las primeras veces que se utilizan técnicas de metagenómica para estudiar dinámicas de comunidades microbianas en suelos contaminados por HAPs.

Desafortunadamente, estas nuevas metodologías continúan dependiendo de la PCR, con los inconvenientes y limitaciones que esto supone, como la amplificación selectiva de los genes del ARNr 16S o de la región ITS o la formación de quimeras durante la PCR, obteniendo resultados engañosos, ya que se puede infravalorar o sobrevalorar la presencia de ciertos microrganismos. Para intentar eliminar el sesgo producido por la utilización de la PCR, el método basado en la amplificación de los genes de los ARNr se está sustituyendo por la secuenciación “shotgun” del ADN de la comunidad. La secuenciación directa del ADN metagenómico se considera el método más sensible para evaluar la biodiversidad real de cualquier emplazamiento (von Mering et al., 2007).

Todas estas metodologías expuestas (Figura 1.13) han supuesto un gran salto en el conocimiento de la biodiversidad microbiana en los más diversos ambientes, aunque se puede considerar que describir la diversidad taxonómica de las comunidades es solo el primer paso en la voluntad de conocer la relación existente entre los ciertos taxones específicos, presentes en los ecosistemas, y las funciones metabólicas que estos desempeñan (Gray & Head, 2001). Las técnicas moleculares nos muestran la diversidad microbiana sin el sesgo del cultivo, pero no nos aportan ningún tipo de información sobre la función de las poblaciones detectadas, mientras que las técnicas dependientes de cultivo nos permiten estudiar la fisiología de microrganismos cultivables de las muestras, aunque es difícil conocer su papel dentro de la comunidad a la que pertenece.

Objectives

OBJECTIVES

The main specific objectives of the present thesis work are:

- *Chapter 1*: To assess the feasibility of several biostimulation and bioaugmentation treatments to soil contaminated with a heavy mineral oil and improve the understanding of the bacterial community dynamics, as well as the ecotoxicological effects, throughout the different bioremediation strategies.
- *Chapter 2*: To carry out an *in situ* pilot scale biostimulation strategy in creosote polluted soil and study the effects on PAHs degradation and toxicity levels.
- *Chapter 3*: To analyse the microbial population involved in the degradation of HMW-PAHs, and the reasons why some target PAHs remain in bioremediated creosote polluted soil.
- *Chapter 4*: To study the evolution of the autochthonous soil microbial communities after fungal bioaugmentation with *Trametes versicolor*, as an alternative to degrade the HMW-PAHs remaining in an aged creosote polluted soil.
- *Chapter 5*: To evaluate the impact of single or combined supplements on both a biostimulation and a fungal bioaugmentation approach to an aged creosote polluted soil in terms of biodegradation outcomes and evolution of the resident microbiota.
- *Chapter 6*: To give a deeper approximation on both fungal and bacterial populations dynamics and community shifts during the different bioremediation treatments carried out in a historically creosote contaminated soil.

Universitat de Barcelona

Informe sobre la participación del doctorando en los artículos presentados en el presente trabajo de tesis doctoral.

1. **S. Lladó, A.M. Solanas, J. de Lapuente, M. Borras and M. Viñas**. 2012. A diversified approach to evaluate biostimulation and bioaugmentation strategies for heavy-oil contaminated soil. *Science of the Total Environment*, 2012, Vol. 435-436: 262-269.

El doctorando ha llevado a cabo toda la parte experimental del trabajo, en la Universidad de Barcelona, que supuso la evaluación de distintos agentes bioestimulantes y la inoculación de consorcios microbianos en la biodegradación de un suelo contaminado por aceites minerales pesados. El seguimiento del proceso supuso llevar a cabo análisis químicos (GC-FID) y ensayos microbiológicos. La única excepción fueros los análisis de toxicidad llevados a cabo por el grupo de Miquel Borras de la Unidad de Toxicología del Parc Científic de Barcelona. Asimismo llevó a cabo la discusión de resultados y la elaboración del manuscrito.

2. **E. Realp, J.A. Doménech, R. Martínez-García, C. Restrepo, S. Lladó, M. Viñas y A.M. Solanas.** 2008. Ensayo piloto de biorremediación por la tecnología de la biopila dinámica para la descontaminación de suelos contaminados por creosotas provenientes de las actividades dedicadas a la preparación de la madera. *Revista Técnica Residuos*. Año 2008. Año nº 18. Número 103: 38-49.

El doctorando se incorporó al final del proceso de biorremediación del suelo de creosota por la tecnología de una biopila, llevando a cabo los análisis tanto microbiológicos como químicos de las últimas muestras. Sin embargo ha contribuido de forma decisiva en el análisis y discusión de los resultados y redactó el manuscrito bajo mi supervisión.

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3. **S. Lladó, N. Jiménez, M. Viñas and A.M. Solanas.** 2009. Microbial populations related to PAH biodegradation in an aged biostimulated creosote-contaminated soil. *Biodegradation*, 2009, Vol. 20(5): 593-601.

El doctorando ha llevado a cabo todo el trabajo experimental realizado en la Universidad de Barcelona que incluyó tanto análisis químicos (GC-FID) como de ecología microbiana clásica y molecular (DGGE). Cabe resaltar su participación en el diseño experimental de utilización de slurries dopados con HAPs de elevado peso molecular. Asimismo participó en la discusión de resultados y elaboración del manuscrito.

4. **S. Lladó, E. Gràcia, A.M. Solanas and M. Viñas.** 2012. Fungal/bacterial interactions throughout bioremediation assays in an aged creosote polluted soil. *Submited to Soil Biology and Biochemistry.*

El doctorando ha llevado a cabo la totalidad del trabajo experimental en la Universidad de Barcelona que ha consistido en la evaluación de la inoculación de *Trametes versicolor* a un suelo enriquecido con HAPs de elevado peso molecular. Cabe resaltar la puesta a punto de la utilización de hongos ligninolíticos, siendo el primero del grupo de investigación en implementar dicha tecnología, así como la metodología del ergosterol (GC-MS) como método químico de seguimiento del crecimiento del hongo ligninolítico. Los análisis de la comunidad eubacteriana y de la comunidad fúngica así como el estudio de sus proporciones (qPCR) han permitido aportar información muy valiosa en relación a la interacción de ambas poblaciones. El doctorando también participó activamente en el diseño experimental, la discusión de resultados y la elaboración del manuscrito.

5. **S. Lladó, S. Covino, A.M. Solanas, M. Viñas, M. Petruccioli and A. D'Annibale.** 2012. Comparative assessment of bioremediation approaches to

highly recalcitrant PAH degradation in a real industrial polluted soil. *Submited to Science of the Total Environment.*

El doctorado ha llevado a cabo la totalidad del trabajo experimental, en la Universidad de Barcelona y en el laboratorio del Profesor Petruccioli de la Universidad della Tuscia (Italia). Cabe resaltar la envergadura del trabajo experimental que ha abarcado análisis de tipo químico (GC-FID y GC-MS), enzimológicos y de seguimiento de las poblaciones tanto eubacterianas como fúngicas por metodologías tanto clásicas como moleculares. Hay que destacar su participación en el diseño experimental, en la discusión de resultados y en la elaboración del manuscrito.

6. **S. Lladó, S. Covino, A.M. Solanas, M. Petruccioli, A. D'Annibale and M. Viñas.** 2012. Combining DGGE and Bar-Coded Pyrosequencing for microbial community characterization throughout different soil bioremediation strategies in an aged creosote-polluted soil. *Submitted to Soil Biology and Biochemistry.*

El doctornado ha llevado a cabo la totalidad del trabajo experimental, en el laboratorio del Dr. Marc Viñas del GIRO CT. Cabe destacar la implementación de la tecnologia de la pirosecuenciación, por parte del doctorando, que ha permitido conseguir niveles de resolución de la biodiversidad, tanto bacteriana como fúngica, presente en un suelo contaminado por creosota, mucho mayores que los visualizados hasta la fecha. Esta potente técnica podrá ser utilitzada en el futuro por los investigadores del grupo. Finalmente, el doctorando ha contribuido de forma decisiva, tanto en el diseño experimental como en la discusión de los resultados y la redacción del manuscrito.

Firmado:

Dra. Anna Maria Solanas Cánovas / Dr. Marc Viñas Canals / Dr. Enric Gràcia Barba

Barcelona, a 17 de septiembre de 2012

Universitat de Barcelona

Informe sobre el factor de impacto de los artículos presentados en el presente trabajo de tesis doctoral.

Las publicaciones que forman parte de la Tesis doctoral presentada por Salvador Lladó Fernández han sido publicadas o se han enviado para su publicación a revistas científicas relevantes en la línea de investigación en que ha participado.

El artículo "A diversified approach to evaluate biostimulation and bioaugmentation strategies for heavy-oil contaminated soil" ha sido publicado en *Science of the Total Environment*, en el presente 2012, siendo su índice de impacto de 3.286 en el 2011 y primer cuartil de su área específica de conocimiento. El artículo "Microbial populations related to PAH biodegradation in an aged biostimulated creosote-contaminated soil" fue publicado en *Biodegradation* en el año 2009, en que esta revista mostró índices de impacto de 1.873. Los artículos "Fungal/bacterial interactions throughout bioremediation assays in an aged creosote polluted soil" y "Combining DGGE and Bar-Coded Pyrosequencing for microbial community characterization throughout different soil bioremediation strategies in an aged creosote-polluted soil" han sido enviados a *Soil Biology and Biochemistry*, que el año pasado presentó un índice de impacto de 3.504 y ocupó la primera posición del primer cuartil, en su área de conocimiento. El artículo "Comparative assessment of bioremediation approaches to highly recalcitrant PAH degradation in a real industrial polluted soil" ha sido enviado también a *Science of the Total Environment*. Finalmente, debido a sus características, el artículo "Ensayo piloto de biorremediación por la tecnología de la biopila dinámica para la descontaminación de suelos contaminados por creosotas provenientes de las actividades dedicadas a la preparación de la madera" fue publicado en la revista no-indexada *Revista técnica Residuos*, siendo importante para hacer llegar nuestros progresos en el campo de la biotecnología ambiental a otros ámbitos más aplicados y técnicos.

Firmado:

Dra. Anna Maria Solanas Cánovas / Dr. Marc Viñas Canals / Dr. Enric Gràcia Barba

Barcelona, a 17 de septiembre de 2012

CAPÍTULO 1 / CHAPTER 1

A diversified approach to evaluate biostimulation and bioaugmentation strategies for heavy-oil contaminated soil

A diversified approach to evaluate biostimulation and bioaugmentation strategies for heavy-oil contaminated soil

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Un estudio multidisciplinar, implicando análisis químicos, microbiológicos y ecotoxicológicos, se llevó a cabo en un suelo contaminado por aceites minerales, con el objetivo de mejorar nuestros conocimientos sobre la biodegradabilidad de los contaminantes, evolución de las poblaciones microbianas y efectos ecotoxicológicos, durante el ensayo de diferentes estrategias de biorremediaición.

Con el propósito de mejorar la degradación de los hidrocarburos presentes en el suelo, los siguientes tratamientos de biorremediación fueron ensayados: la adición de nutrientes inorgánicos y del biosurfactante MAT10, la inoculación de un consorcio bacteriano especializado en la degradación de hidrocarburos alifáticos, así como la inoculación de una cepa del hongo de podredumbre blanca *Trametes versicolor*, previamente descrito como degradador de hidrocarburos.

Después de 200 días de incubación en microcosmos, en todos los tratamientos se observaron degradaciones de entre el 30 y el 50%, de los hidrocarburos totales del petróleo (TPH), respecto al contenido inicial del suelo, siendo la inoculación de *Trametes versicolor* el tratamiento que obtuvo mejores resultados.

Tanto la bioestimulación del suelo como la inoculación de *Trametes versicolor*, se relacionaron con el aumento del género bacteriano *Brevundimonas,* así como otros pertenecientes a las familias: α-proteobacteria, β-proteobacteria, y al grupo Cytophaga-Flexibacter-Bacteroidetes (CFB). Sin embargo, es destacable que con la inoculación de *Trametes versicolor*, estrategia donde se observó la mayor degradación de hidrocarburos, grupos de bacterias Gram-positivas autóctonas, como Firmicutes y Actinobacteria vieron aumentada, de forma notable, su prevalencia en el suelo.

El test de toxicidad de lumbrícidos, utilizando *Eisenia foetida,* confirmó la mejoría en la calidad del suelo después de todos los tratamientos de bioestimulación y bioaumento.

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A diversified approach to evaluate biostimulation and bioaugmentation strategies for heavy-oil-contaminated soil

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HIGHLIGHTS

- A diversified approach during bioremediation of oil-polluted soil is provided.
- Microbial community during biostimulation and bioaugmentation is assessed.
- Acute toxicity and genotoxicity throughout bioremediation lab tests are assessed.
- Inoculation of *Trametes versicolor* promotes autochthonous hydrocarbon-degraders.
- The lowest soil acute toxicity is achieved after *T. versicolor* bioaugmentation.

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ABSTRACT

A diversified approach involving chemical, microbiological and ecotoxicity assessment of soil polluted by heavy mineral oil was adopted, in order to improve our understanding of the biodegradability of pollutants, microbial community dynamics and ecotoxicological effects of various bioremediation strategies.
With the aim of improving hydrocarbon degradation, the following bioremediation treatments were assayed: i) addition of inorganic nutrients; ii) addition of the rhamnolipid-based biosurfactant M_{AT10}; iii) inoculation of an aliphatic hydrocarbon-degrading microbial consortium (TD); and iv) inoculation of a known hydrocarbon-degrading white-rot fungus strain of *Trametes versicolor*.
After 200 days, all the bioremediation assays achieved between 30% and 50% total petroleum hydrocarbon (TPH) biodegradation, with the *T. versicolor* inoculation degrading it the most. Biostimulation and *T. versicolor* inoculation promoted the *Brevundimonas* genus concurrently with other α-proteobacteria, β-proteobacteria and Cytophaga-Flexibacter-Bacteroides (CFB) as well as Actinobacteria groups. However, *T. versicolor* inoculation, which produced the highest hydrocarbon degradation in soil, also promoted autochthonous Gram-positive bacterial groups, such as Firmicutes and Actinobacteria. An acute toxicity test using *Eisenia fetida* confirmed the improvement in the quality of the soil after all biostimulation and bioaugmentation strategies.

1. Introduction

The application of bioremediation technologies to soils contaminated by light oil products, such as petrol or diesel, is feasible. However, decontaminating soils polluted with mineral oils that comprise the heaviest hydrocarbon fractions is still a challenge because of the low bioavailability and complex chemical composition of these products (Lee et al., 2008; Sabaté et al., 2004). In addition, an excessive residual concentration of hydrocarbons and possible oxidative metabolites with unacceptable human health risks may remain in the soil after bioremediation (Nocentini et al., 2000).

The aliphatic fraction of an oil product is formed mainly of alkanes, branched alkanes and isoprenoids, and to a lesser extent by cycloalkanes. Alkanes are more easily biodegraded than branched alkanes and biodegradability decreases with an increase in the number of carbon atoms. This pattern of hydrocarbon biodegradation has been described for bacterial and fungus metabolism (Colombo et al., 1996). Heavy-oil products have a considerable fraction of the so-called unresolved complex mixture (UCM) on the basis of its chromatographic profile. In fact, little is known about the composition of the UCM despite it being the main component of fuel oils (Wang and Fingas, 2003) that harbor branched and cyclic aliphatic and aromatic hydrocarbons, characterized by high resistance to biodegradation (Nievas et al., 2008). Furthermore, increases in the UCM after oil

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biodegradation processes have been reported in several studies (Ross et al., 2010).

Given this biodegradability pattern, the residual hydrocarbons in a soil contaminated with a heavy-oil product after bioremediation are complex mixtures rich in high-molecular-weight (HMW) hydrocarbons with a substantial proportion of a UCM. Because of this and as it is particularly difficult to decrease the concentration of total petroleum hydrocarbons (TPH) below the limits established by legislation in soils contaminated with heavy-oil products, efforts should be made to minimize the presence of such compounds and to better understand their effect on soil ecotoxicity. To improve understanding and efficacy, both chemical biodegradation and the predominant microbial populations need to be assessed during bioremediation processes. Previous studies have focused on microbial communities responsible for degrading heavy fuel in marine environments (Alonso-Gutierrez et al., 2009) but little is known about oil-degrading communities in industrially polluted soils (MacNaughton et al., 1999; Mishra et al., 2001; Zucchi et al., 2003).

Ecotoxicological tests have successfully been used as a complementary tool to monitor bioremediation efficiency in soil, which is important to assess ecological risks at polluted sites (Wang et al., 2010). However, very few studies combine these toxicological tests with a detailed study of the microbial communities in historically oil-polluted soils (Liu et al., 2010; Sheppard et al., 2011). To ensure proper risk assessment of contaminated sites and the monitoring of bioremediation processes, toxicity assays, chemical analyses and molecular microbial ecology studies of the microbial populations in polluted areas should be combined (Plaza et al., 2010).

Here we evaluated the feasibility of several biostimulation and bioaugmentation agents in soil contaminated with a heavy mineral oil (C_{15}–C_{35}). To this end, we tested the following strategies: i) addition of the biosurfactant M_{AT10}, obtained by cultivating the strain *Pseudomonas aeruginosa* AT10 (Abalos et al., 2004); ii) addition of glucose; iii) inoculation of a microbial consortium (TD) that is specialized in the biodegradation of the aliphatic fraction of crude oil (Viñas et al., 2002); and iv) inoculation of a hydrocarbon-degrading strain of the ligninolytic fungus *Trametes versicolor* (Borràs et al., 2010). In addition, to better understand potential metabolic strategies and their final effects on soil toxicity, we studied toxicity and characterized the microbial community during biodegradation by means of multiple culture-independent techniques.

2. Material and methods

2.1. Soil analysis

Oil-contaminated soil was sampled from a former screw manufacturing metallurgic facility in the city of Barcelona (Spain) which was decommissioned in 1990. The soil has been subjected to contamination during a period of 20 years. A cutting oil-contaminated soil from a former screw manufacturing metallurgic facility in the city of Barcelona (Spain) was affected by a previous period pollution of 20 years which was decommissioned in 1990. The upper part of the soil (1.5 m) was excavated and disposed into a landfill in 2005. In the present study a composite soil sample (50 kg) was obtained from the top soil layer (0–20 cm) and sieved (<6 mm) after soil excavation. Inorganic nutrients were determined by ion chromatography in a 1:5 (w/w) soil:water slurry with double deionized water. Nitrite, nitrate and phosphate were measured in a chromatographic system equipped with a Waters 515 pumping system, a Waters IC-PAK Anion column (Waters Corporate, Milford, USA), a UV/V Kontron model 332 detector (Kontron Instruments, Milan, Italy) and a Wescan conductivity meter (Wedan Instruments, Santa Clara, USA). The ammonium concentration was assessed using the automated phenate method (Standard Method 4500-NH3 H, American Public Health Association, 1992) in a Technicon Autoanalyzer II (Bran and Luebbe Analyzing Technologies Inc., Elmsford, USA). The pH was measured in a 1:2.5 (w/v) soil:water slurry with a Crison micro pH 2000 meter (Crison, Barcelona, Spain). Conductivity was determined with a Crison conductimeter model 522 in a 1:10 (w/v) soil:water slurry. Other physicochemical parameters such as soil moisture and water-holding capacity (WHC) were determined as described elsewhere (Sabaté et al., 2004).

2.2. Soil microcosm experiments

Initially the soil was treated with water and aerated by means of mechanical mixing with a glass rod twice a week for 100 days. Afterwards, the soil was subjected to different treatments for an additional 180 days. For each treatment, three independent replicates (200-ml glass receptacles covered with perforated parafilm) were prepared as microcosms, each containing 60 g of sieved (<6 mm) soil. In all the treatments, the water content was adjusted to 60% of WHC. Twice a week, the microcosm contents were mixed and the soil water content was restored by controlling the weight.

Seven different treatments were applied in triplicate:

1) *Basic treatment (H)*: soil was aerated by mixing every week and water added to maintain at 60% of the WHC. This basic treatment (H) was applied to all the samples except the air dried control.
2) *Inorganic nutrient treatment (H + N)*: NH_4NO_3 and K_2HPO_4 were added during the first 30 days, to produce a final C:N:P molar concentration equivalent to 300:10:1.
3) *Easily biodegradable substrate (H + N + G)*: inorganic nutrients and 0.2% w/w glucose were added.
4) *Bioaugmentation I: (H + N + TD)*: nutrients and the bacterial consortium TD, as a gas–oil degrading inoculum, were inoculated into the soil to reach 10^8 microorganisms · g^{-1} of soil (Abalos et al., 2004). Consortium TD is capable of extensively degrading Casablanca crude oil by using both the linear aliphatic fraction and the branched alkanes to a high degree (Viñas et al., 2002). The mixture has been maintained using diesel as the sole carbon and energy source for 10 years.
5) *Bioaugmentation II: (H + N + F)*: the ligninolytic fungus *T. versicolor* strain ATCC#42530 pre-grown on 3.5 g of rice straw, previously described as a PAH-degrading inoculum (Borràs et al., 2010), was inoculated into the soil. The fungus was previously grown with the rice straw for seven days. Once the mycelium colonized the straw, the mycelium and the straw were crushed together and mixed with the soil to generate many different points of fungal colonization.
6) *Biosurfactant treatment (H + N + BS)*: nutrients and the biosurfactant M_{AT10} were added to the soil in two different concentrations: 10 and 100 times above its critical micelle concentration (CMC) defined as 39 mg/l (Abalos et al., 2004). M_{AT10} rhamnolipids were harvested from the supernatant of a cell culture of *P. aeruginosa* AT10 grown in a mineral medium with soybean oil, as previously described (Abalos et al., 2004).
7) *Air dried soil* (1% (w/w) water content) was used as a biodegradation control.

2.3. Analysis of TPH

At days 0, 100, 190, and 280, a 30-g soil sample was taken from each microcosm in triplicate. The samples were sieved (2-mm grid) and dried for 16 h at room temperature. Organic pollutants were extracted from 10 g of the soil. Before the extraction, *o*-terphenyl (50 μg) was added in acetone solution as a surrogate internal standard. The acetone was allowed to evaporate and 10 g of anhydrous Na_2SO_4 was added and mixed. Soxhlet extraction was performed on this mixture with dichloromethane:acetone (1:1 (v/v)) for 6 h. The extract was dehydrated through a Na_2SO_4 column and concentrated to 1 ml with a rotary evaporator. The TPH fraction was obtained

with an alumina chromatographic column following the EPA3611 method (U.S. Environmental Protection Agency). The TPH fraction was analyzed by gas chromatography with flame ionization detection (Lladó et al., 2009). The TPH content was calculated from the total area compared to that of an aliphatic standard (AccuStandard, New Haven, USA) calibration curve.

2.4. Counting of total heterotrophic and hydrocarbon-degrading microbial populations

Heterotrophic and alkane-degrading microbial populations were enumerated throughout the microcosm experiments by the miniaturized most-probable-number (MPN) technique (Wrenn and Venosa, 1996). The heterotrophic microbial populations were enumerated on Trypticase Soy Broth. The mineral medium with the aliphatic saturated fraction (F1) of Casablanca crude oil was used as the sole source of carbon and energy for the alkane degraders (Aceves et al., 1988).

2.5. Microbial community characterization by means of denaturing gradient gel electrophoresis (DGGE)

2.5.1. DNA extraction

Soil samples were collected from each microcosm for DNA extraction at days 0, 100 and 280 in sterile Eppendorf tubes and stored at −20 °C prior to analysis. To ascertain the repeatability of the DNA extraction process and PCR protocols, a set of replicates was analyzed by means of DGGE. This showed a high degree of repeatability of the sampling and molecular protocols (DNA extraction and PCR) among replicates (Fig. 3B). Hence, DNA was extracted from a composite 0.75-g sample containing 0.25 g from each microcosm replicate. Total community DNA was extracted from the soil microcosms following a bead beating protocol using the Power Soil DNA extraction kit (MoBio Laboratories, Solano Beach, USA), according to the manufacturer's instructions. A further clean-up step was necessary to avoid PCR inhibition; we performed this using Clean DNA Wizard kit (Promega, Madison, USA).

PCR: The V3–V5 hypervariable regions of the 16S rRNA gene were amplified from total community DNA by PCR using primers F341-GC and R907 (Yu and Morrison, 2004). The primer F341-GC included a GC clamp at the 5′ end (5′-CGCCCGCCGCGC CCCGCGCCCGTCCCGCCG CCCCCGCCCG-3′). All PCR reactions were performed in a Mastercycler personal thermocycler (Eppendorf, Hamburg, Germany). Fifty ml of the PCR mixture contained 2.5 U Takara Ex Taq DNA Polymerase (Takara Bio, Otsu, Shiga, Japan), 25 mM TAPS (pH 9.3), 50 mM KCl, 2 mM $MgCl_2$, 200 μM of each deoxynucleoside triphosphate, 0.5 μM of each primer, and 100 ng of template DNA quantified by means of the Low DNA Mass Ladder (Gibco BRL, Rockville, USA). After 9 min of initial denaturation at 95 °C, a touchdown thermal profile protocol was performed and the annealing temperature was decreased by 1 °C per cycle from 65 °C to 55 °C, at which temperature 20 additional cycles were carried out. Amplification was carried out with 1 min of denaturation at 94 °C, 1 min of primer annealing and 1.5 min of primer extension at 72 °C. The last step involved a 10-min extension at 72 °C.

2.5.2. DGGE gel

Approximately 800 ng of purified PCR-16SrRNA amplicon product was loaded onto a 6% (wt/vol) polyacrylamide gel, 0.75 mm thick (to obtain better resolution) with denaturing chemical gradients of formamide and urea ranging from 40% to 60% (100% denaturant contains 7 M urea and 40% formamide). The Low DNA Mass Ladder was used for quantification. DGGE was performed in 1× TAE buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, pH 7.4) using a DGGE-2001 System (CBS Scientific Company, Del Mar, USA) at 100 V and 60 °C for 16 h.

The gels were stained for 45 min in 1× TAE buffer containing SybrGold (Molecular probes, Inc., Eugene, USA), then scanned using a Bio-Rad molecular imager FX Pro Plus multi-imaging system (Bio-Rad Laboratories, Hercules, USA) in DNA stain gel mode for SybrGold at medium sample intensity. Images of the DGGE gels were digitalized and the DGGE bands were processed using Quantity-one image analysis software, version 4.1 (Bio-Rad Laboratories) and corrected manually.

2.6. Sequencing and phylogenetic analysis

Predominant DGGE bands were excised with a sterile razor blade, resuspended in 50 μl sterilized MilliQ water and stored at 4 °C overnight. An aliquot of the supernatant (2 μl) was used to reamplify the DGGE bands with primers F341, without the GC clamp, and R907, under the same conditions. Band-PCR products were further purified for sequencing using a Wizard SV Gel and PCR Clean-Up System (Promega) according to the manufacturer's instructions. The DNA sequencing reaction was carried out in a thermocycler (Mastercycler) using an ABI Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) as specified by the manufacturer. The primers used were F341 and R907 and the conditions of the amplification were as follows: an initial denaturing step of 1 min at 96 °C, followed by 25 cycles of 10 s at 96 °C, 5 s at 55 °C and 4 min at 60 °C. The sequencing reaction was analyzed by the Scientific-Technical Services of the University of Barcelona (SCT-UB) using an ABI Prism 3700 DNA Analyzer (Applied Biosystems).

Raw sequence data were checked and analyzed with the BioEdit (version 7.0) software package (Ibis Biosciences, Carlsbad, USA), inspected for the presence of ambiguous base assignments and subjected to the Chimera check with Bellerophon version 3 (Huber et al., 2004). Sequences were compared with those deposited in the GenBank (NCBI) database using alignment tool comparison software (BLASTn and RDP) to find the closest sequence match and taxonomic affiliation.

The 18 nucleotide sequences (DGGE bands 1–18) identified in this study were deposited in the GenBank database under accession numbers JN795892 to JN795909.

2.7. Acute toxicity test in Eisenia fetida

Worms were selected from a lab-reared population destined for experimentation. The individuals were more than 3 months old, with well-developed clitellum and a weight of from 0.25 to 0.4 g per animal.

Toxicity testing was performed according to the Organisation for Economic Cooperation and Development (OECD) guideline 207 for acute soil toxicity testing in its “artificial soil” modality (OECD, 1984). The artificial soil test yields toxicity data that is more representative of natural earthworm exposure to chemicals than the “simple contact” test, which is easier to perform. The OECD guideline uses an artificial soil both as a control in the toxicity assays and also to obtain the polluted soil dilutions for the assay.

Ten earthworms were cultivated in 200 g of each treatment and in control soils for 14 days. The dilutions of the experimental soil were carried out using dry weight of test artificial soil according to the OECD guideline 207 (70% sand, 20% kaolin clay and 10% sphagnum peat). The soil moisture was adjusted every three days to 35% with deionized water. The temperature of the assay was 21 °C ± 3 °C and the photoperiod was of 16 h light:8 h dark. A range-finding test using 37.5%, 50%, 75%, 87.5% and 100% of polluted soil was initially performed to determine the concentrations at which 0% and 100% mortality occurred, and to establish lethal concentration 50 (LC_{50}). Two full assays were then carried out at 100 and 190 days with the treatments described previously in Section 2.2. In each period of exposure, a parallel negative control of the artificial soil was performed (OECD, 1984). After 7 and 14 days, the weight of each earthworm was recorded as well as the number of casualties and any comments.

2.8. Comet assay

After exposure to the treatment soils, coelomocytes were obtained from the surviving earthworms using the extrusion method as previously described (Eyambe et al., 1991). Genotoxicity was determined using the comet assay (Singh et al., 1988). The cell suspensions from each animal, in each exposure, were included in low-melting-point agarose and extended on coded slides previously treated with a layer of agarose of normal melting point. After solidification at 4 °C for 10 min, the cover slides were removed and the slides were immersed in lysis buffer (4 °C, 2.5 M NaCl, 100 mM disodium EDTA and 10 mM Tris; and 1% Triton X-100 just before use, pH 10) for 2 h. The slides were placed in an electrophoresis tank with electrophoresis buffer (4 °C, 1 mM disodium EDTA and 300 mm NaOH, pH > 13) for 20 min to facilitate the unwinding of the cell DNA. The electrophoresis was then run for 20 min at 25 V and 300 mA. After the electrophoresis, the DNA was fixed with 0.4 M Tris buffer pH 7.5 with 3 changes of 5 min each at 4 °C. The samples were stained with DAPI (4′,6-diamidino-2-phenylindole) and 50 cells from each individual were analyzed, whenever possible, with Analysis® software.

2.9. Statistical analysis

The statistical significance of the TPH data from the biodegradation experiments was evaluated by analysis of variance (ANOVA) and Tukey's multiple comparison test. The data were considered to be significantly different if $P \leq 0.05$. The effect of the main biostimulation and bioaugmentation treatments on the microbial diversity of the soil was assessed by comparing the DGGE profiles using a similarity cluster analysis. A dendogram was constructed, using the group average method with the Pearson product–moment correlation coefficient. Version 5.1 of Statgraphics Plus (Statistical Graphics Corp.) was used for all chemical and microbiological assay statistical analyses.

The comet assay was statistically evaluated using the SPSS 15.0 statistical package (SPSS Inc., Chicago, USA). Each encoded sample was considered as independent and duplicates were performed.

3. Results and discussion

3.1. Soil description

The soil used (sandy-loam texture) was from the site of a former screw plant, which had been operating for several decades before this study. Thus, information about the kind of contaminating products present was obtained from the chromatographic profile. The TPH profile was of a heavy-oil product (mineral oil), in the hydrocarbon range of C_{15}–C_{35}, with a considerable UCM, which might well correspond to a heavy mineral oil, such as drilling/cutting oil (Fig. 1). First, to establish the feasibility of applying bioremediation technology to this soil, we performed a bio-feasibility assay, as previously described (Sabaté et al., 2004). Also, the optimum water content of the soil for the microcosm experiments was defined as 60% of WHC. Table 1 shows the main physical, chemical and microbiological characteristics of the soil studied. It contained a significant amount and proportion of an alkane (saturated) fraction-degrading population (0.2%), thereby indicating that biostimulation and bioaugmentation strategies were suitable for this matrix.

3.2. Biostimulation and bioaugmentation microcosm assays

Microcosm experiments were carried out for 280 days. During the first 100 days, biostimulation was only by means of aeration at optimal humidity (60% WHC). This process caused a 15% depletion of the soil TPH content.

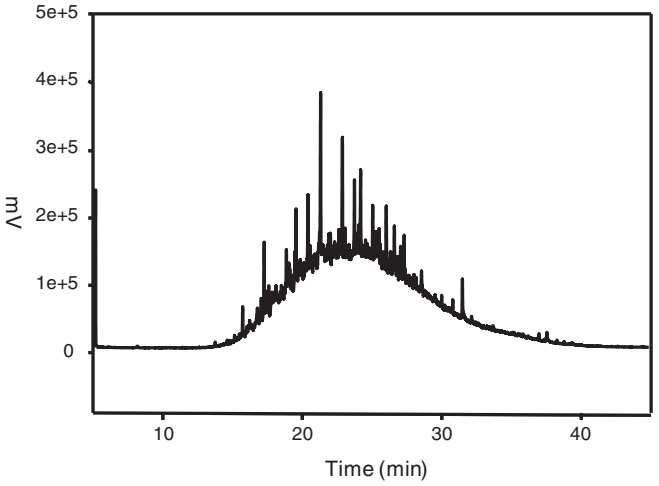

Fig. 1. GC-FID chromatographic profile of the TPH content of the original heavy-oil-polluted soil.

After biostimulation and bioaugmentation strategies applied for the following 180 days, TPH biodegradation ranging from 30% to 50% was achieved, depending on the treatment (Fig. 2). Neither the nutrient additions nor the nutrient additions plus the TD consortium improved the hydrocarbon degradation achieved by the autochthonous microbial population biostimulated by optimal soil water content. This finding is consistent with other studies reporting no benefit from bacterial inocula in hydrocarbon-contaminated soil (Jorgensen et al., 2000). It is important to point out that the highest TPH degradation was reached after *T. versicolor* inoculation, with a reduction of 50% of TPH ($P < 0.05$) accompanied by a considerable decrease in the UCM and a significant shift in the microbial population's diversity (Fig. 3), promoting hydrocarbon-degrading microbial populations (Fig. 4). Ligninolytic fungi have traditionally been used to enhance the biodegradation of recalcitrant compounds with structural similarities to lignin, such as polycyclic aromatic hydrocarbons (PAHs) (Chupungars et al., 2009). Nevertheless, the degradation of TPH by *Phanerochaete chrysosporium*, *Pleurotus ostreatus* and *Coriolus versicolor* has also been reported (Yateem et al., 1997). Several studies have shown degradation of TPH in crude oil by *T. versicolor*, but only in liquid biodegradation assays (Colombo et al., 1996). Furthermore, the filamentous fungus *Penicillium simplicissimum* YK degrades long-chain alkanes comprising up to 50 carbon atoms (Yamada-Onodera et al., 2002). While most previous *Trametes* bioaugmentation studies of polluted soils focus mainly on PAH biodegradation, its effect on a non-sterile industrial mineral-oil-polluted soil including active autochthonous microbial populations

Table 1
Physical, chemical and microbiological characteristics of the contaminated soil.

Main characteristics	Values
TPH ($mg \cdot kg^{-1}$)	1727
pH	7.5
Conductivity ($\mu S \cdot cm^{-1}$)	322
WHC (% humidity w/w)[a]	33.7
Humidity (% WHC)	58.8
$N\text{–}NH_4$ ($mg \cdot kg^{-1}$)	45.8
$N-(NO_3+NO_2)$ ($mg \cdot kg^{-1}$)	1.7
Heterotrophs (MPN $\cdot kg^{-1}$)[b]	$8.0 \cdot 10^8$
F1 degraders (MPN $\cdot kg^{-1}$)[c]	$2.1 \cdot 10^6$

[a] WHC: Water Holding Capacity.
[b] MPN: Most Probably Number.
[c] F1: aliphatic saturated fraction of the Casablanca crude oil.

Table 2
Properties of DGGE bands: designations and accession numbers for the band sequences and levels of similarity to related organisms.

Band	Band detection[a]						Closest organism in GenBank database (accession no.)	% similarity[b]	Phylogenetic group[c]
	L1	L2	L3	L4	L5	L6			
B1 = B3 = B4 = B13	+	+	+	+	+	+	*Brevundimonas vesicularis* (JN084130)	96%	*Caulobacteraceae* (α)
B2	+	−	−	−	−	−	*Dietzia maris* (JF505994)	100%	*Corynebacterineae* (Actinobacteria)
B5	−	−	+	−	−	−	*Rhizobium* sp. (Y12350)	90%	*Rhizobiaceae* (α)
B6	−	−	+	−	−	−	*Flavobacterium* sp. (EU037956)	99%	*Flavobacteriaceae* (CFB group)
B7	−	−	−	+	+	−	*Altererythrobacter* sp. (FN397680)	94%	*Erythrobacteraceae* (α)
B8	−	−	−	+	+	−	*Parasegitibacter luojiensis* (NR_044576)	97%	*Chitinophagaceae* (CFB group)
B9	−	−	+	+	+	+	Uncultured *Sphingobacteriales* (AM934931)	98%	*Sphingobacteriales* (CFB group)
B10	−	−	−	+	+	+	*Comamonadaceae* bacterium (GQ454852)	95%	*Comamonadaceae* (β)
B11	+	+	+	+	+	−	Uncultured *Sphingobacteriales* (AM936239)	88%	*Sphingobacteriales* (CFB group)
B12	−	−	−	+	+	+	*Ramlibacter* sp. (AM411936)	97%	*Comamonadaceae* (β)
B14	−	−	−	−	−	+	*Herbaspirillum* sp. (AB545652)	94%	*Oxalobacteraceae* (β)
B15 = B16	−	−	−	−	−	+	*Bacillus selenatarsenatis* (JN624922)	100%	*Bacillaceae* (Firmicutes)
B17	−	−	−	+	+	+	*Arthrobacter sulfonivorans* (HQ824849)	99%	*Micrococcaceae* (Actinobacteria)
B18	−	−	−	−	−	+	*Streptomyces* sp. (JN572690)	98%	*Streptomycetaceae* (Actinobacteria)

[a] Band detection (+) above 1% of relative intensity.
[b] Sequences were aligned against the GenBank database with the BLAST search alignment tool.
[c] Phylogenetic groups were defined by using the Ribosomal Data Project (RDP) Naive Bayesian Classifier (Wang et al., 2007). Family is represented. α, β, represent α-proteobacteria and β-proteobacteria, respectively.

has rarely been reported (Yateem et al., 1997). Yateem et al. (1997) described significant enhancement of heavy-oil biodegradation, but, as in other fungal bioaugmentation studies of industrially polluted soils, reported no information about its effect on either the autochthonous microbial community or soil ecotoxicity. In contrast, among the biostimulation agents, the addition of the rhamnolipids produced by the strain AT10 from *P. aeruginosa* did not improve the biodegradation achieved by the treatments. In a previous paper we described, in a liquid culture, a considerable improvement in the biodegradation of a crude oil by a microbial consortium specializing in degrading polycyclic aromatic hydrocarbons in the presence of the same biosurfactant as that used in the present study (Abalos et al., 2004). The interactions between the surfactant, the solid matrix, the contaminant and the microbial populations in a soil are highly complex and give rise to a lot of controversy (Elliot et al., 2010; Whang et al., 2008). The preferential use of surfactants as a carbon source by hydrocarbon degraders could explain the inhibited biodegradation of the pollutants (Deschenes et al., 1996).

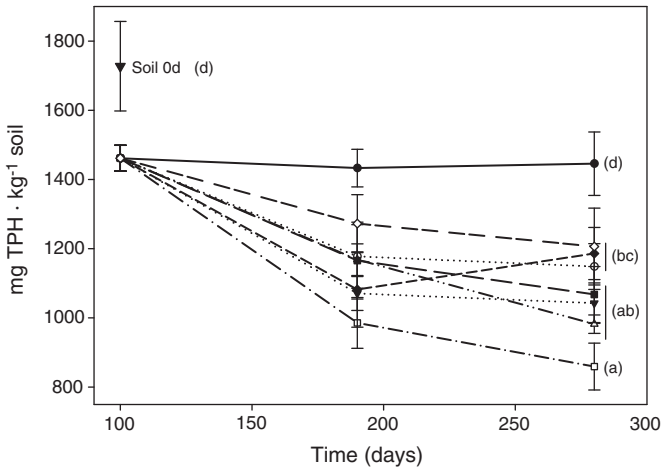

Fig. 2. Residual concentration of TPH after bioremediation treatments. ●, control (air-dried soil); ○, basic (H); ▼, nutrients (H + N); △, nutrients and glucose (H + N + G); ■, nutrients and TD consortium (H + N + TD); □, nutrients and *Trametes versicolor* (H + N + F); ◆, nutrients and surfactant (H + N + BS) at 10 times its critical micelle concentration (CMC); ◊, nutrients and surfactant (H + N + BS) at 100 times its CMC. Different letters in brackets indicate significant differences among the treatments ($P<0.05$). Vertical bars represent the standard deviation of three independent replicates ($n=3$).

3.3. Monitoring of heterotrophic and hydrocarbon-degrading microbial populations

The MPN results show that, from day 100, the presence of heterotrophic populations decreased due to almost all the treatments (Fig. 4). This finding suggests a reduction in organic matter that can be easily assimilated during incubation. In contrast, the population of aliphatic hydrocarbon degraders increased from one-fold to five-fold in all the biostimulation and bioaugmentation treatments, with the highest values reached when *T. versicolor* was inoculated. A similar phenomenon has been described in other historically polluted soils, which suggests that it is a common trend in bioremediation processes for this matrix (Liu et al., 2010). This is consistent with the gradual depletion of TPH detected in the soil.

In the treatment with *T. versicolor*, the hydrocarbon-degrading population was higher than in the other treatments, reaching 100% of the heterotrophic population after 280 days. This increase in the specialized population as a consequence of fungal bioaugmentation, which was concomitant with a marked change in the eubacterial diversity detected by PCR-DGGE analyses (Fig. 3), may explain the TPH biodegradation efficiency. The change in the eubacterial community could be explained by the presence of the ligninolytic substrate in the soil, the use of fungal exudates as a nutrient source (Boer et al., 2005) or the antimicrobial compounds produced by the inoculated fungus (Vázquez et al., 2000). Furthermore, the heterotrophic population was also approximately double that in the other treatments. A significant part of this bacterial growth could be attributed to the presence of the fungal ligninolytic substrate in the microcosms, as well as changes in the microbial population (Federici et al., 2007). Nonetheless, mycoremediation was enhanced by the presence of active autochthonous microbial populations. Positive and negative interactions between the indigenous microbial populations and inoculated fungi have been described. Thus, fungi could participate in the transformation of some HMW hydrocarbons into readily biodegradable substrates by bacteria. In keeping with this, an increase in heterotrophic cultivable bacteria in soils inoculated with *Irpex lacteus* and *P. ostreatus* has been reported (Leonardi et al., 2008). In contrast, the growth of certain white-rot fungus is commonly suppressed by indigenous soil microbes and by abiotic features of soil compounds (Tucker et al., 1995).

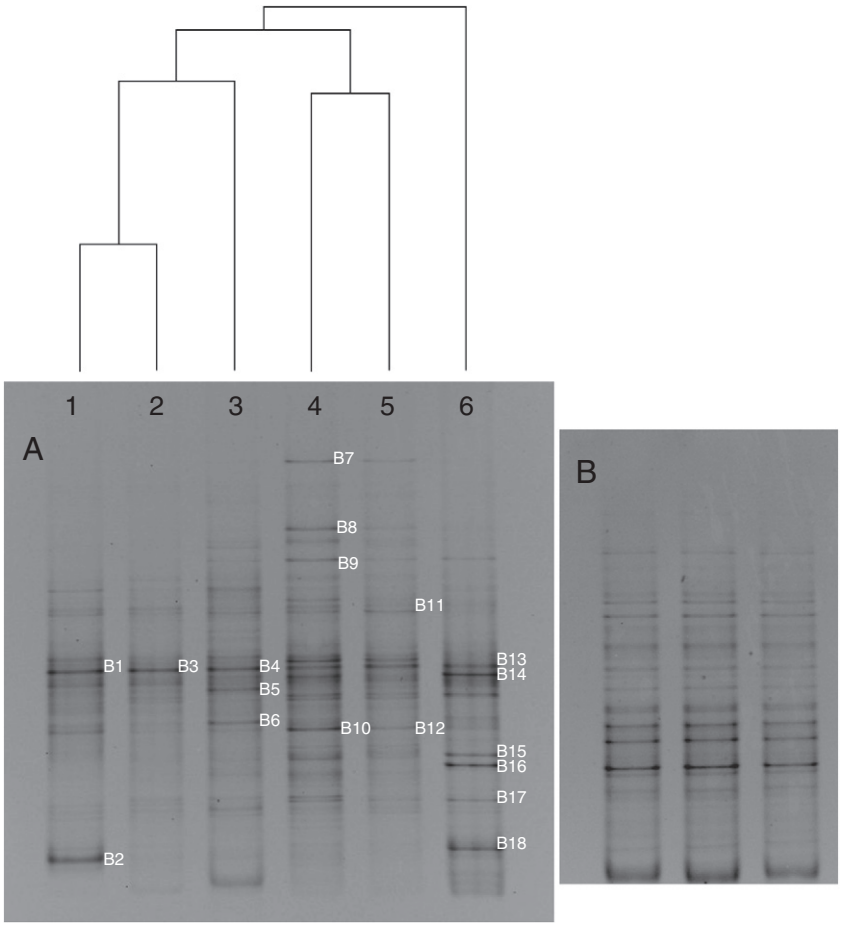

Fig. 3. (A) Denaturing gradient gel electrophoresis (40% to 60% denaturant) profiles and cluster analysis (group average method; squared Euclidean distance) of eubacterial biodiversity from the original and five treated soils. From left to right: lane 1, 0 days; lane 2, 100 days; lane 3, 100 days plus rice straw; lane 4, basic treatment at 280 days; lane 5, nutrient treatment at 280 days; lane 6, nutrient and *Trametes versicolor* treatment at 280 days. Numbered DGGE bands were successfully excised and sequenced and are shown in Table 2. (B) DGGE (20% to 80% denaturant) from a set of independent samples in triplicate (sample: 100 days plus rice straw addition).

3.4. Microbial community assessment

To analyse the initial microbial population in the soil and its response to different bioremediation treatments, we performed a PCR-DGGE analysis (Fig. 3).

A DGGE profile of the initial polluted soil showed little diversity, which is a common result of the DGGE technique and is also common in polluted environments. Two predominant DGGE bands were detected. Band B1 was found in all the treatments. On the basis of partial *16SrRNA* gene sequences, band B1 was found to be very similar to the *Brevundimonas* genus, while band B2 was very similar to the *Dietzia* genus. Although *Brevundimonas* and *Dietzia* are microbial genera commonly found in pristine soil environments, some members isolated from polluted environments show aliphatic hydrocarbon-degrading capability as well (Bodtker et al., 2009; Xiao et al., 2010).

The DGGE profiles from the first 100 days of biostimulation (Fig. 3; lanes 2 and 3) were not very different. The addition of rice straw on day 100 did not alter the soil population substantially, either. However, during the following 180 days of treatment, biodiversity increased considerably in the three profiles (basic biostimulation, inorganic nutrients and *T. versicolor* inoculation). This finding could be attributable to the late growth of bacterial species that are adapted to the use of more recalcitrant hydrocarbons as a carbon source.

Soil biostimulation with water or water plus nutrients for 280 days resulted in similar DGGE profiles and TPH degradation rates (lanes 4 and 5 in Fig. 3). However, other studies report that the DGGE profiles for a hydrocarbon-polluted soil biostimulated with water or water plus nutrients differ greatly (Wu et al., 2008). These distinct diversity patterns suggest that similar biostimulation treatments produce population changes that differ, depending on the polluted soil matrix and the microbial community involved.

At the end of the bioaugmentation experiments involving *T. versicolor* inoculation, five additional bands (B14, B15, B16, B17 and B18) appeared in the 16SrRNA-DGGE. B14 corresponded to *Herbaspirillum* sp., B18 to *Streptomyces* sp., and B15 and B16 to *Bacillus* sp. All these genera have been associated with recalcitrant hydrocarbon biodegradation (Chaudhary et al., 2011; Das and Mukherjee, 2007; Ross et al., 2010). Finally, B17 corresponded to the genus *Arthrobacter*, which produces extracellular emulsifier factors with the capacity to emulsify light petroleum oil, diesel oil and a variety of crude oils and gas oils (Rosenberg et al., 1979).

These results confirm that the presence of *T. versicolor* and its ligninolytic substrate in the soil substantially changed the bacterial biodiversity over the 180 days of incubation, promoting the enrichment of Gram-positive bacteria belonging to the *Actinobacteria* and *Bacillus* groups. It is important to point out that microbial diversity changes promoted after *T. versicolor* inoculation were concomitant with both the high proportion of hydrocarbon degraders encountered in the MPN assays and the higher TPH biodegradation observed in the white-rot fungus bioaugmentation treatment.

3.5. Acute toxicity test in E. fetida

Filtering organisms in ecosystems reflect the health of the environment; in particular, *E. fetida* is one of the clearest cases of this.

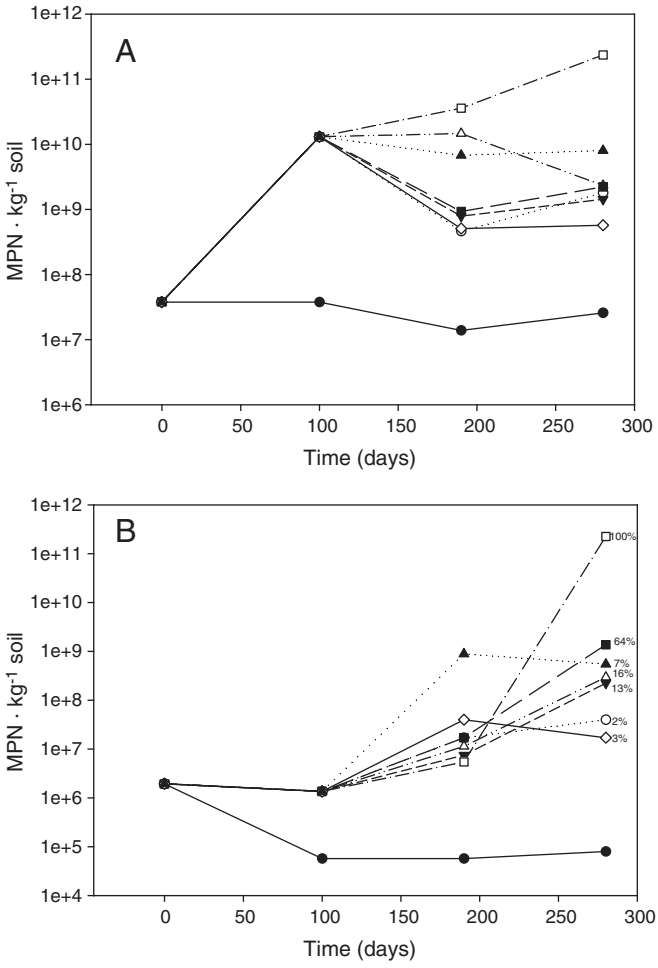

Fig. 4. Heterotrophic (A) and F1-degrading (B) populations in soil treatments over the 280 days of incubation in microcosms. ●, control (air-dried soil); ○, basic (H); ▼, nutrients (H + N); △, nutrients and glucose (H + N + G); ■, nutrients and TD consortium (H + N + TD); □, nutrients and *Trametes versicolor* (H + N + G); ◊, nutrients and surfactant (H + N + BS) at 10 times its CMC; ▲, nutrients and surfactant (H + N + BS) at 100 times its CMC. Panel B shows the percentage of the heterotrophic population represented by the aliphatic (F1)-degrading population.

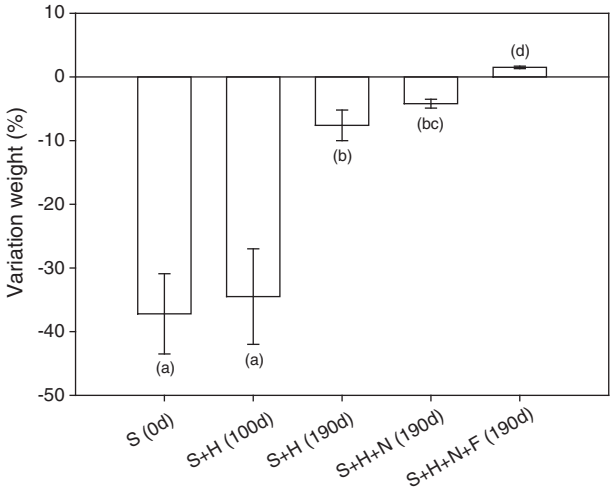

Fig. 5. Evolution of *Eisenia fetida* weight during the soil experiment. From left to right: S(0), soil at 0 days; S + H(100d), soil + humidity at 100 days; S + H(190d), soil + humidity at 190 days; S + H + N(190d), soil + humidity + nutrients at 190 days; S + H + N + F(190d), soil + humidity + nutrients + fungus at 190 days. Different letters in brackets indicate significant differences between the treatments ($P<0.05$). Vertical bars represent the standard deviation ($n=10$).

This is why the organism has been used as an indicator of pollution in many studies and is the experimental system of choice in the Organisation for Economic Cooperation and Development guidelines for soil assessment (OECD, 1984).

No *E. fetida* mortality was observed in the range finding test (Section 2.7), at any polluted soil dilution tested. Therefore no LC_{50} could be established for the contaminated soil.

Undiluted soil was used for the subsequent worm weight assessment and acute toxicity tests for the most significant bioremediation treatments (Fig. 5). No lethality was observed at day 100 or in three of the assays at day 190 (H, H + N and H + N + F); none of the exposure patterns tested affected *E. fetida* mortality. This finding could be explained by the low bioavailability of the pollutant. However, bioremediation treatments altered worm weight during the incubation period in relation to controls. Other studies have reported decreasing toxicity in polluted soils during bioremediation treatments (Liu et al., 2010). At day 190, the individuals in all three of the treatment groups showed lower weight losses than after 0 or 100 days, and there was even a weight increase in the H + N + F group. This finding suggests positive correlation between the length of treatment and the health of the organisms (expressed as weight). At 190 days, the treatments increased soil quality in the order: H+N+F > H+N > H. The increased eubacterial biodiversity in the degrading population detected through DGGE in the bioaugmentation with *T. versicolor* may be related to the increased detoxifying potential.

3.6. Comet assay in coelomocytes of *E. fetida*

We performed a comet assay using coelomocytes from surviving worms from the different biotreated soil samples after the acute toxicity tests. DNA degradation, ranging from 33% to 47%, was observed in all the treatments. However, no significant differences on the basis of DNA fragmentation were observed between treatments over time compared to their respective controls ($P>0.05$). This result suggests that the aliphatic compounds present in the polluted soil were not genotoxic. This notion is supported by the lack of evidence in the literature of genotoxicity caused by aliphatic hydrocarbons. However, a genotoxicity evaluation should be performed because, although the parental compounds are non-genotoxic, intermediate metabolites produced by the microbial metabolism could contribute to increased soil genotoxicity (Cao et al., 2009).

4. Conclusions

This study confirms that mycoremediation by means of allochthonous bioaugmentation with a white-rot fungus such as *T. versicolor* is an effective remediation and detoxifying strategy, not only for PAH-polluted soils, but also for soils contaminated with heavy mineral oil.

The study also highlights the importance of carrying out an in-depth microbiological assessment through bioremediation experiments involving historically polluted soils, in order to gain insight into bacteria–fungi interactions. Here we report that the use of an external fungal inoculum produces a significant increase and shift in the detectable biodiversity of the autochthonous bacterial community, promoting more hydrocarbon-degrading microbial populations in the soil than other biostimulation treatments do.

Finally, we recommend a diversified approach in bioremediation tests at the bench scale by combining TPH degradation, microbial ecology, acute toxicity and genotoxicity assessment in order to clarify biodegradation processes and ensure reliable risk assessment throughout the bioremediation of industrially polluted soils.

Acknowledgments

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References

Abalos A, Viñas M, Sabate J, Manresa MA, Solanas AM. Enhanced biodegradation of Casablanca crude oil by a microbial consortium in presence of a rhamnolipid produced by *Pseudomonas aeruginosa* AT10. Biodegradation 2004;15:249–60.

Aceves M, Grimalt J, Albaiges J, Broto F, Comellas L, Gassiot M. Analysis of hydrocarbons in aquatic sediments. II. Evaluation of common preparative procedures for petroleum and chlorinated hydrocarbons. J Chromatogr 1988;436:503–9.

Alonso-Gutierrez J, Figueras A, Albaiges J, Jimenez N, Viñas M, Solanas AM, et al. Bacterial communities from shoreline environments (costa da morte, northwestern Spain) affected by the prestige oil spill. Appl Environ Microbiol 2009;75:3407–18.

Association American Public Health. Standard methods for the examination of water and wastewater. In: Clesceri LS, Greenberg AE, Eaton AD, editors. American Public Health Association. Washington, DC, USA: American Water Works Association and the Water Environment Federation; 1992.

Bodtker G, Hvidsten IV, Barth T, Torsvik T. Hydrocarbon degradation by *Dietzia* sp. A14101 isolated from an oil reservoir model column. Antonie Van Leeuwenhoek 2009;96:459–69.

Boer W, Folman LB, Summerbell RC, Boddy L. Living in a fungal world: impact of fungi on soil bacterial niche development. FEMS Microbiol Rev 2005;29:795–811.

Borràs E, Caminal G, Sarrà M, Novotný C. Effect of soil bacteria on the ability of polycyclic aromatic hydrocarbons (PAHs) removal by *Trametes versicolor* and *Irpex lacteus* from contaminated soil. Soil Biol Biochem 2010;42:2087–93.

Cao B, Nagarajan K, Loh KC. Biodegradation of aromatic compounds: current status and opportunities for biomolecular approaches. Appl Microbiol Biotechnol 2009;85:207–28.

Chaudhary P, Sharma R, Singh SB, Nain L. Bioremediation of PAH by *Streptomyces* sp. Bull Environ Contam Toxicol 2011;86:268–71.

Chupungars K, Rerngsamran P, Thaniyavarn S. Polycyclic aromatic hydrocarbons degradation by *Agrocybe* sp. CU-43 and its fluorene transformation. Int Biodeter Biodegr 2009;63:93–9.

Colombo JC, Cabello M, Arambarri AM. Biodegradation of aliphatic and aromatic hydrocarbons by natural soil microflora and pure cultures of imperfect and lignolitic fungi. Environ Pollut 1996;94:355–62.

Das K, Mukherjee AK. Crude petroleum–oil biodegradation efficiency of *Bacillus subtilis* and *Pseudomonas aeruginosa* strains isolated from a petroleum–oil contaminated soil from North-East India. Bioresour Technol 2007;98:1339–45.

Deschenes L, Lafrance P, Villeneuve JP, Samson R. Adding sodium dodecyl sulfate and *Pseudomonas aeruginosa* UG2 biosurfactants inhibits polycyclic aromatic hydrocarbon biodegradation in a weathered creosote-contaminated soil. Appl Microbiol Biotechnol 1996;46:638–46.

Elliot R, Singhal N, Swift S. Surfactants and bacterial bioremediation of polycyclic aromatic hydrocarbon contaminated soil—unlocking the targets. Crit Rev Environ Sci Technol 2010;41:78-124.

Eyambe GS, Goven AJ, Fitzpatrick LC, Venables BJ, Cooper EL. A non-invasive technique for sequential collection of earthworm (*Lumbricus terrestris*) leukocytes during subchronic immunotoxicity studies. Lab Anim 1991;25:61–7.

Federici E, Leonardi V, Giubilei MA, Quaratino D, Spaccapelo R, D'Annibale A, et al. Addition of allochthonous fungi to a historically contaminated soil affects both remediation efficiency and bacterial diversity. Appl Microbiol Biotechnol 2007;77:203–11.

Huber T, Faulkner G, Hugenholtz P. Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. Bioinformatics 2004;20:2317–9.

Jorgensen KS, Puustinen J, Suortti AM. Bioremediation of petroleum hydrocarbon-contaminated soil by composting in biopiles. Environ Pollut 2000;107:245–54.

Lee S-H, Oh B-I, J-g Kim. Effect of various amendments on heavy mineral oil bioremediation and soil microbial activity. Bioresour Technol 2008;99:2578–87.

Leonardi V, Giubilei MA, Federici E, Spaccapelo R, Sasek V, Novotny C, et al. Mobilizing agents enhance fungal degradation of polycyclic aromatic hydrocarbons and affect diversity of indigenous bacteria in soil. Biotechnol Bioeng 2008;101:273–85.

Liu W, Luo Y, Teng Y, Li Z, Ma LQ. Bioremediation of oily sludge-contaminated soil by stimulating indigenous microbes. Environ Geochem Health 2010;32:23–9.

Lladó S, Jimenez N, Viñas M, Solanas AM. Microbial populations related to PAH biodegradation in an aged biostimulated creosote-contaminated soil. Biodegradation 2009;20:593–601.

MacNaughton SJ, Stephen JR, Venosa AD, Davis GA, Chang YJ, White DC. Microbial population changes during bioremediation of an experimental oil spill. Appl Environ Microbiol 1999;65:3566–74.

Mishra S, Jyot J, Kuhad RC, Lal B. Evaluation of inoculum addition to stimulate in situ bioremediation of oily-sludge-contaminated soil. Appl Environ Microbiol 2001;67:1675–81.

Nievas ML, Commendatore MG, Esteves JL, Bucala V. Biodegradation pattern of hydrocarbons from a fuel oil-type complex residue by an emulsifier-producing microbial consortium. J Hazard Mater 2008;154:96-104.

Nocentini M, Pinelli D, Fava F. Bioremediation of a soil contaminated by hydrocarbon mixtures: the residual concentration problem. Chemosphere 2000;41:1115–23.

OECD. Guideline for testing chemicals. Earthworm acute toxicity tests Paris, 207. Organisation for Economic Cooperation and Development; 1984.

Plaza GA, Nalecz-Jawecki G, Pinyakong O, Illmer P, Margesin R. Ecotoxicological and microbiological characterization of soils from heavy-metal- and hydrocarbon-contaminated sites. Environ Monit Assess 2010;163:477–88.

Rosenberg E, Zuckerberg A, Rubinovitz C, Gutnick DL. Emulsifier of *Arthrobacter* RAG-1: isolation and emulsifying properties. Appl Environ Microbiol 1979;37:402–8.

Ross AS, Farrimond P, Erdmann M, Larter SR. Geochemical compositional gradients in a mixed oil reservoir indicative of ongoing biodegradation. Org Geochem 2010;41:307–20.

Sabaté J, Viñas M, Solanas A. Laboratory-scale bioremediation experiments on hydrocarbon-contaminated soils. Int Biodeter Biodegr 2004;54:19–25.

Sheppard P, Adetutu E, Makadia T, Ball A. Microbial community and ecotoxicity analysis of bioremediated, weathered hydrocarbon-contaminated soil. Soil Res 2011;49:261–9.

Singh N, McCoy M, Tice R, Schneider E. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res 1988;175:184–91.

Tucker B, Radtke C, Kwon I, Anderson A. Suppression of bioremediation by *Phanerochaete chrysosporium* by soil factors. J Hazard Mater 1995;41:251–65.

Vázquez MM, César S, Azcón R, Barea JM. Interactions between arbuscular mycorrhizal fungi and other microbial inoculants (*Azospirillum*, *Pseudomonas*, *Trichoderma*) and their effects on microbial population and enzyme activities in the rhizosphere of maize plants. Appl Soil Ecol 2000;15:261–72.

Viñas M, Grifoll M, Sabate J, Solanas AM. Biodegradation of a crude oil by three microbial consortia of different origins and metabolic capabilities. J Ind Microbiol Biotechnol 2002;28:252–60.

Wang Z, Fingas MF. Development of oil hydrocarbon fingerprinting and identification techniques. Mar Pollut Bull 2003;47:423–52.

Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol. 2007;73:5261–7.

Wang SJ, Yan ZG, Guo GL, Lu GL, Wang QH, Li FS. Ecotoxicity assessment of aged petroleum sludge using a suite of effects-based end points in earthworm *Eisenia fetida*. Environ Monit Assess 2010;169:417–28.

Whang LM, Liu PW, Ma CC, Cheng SS. Application of biosurfactants, rhamnolipid, and surfactin, for enhanced biodegradation of diesel-contaminated water and soil. J Hazard Mater 2008;151:155–63.

Wrenn BA, Venosa AD. Selective enumeration of aromatic and aliphatic hydrocarbon degrading bacteria by a most-probable-number procedure. Can J Microbiol 1996;42:252–8.

Wu Y, Luo Y, Zou D, Ni J, Liu W, Teng Y, et al. Bioremediation of polycyclic aromatic hydrocarbons contaminated soil with *Monilinia* sp.: degradation and microbial community analysis. Biodegradation 2008;19:247–57.

Xiao J, Guo L, Wang S, Lu Y. Comparative impact of cadmium on two phenanthrene-degrading bacteria isolated from cadmium and phenanthrene co-contaminated soil in China. J Hazard Mater 2010;174:818–23.

Yamada-Onodera K, Mukumoto H, Katsuyama Y, Tani Y. Degradation of long-chain alkanes by a polyethylene-degrading fungus, *Penicillium simplicissimum* YK. Enzyme Microb Technol 2002;30:828–31.

Yateem A, Balba M, Al-Awadhi N, El-Nawawy A. White rot fungi and their role in remediating oil-contaminated soil. Environ Int 1997;24:181–7.

Yu Z, Morrison M. Comparisons of different hypervariable regions of rrs genes for use in fingerprinting of microbial communities by PCR-denaturing gradient gel electrophoresis. Appl Environ Microbiol 2004;70:4800–6.

Zucchi M, Angiolini L, Borin S, Brusetti L, Dietrich N, Gigliotti C, et al. Response of bacterial community during bioremediation of an oil-polluted soil. J Appl Microbiol 2003;94:248–57.

CAPÍTULO 2 / CHAPTER 2

Ensayo piloto de biorremediación por la tecnología de la biopila dinámica para la descontaminación de suelos contaminados por creosotas provenientes de las actividades dedicadas a la preparación de la madera

Ensayo piloto de biorremediación por la tecnología de la biopila dinámica para la descontaminación de suelos contaminados por creosotas provenientes de las actividades dedicadas a la preparación de la madera

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Se ha llevado a cabo un ensayo piloto de biorremediación in-situ, mediante la estrategia de una biopila dinámica, para estudiar la biodegradabilidad de los HAPs presentes en un suelo contaminado por creosota. Al mismo tiempo, a lo largo de los 180 días de tratamiento, se estudió la población microbiana presente en el suelo, tanto heterótrofa como degradadora de HAPs y se monitorizó la concentración de los HAPs de 3, 4 y 5 anillos aromáticos. Además, se evaluó la toxicidad aguda mediante el ensayo de Microtox.

Se decidió proceder con el ensayo piloto porque, con anterioridad, se obtuvieron resultados satisfactorios con los ensayos de tratabilidad llevados a cabo en el laboratorio, con el mismo suelo, observándose que el tratamiento con mejores resultados fue el llevado a cabo con aireación y humedad óptima. Como consecuencia, se construyó una biopila de 12m^2 x 0,5 m de alto, con una cubierta con plástico permeable para los gases. Durante el proceso, la tierra se mezclo cada 2 o 3 semanas para prevenir valores de oxígeno por debajo del 5%.

En los resultados del ensayo piloto se pudo observar la alta reproducibilidad en el campo de los estudios anteriores realizados en el laboratorio. Los HAPs de 3 o menor número de anillos bencénicos fueron totalmente degradados por la flora bacteriana autóctona, mientras que los HAPs de 4 se degradaron en menor medida, aunque el elevado número de poblaciones degradoras de HAPs presentes en el suelo a los 180 días y las cinéticas de degradación, hacen pensar que la degradación de este tipo de hidrocarburos hubiera llegado a cotas todavía más bajas. Por otro lado, solo se observó una degradación muy ligera en los hidrocarburos de 5 anillos aromáticos, debido a su elevada recalcitrancia y a una menor biodisponibilidad.

A la vista de los resultados se consideró que los valores absolutos de las concentraciones de los HAPs no pueden ser el único criterio para establecer el nivel de

riesgo de contaminación en un suelo. Otros factores como la biodisponibilidad y la toxicidad deberían ser tomados en mucha más consideración.

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Summary

An in situ bioremediation pilot test of creosote contaminated soil was carried out using dynamic biopile technology. Over the 180 days of treatment, the eubacterial community, the number of heterotrophs and polycyclic aromatic hydrocarbon (PAH) degraders were monitored, as well as the concentration of 3, 4, 5-ringed PAHs. In addition, the Microtox assay was used to evaluate acute toxicity. In the treatability assays, carried out previously in the laboratory, it was noted that the most effective treatment was based on good aeration, maintenance of optimal humidity and no added nutrients.

A 12 m^2 x 0.50 m-high biopile covered with a gas-permeable plastic cover was built. During the process it was turned every 2 or 3 weeks to keep the oxygen levels above 5%. The results revealed high reproducibility similar to that obtained in the laboratory. The 2 and 3-ringed PAHs were completely degraded by indigenous bacterial flora. The 4-ringed PAHs were significantly degraded, and the kinetics of decline as well as the high microbial population of HAPs degraders apparent at the end of the process indicated that a further decline would take place. The 5-ringed PAHs revealed only a slight decrease due to their proven recalcitrance and lower bioavailability.

From the results of this work it is evident that the absolute values of the concentrations of PAWs cannot be the only criterion taken for establishing the level of risk of soil contamination. Other factors such as bioavailability and toxicity should be taken into greater consideration.

Resumen

Se realizó un ensayo piloto de biorremediación in situ de suelos contaminados por creosotas mediante el uso de la tecnología de la biopila dinámica. Durante los 180 días de tratamiento se observó la población microbiana heterótrofa y degradadora de hidrocarburos aromáticos policíclicos (HAP), así como la concentración de HAP de 3, 4 y 5 anillos. Además, se evaluó la toxicidad aguda mediante el ensayo de Microtox. En los ensayos de tratabilidad, desarrollados previamente en el laboratorio, se demostró que el mejor tratamiento fue el basado en una buena aireación, el mantenimiento de una humedad óptima y sin la adición de nutrientes.

Se instaló una biopila de 12 m^2 x 0,50 metros de alto cubierta por un plástico permeable a los gases. Durante el proceso, se volteó cada dos o tres semanas para mantener los valores de oxígeno por encima del 5%. Los resultados relevaron una gran reproducibilidad similar a los resultados obtenidos en el laboratorio. Los HAP de 2 y 3 anillos fueron completamente degradados por las poblaciones microbianas. Los HAP de 4 anillos presentaron una importante degradación, y tanto la cinética de disminución como la presencia de una elevada población degradadora al final del proceso fueron indicativos de que la biodegradación podía continuar. Los HAP de 5 anillos presentaron una degradación leve debido a su baja y recalcitrante biodisponibilidad.

Los resultados de este trabajo demuestran que los valores absolutos de las concentraciones de los HAP no pueden ser el único criterio para establecer el nivel de riesgo de contaminación de un suelo. Otros factores, como la biodisponibilidad y la toxicidad pueden ser muy importantes y deberían tomarse en consideración.

PLANTEAMIENTO

En un emplazamiento de creosotado de maderas, se llevó a cabo un ensayo piloto de aplicación de la tecnología de la biorremediación, mediante la estrategia de biopila dinámica. Las condiciones de bioestimulación que se aplicaron fueron las que se mostraron como óptimas en los ensayos de tratabilidad realizados a nivel de laboratorio por el Departamento de Microbiología de la Universidad de Barcelona.

La creosota

La creosota es un producto líquido viscoso de textura aceitosa, utilizado para la conservación de la madera que habitualmente se emplea en el tratamiento de traviesas de ferrocarril, en postes de la red de telefonía y de transmisión de energía eléctrica y en cercados o puentes.

Se obtiene fundamentalmente por procesos de destilación entre 200 y 400 °C de alquitranes procedentes de la combustión (900-1.200 °C) de carbones grasos (hulla). Su composición química es compleja, estando formada por 150-200 componentes químicos diferentes, de los cuales un 85% son hidrocarburos aromáticos policíclicos (HAP) de origen pirolítico de 2 hasta 5 anillos aromáticos (**tabla 1**); un 10% son compuestos fenólicos y un 5% son compuestos heterocíclicos (N-, S-, y O-). Asimismo, es importante destacar que más del 50% de la composición de la creosota está representado por HAP de dos y tres anillos, y que además, los HAP, al ser mayoritariamente de origen pirolítico, están mayormente representados por HAP no alquilados.

Las propiedades fisicoquímicas de los diferentes componentes de la creosota condicionan su destino ambiental. De esta forma, los compuestos fenólicos y algunos hidrocarburos heterocíclicos presentan elevadas solubilidades (3 a 4 órdenes de magnitud superiores a los HAP de 3 o más anillos) y por lo tanto pueden ser movilizados en las fases acuosas del suelo y, en consecuencia, pueden afectar a sistemas acuáticos colindantes al suelo contaminado (superficial o subterráneo). Asimismo, los componentes más volátiles (HAP de 2 anillos, compuestos fenólicos y heterociclos de bajo peso molecular) pueden disminuir paulatinamente del suelo pasando a la atmósfera.

Tabla 1 Hidrocarburos aromáticos policíclicos (HAP) predominantes en la creosota

HAP	PM*	Proporción**	Solubilidad (mg l^{-1})***
Naftaleno	128	13%	31,7
2-metilnaftaleno	142	13%	25,4
Fenantreno	178	13%	1,3
Antraceno	178	13%	0,07
1-metilnaftaleno	142	8%	28,5
Bifenil	154	8%	7,5
Fluoreno	166	8%	2,0
2,3-dimetil naftaleno	156	4%	3,0
2,6- dimetilnaftaleno	156	4%	2,0
Acenafteno	154	4%	3,9
Fluoranteno	202	4%	0,26
Pireno	202	2%	0,14
Criseno	228	2%	0,002
Antraquinona	208	1%	ND
2-metilantraceno	192	1%	0,04
2,3-benzo(b)fluoreno	216	1%	0,002
Benzo(a)pireno	252	1%	0,003

* PM: Peso molecular
** Proporción respecto a los HAP totales
*** Solubilidad en agua a 25 °C

UN SUELO CON CONTAMINACIÓN REMOTA (MESES-AÑOS) PUEDE PRESENTAR UNA MAYOR PROPORCIÓN RELATIVA DE HAP PESADOS.

En consecuencia, los compuestos presentes en suelos contaminados con creosota pueden ser diferentes en función del tiempo transcurrido desde el episodio de contaminación. Así, un suelo con contaminación reciente de creosota se caracteriza por presentar compuestos contaminantes parecidos a los descritos en la **tabla 1**, mientras que un suelo con contaminación remota (meses-años) puede presentar una mayor proporción relativa de HAP pesados (de tres o más anillos) y una menor proporción de compuestos fenólicos e hidrocarburos heterocíclicos.

La toxicidad y mutagenicidad intrínseca de los componentes que forman la creosota obligó a la Unión Europea a redactar una Directiva Comunitaria en el año 2001 (2001/90/CE), donde se prohibió el uso de maderas tratadas con creosota en cualquier tipo de obra que estuviera en contacto directo con la población. Solamente se permitió la aplicación de creosota, con unas concentraciones de benzo(a)pireno y fenol inferiores a 50 y 30.000 mg kg^{-1}, en traviesas de ferrocarril, postes eléctricos y de telecomunicaciones, cercados y en puertos y vías navegables.

BIOTECNOLOGÍA DE LA BIORREMEDIACIÓN DE SUELOS CONTAMINADOS POR HIDROCARBUROS

La biorremediación es el proceso que se basa en la utilización de sistemas biológicos para eliminar o producir rupturas o cambios moleculares de tóxicos, contaminantes y sustancias de importancia ambiental en suelos, aguas y aire, generando compuestos de menor o ningún impacto ambiental. Estas degradaciones o cambios ocurren usualmente en la naturaleza (en ese caso se denomina “atenuación natural”), aunque la velocidad de tales cambios suele ser excesivamente baja. Mediante una

Figura 1
visión panorámica y detallada de los acopios de troncos tratados con creosota durante el proceso de secado

adecuada manipulación, estos sistemas biológicos pueden ser optimizados para aumentar la velocidad de cambio o degradación. Al convertir el contaminante en un producto inocuo, la biorremediación supone una solución definitiva al problema pues no genera un residuo final como puede ocurrir en otro tipo de tratamientos. Además, supone una ventaja por su bajo coste. Los microorganismos son los principales responsables de la degradación de hidrocarburos en los ecosistemas acuáticos y terrestres. La optimización de la biodegradación microbiana es la base para las distintas tecnologías de biorremediación, el uso de las cuales ha sido efectivo y descrito en diferentes estudios en el tratamiento de suelos contaminados por hidrocarburos (Alexander, 1999).

Nuestro país dispone de una reciente legislación en materia de suelos contaminados (R.D. 9/2005), en la que se priorizan las técnicas de remediación in situ que eviten el traslado de tierras a depósitos controlados. Es de esperar que después de superar las etapas de caracterización y evaluación de riesgos, la tecnología de la biorremediación cobre una mayor importancia. Actualmente, estas tecnologías representan solo un 2-3%, por lo que indiscutiblemente se deben ver ampliadas en un futuro próximo.

Cuando se llevan a cabo acciones encaminadas al aumento del número y de la actividad metabólica de las poblaciones microbianas existentes en el propio emplazamiento hablamos de bioestimulación, mientras que si introducimos cepas o consorcios microbianos obtenidos en el laboratorio hablamos de biorrefuerzo.

En emplazamientos contaminados por hidrocarburos, especialmente en zonas con exposición larga a los contaminantes, la población microbiana indígena, habitualmente, responde de forma favorable a estrategias de bioestimulación, multiplicándose y metabolizando el residuo de interés. Sin embargo, en otros casos, cuando la población microbiana degradadora de hidrocarburos es baja, el biorrefuerzo puede ser beneficioso.

Si bien una de las limitaciones de la técnica es que el tiempo para conseguir una biodegradación aceptable, de acuerdo a las normativas, de determinados hidrocarburos puede ser en algunos casos excesivamente largo, en la actualidad diversos grupos de investigación están intentando disminuir el tiempo del proceso mediante ensayos basados en un mejor conocimiento de las poblaciones microbianas implicadas, de mejora de la biodisponibilidad o mediante la utilización de microorganismos con mayores capacidades catabólicas.

La típica cinética de eliminación de los contaminantes en un proceso de biorremediación sigue la denominada cinética de palo de "hockey". Una fase inicial de descenso muy rápido seguido de una etapa de ralentización debido al enriquecimiento del suelo en componentes más recalcitrantes o por una disminución de la biodisponibilidad de los contaminantes.

FACTORES QUE AFECTAN A LA BIORREMEDIACIÓN EN SUELOS

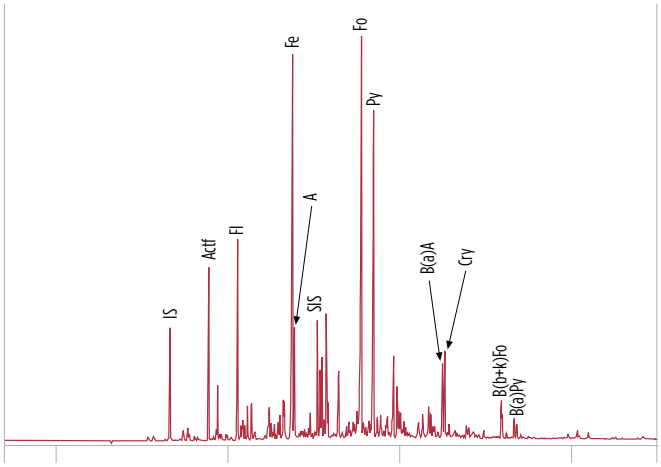
La biorremediación es un proceso complejo y su éxito, tanto cualitativo como cuantitativo, se puede ver condicionado por las condiciones ambientales, por la naturaleza del propio contaminante o por las poblaciones microbianas implicadas.

Condiciones ambientales

Si la eliminación de los contaminantes es consecuencia del metabolismo de los microorganismos presentes en el suelo, como sucede con cualquier proceso metabólico, estará condicionado por las condiciones ambientales de la zona donde se encuentren.

En el caso de los hidrocarburos, la degradación aeróbica es la ruta más favorable de degradación. Aunque se han descrito degradaciones anaerobias, el principal proceso para eliminarlos es la respiración aeróbica de los microorganismos implicados (Van Hamme et ál., 2003). La tasa de degradación es directamente proporcional a la disponibilidad de oxígeno, ya que es utilizado como aceptor final de electrones y también como sustrato en las

Figura 2
perfil cromatográfico (GC-FID) del extracto de TPH del suelo



IS: estándar interno
SIS: estándar interno surrogate
Acft: acenafteno
Fl: fluoreno
Fe: fenantreno
A: antraceno
Fo: fluoranteno
Py: pireno
B(a)A: benzo(a)antraceno
Cry: criseno
B(b+k)Fo: benzo(b)fluoranteno y benzo(k)fluoranteno
B(a)Py: benzo(a)pireno

reacciones catalizadas por las oxigenasas, enzimas implicadas en la ruta aeróbica bacteriana de degradación de los hidrocarburos. En el suelo, el oxígeno suele ser un factor limitante. En la zona no saturada, los suelos arcillosos presentan una mayor resistencia a la difusión de oxígeno que los más arenosos, y en la zona saturada, encontramos, muchas veces, condiciones próximas a la anoxia.

En suelos, la humedad puede ser crítica para la biodegradación, ya que los microorganismos requieren una humedad óptima para sobrevivir. La capacidad de campo es la cantidad de agua que puede retener un suelo. Este parámetro depende del tipo de suelo, de la permeabilidad y de la concentración de contaminantes. Valores muy bajos pueden significar una inactividad metabólica de los microorganismos, así como una reducción del transporte de nutrientes y contaminantes, es decir, de su biodisponibilidad. Un exceso de agua, por el contrario, podría suponer una disminución en la circulación del aire y, por tanto, una disminución de la disponibilidad del oxígeno. El rango óptimo de contenido de agua en un suelo, para la biodegradación, suele estar entre el 30 y el 80% de la capacidad de campo.

En la biodegradación, en la mayoría de los casos, el contaminante actúa como fuente de carbono y energía. Por otro lado, las células microbianas requieren de otros macronutrientes y también de nutrientes traza para la correcta metabolización de los contaminantes. Los niveles de nitrógeno y fósforo suelen ser limitantes para la biodegradación, ya que la mayoría de contaminantes sólo están formados por carbono e hidrógeno.

La temperatura óptima para el proceso de biodegradación varía según el clima y el tipo de poblaciones presentes, pero un rango de temperatura mesofílico, entre 20 y 40 °C, es el más adecuado. De todos modos, su fluctuación representa un obstáculo para la biorremediación.

La variación de pH afecta tanto a la actividad microbiana como a la solubilidad y adsorción de contaminantes. El rango óptimo para la degradación de hidrocarburos es de entre 7.4 y 7.8.

Naturaleza de los contaminantes

Para relacionar los contaminantes con la biodegradación es muy importante tener en cuenta su estructura química, las posibles interacciones entre ellos y su biodisponibilidad.

Conocer la estructura de los hidrocarburos es importante para prever su biodegradación, pero la tasa de degradación también depende de la biodisponibilidad que, al mismo tiempo, depende de las características químicas, de la naturaleza del suelo y de la presencia de agentes solubilizantes. Cuanto más recalcitrantes sean los compuestos, menos biodisponibles estarán y menor será su tasa de degradación. Por este motivo, en muestras complejas, se produce una degradación selectiva y, como consecuencia, un enriquecimiento en compuestos recalcitrantes. Esta recalcitrancia conduce a que los compuestos no sean accesibles a los microorganismos degradadores. A causa de su hidrofobicidad, su solubilidad en agua es extremadamente baja y pueden quedar adsorbidos a las partículas inorgánicas de suelo, absorbidos a la materia orgánica o incluidos en nanoporos. Por estos motivos resulta compleja la biorremediación en un suelo con creosota, ya que casi un 85% del total de sus hidrocarburos son HAP. Además, buena parte de estos HAP son de elevado peso molecular y esto todavía hace aumentar más su hidrofobicidad y, por lo tanto, disminuye su biodisponibilidad y aumenta su recalcitrancia.

Tabla 2 Características fisicoquímicas y microbiológicas del suelo

Textura	Arcilloso/Franco-arcilloso
Arcillas (%)	40
Limos (%)	28
Arenas (%)	32
Humedad (%)	1,6
100% CC (% humedad)	27,7
pH $_{(1:2,5)}$	7,5
Conductividad ($\mu S\ cm^{-1}$)	228
Nitrógeno Total (%)	0,15 ± 0,01
Nitrato (mg kg^{-1} suelo)	17,2 ± 0,85
Nitrito (mg kg^{-1} suelo)	< 0,5
Amonio (mg kg^{-1} suelo)	2,8 ± 0,80
Fosfato (mg kg^{-1} suelo)	< 0,5
COT (%)	4,22 ± 0,23
EOT[2] (mg kg^{-1} suelo)	12.510 ± 452
TPH $_{gra}$ (mg kg^{-1} suelo)	8.615 ± 510
TPH $_{GC-FID}$ (mg kg^{-1} suelo)	8.196 ± 480
Heterótrofos totales (NMP g^{-1} suelo)	1,5 x 10^6
Degradadores de HAP (NMP g^{-1} suelo)	1,8 x 10^5

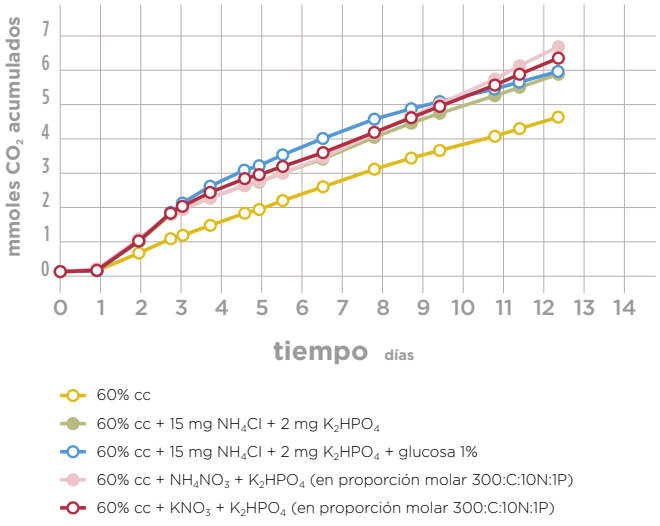
LOS ENSAYOS DE TRATABILIDAD EN LA BIORREMEDIACIÓN DE SUELOS CONTAMINADOS

Para evaluar si la biorremediación es apropiada para la descontaminación de un suelo, es necesario caracterizar tanto las poblaciones microbianas como la biodegradabilidad de los contaminantes del suelo, así como valorar la influencia de los factores ambientales que afectan al proceso de biodegradación. Para ello se diseñan los ensayos de tratabilidad o factibilidad, que se definen como un conjunto de experimentos realizados a escala de laboratorio, previos a la implementación de cualquier tecnología de biorremediación a un suelo contaminado. En este caso aplicamos el ensayo de tratabilidad diseñado por nosotros y publicado en RESIDUOS Revista Técnica (59, 78-82.)

ENSAYOS DE TRATABILIDAD REALIZADOS CON EL SUELO DE CREOSOTA

El suelo utilizado para llevar a cabo los ensayos de tratabilidad procede de los primeros 20 cm. Los resultados de la caracterización fisicoquímica se muestran en la **tabla 2**.

Figura 3
producción de CO_2 acumulada en 10 gramos de suelo en diferentes condiciones de bioestimulación



El suelo presentaba una granulometría con un elevado contenido en arcillas y limos y, en consecuencia, una elevada capacidad de campo (27%). Sin embargo, en el momento del muestreo, el suelo contenía una cantidad muy escasa de agua (1,6%). La conductividad era baja como también lo eran las concentraciones de nitratos, nitritos, amonio y fosfato. El suelo presentaba un contenido de 8.000 ppm de TPH. Las fuentes inorgánicas de nitrógeno estaban en una proporción molar C:N de 1.100:1, proporciones molares inferiores a la óptima. El pH era neutro-básico, favorable para procesos de biodegradación, y había la presencia de una elevada población microbiana degradadora de HAP (8 x 10^5 NMP g^{-1} suelo) que representaba un 12% de la población heterótrofa total.

El suelo presentaba una elevada concentración inicial de TPH, de los cuales, el 90% pertenecían a la fracción aromática.

Los HAP de 3 y 4 anillos fueron los más abundantes, superando en todos los casos los umbrales de los niveles de referencia para suelos de uso industrial definidos en el Real Decreto 9/2005. Las concentraciones de fenantreno, fluoranteno y pireno representaban el 51% del total de los 16 HAP que la Environmental Protection Agency (EPA) considera prioritarios. También se encontraron HAP de 5 anillos, como el benzo(a)pireno.

La elevada proporción de población degradadora y la presencia de fenantreno (HAP poco volátil) en unas concentraciones inferiores a las del fluoranteno, indicaban la posible existencia de procesos de biodegradación llevados a cabo por la microbiota autóctona del suelo.

Para determinar la actividad metabólica de la población microbiana y la biodegradabilidad de la matriz contaminante, se llevaron a cabo una serie de ensayos respirométricos (medición de la producción de CO_2). Sorprendentemente, como puede observarse en la **figura 3**, la población microbiana del suelo mostró, en todos los casos, una gran producción de CO_2. La bioestimulación con adición de nutrientes produjo una respuesta sólo ligeramente superior a la bioestimulación sin nutrientes y la adición de glucosa no incrementó la producción de CO_2.

La elevada producción de CO_2 coincidió con un incremento de las poblaciones microbianas entre 1 y 2 órdenes de magnitud en la población heterótrofa total y en la degradadora de HAP.

Una vez comprobado que el suelo contaminado con creosota presentaba una notable población microbiana degradadora de HAP, metabólicamente muy activa y bioestimulable, y que la matriz contaminante era biodegradable en las condiciones fisicoquímicas del suelo, se procedió a la segunda fase del estudio de tratabilidad. En esta fase se evaluó, en microcosmos, el efecto de diferentes factores fisicoquímicos y biológicos en la biodegradación de la creosota, la dinámica y la estructura de las poblaciones microbianas implicadas y la ecotoxicidad del suelo durante el proceso de biorremediación.

Para determinar la humedad óptima del suelo se evaluaron 5 contenidos de agua, en condiciones de bioestimulación (agua con aireación y con KNO_3 y K_2HPO_4 como fuentes de N y P) en microcosmos miniaturizados (60 gramos de suelo en viales de vidrio de 100 ml de volumen). Los mejores resultados se obtuvieron con una capacidad de campo entre el 40% y el 60%. Teniendo en cuenta la textura franco-arcillosa del suelo, se eligió el 40% para llevar a cabo los diferentes tratamientos en microcosmos.

Se utilizó suelo sin tratar como control, para calcular la biodegradación de los TPH y los HAP diana en los diferentes tratamientos. Se evaluó la adición o no de nutrientes, la adición de un tensioactivo, la adición de un consorcio microbiano con unas

A LO LARGO DEL PROCESO DE BIORREMEDIACIÓN, SE ESTUDIÓ LA TOXICIDAD AGUDA MEDIANTE EL ENSAYO MICROTOX (*VIBRIO FISHERI*).

potentes capacidades catabólicas sobre los HAP y la adición de substratos fácilmente degradables. Todos los tratamientos de bioestimulación, tras los 200 días de incubación, degradaron los TPH de forma muy significativa, llegando en algunos casos al 79% de degradación. El tratamiento de biorrefuerzo no mostró una mayor degradabilidad que la alcanzada por la flora microbiana autóctona del propio emplazamiento.

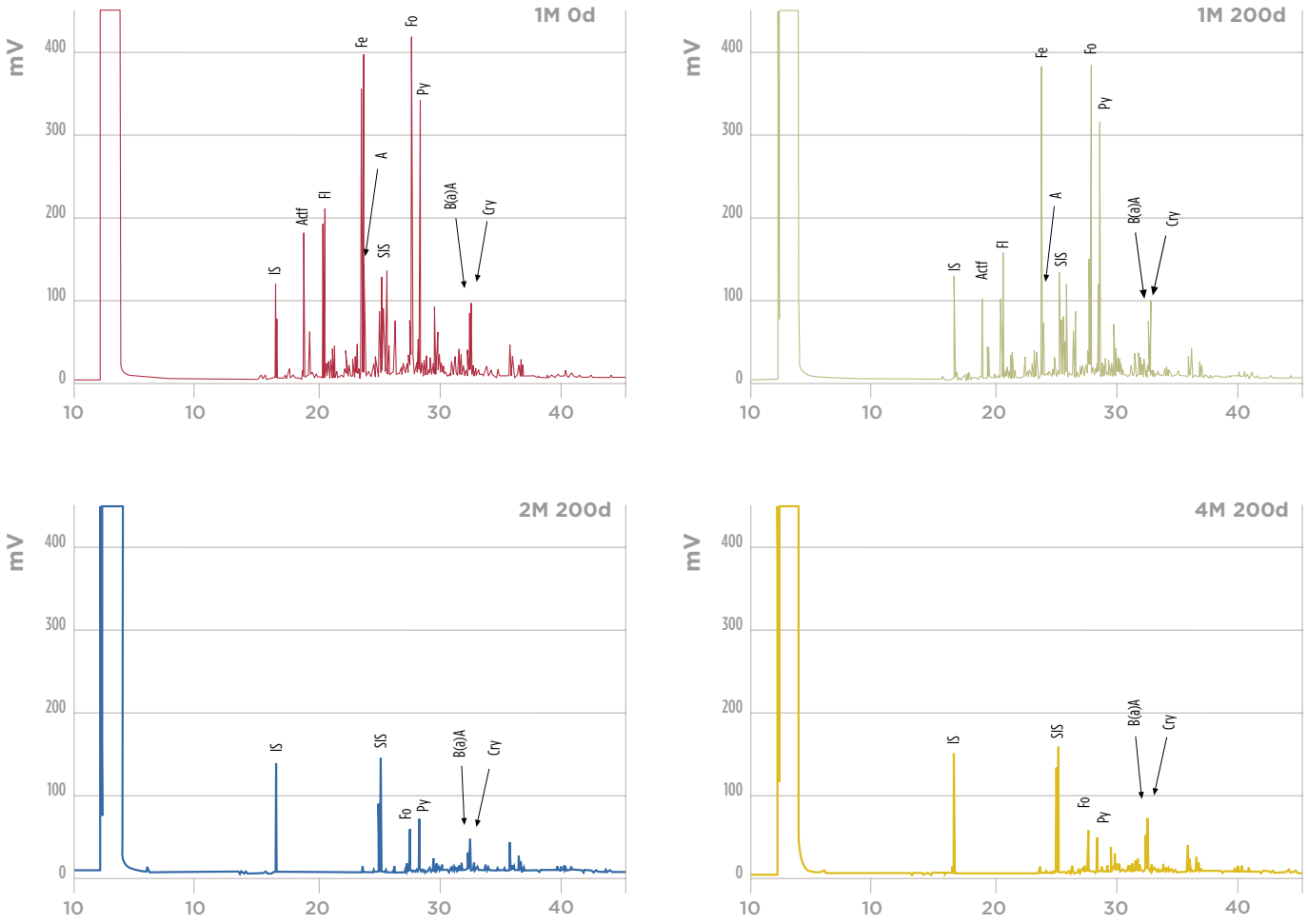
Durante los primeros 45 días, la tasa de degradación, en la bioestimulación sin nutrientes, fue ligeramente inferior que la observada en los tratamientos con nutrientes. Sin embargo, la tasa de biodegradación de TPH a largo plazo (90-200 días) fue superior en el tratamiento sin nutrientes, coincidiendo con una mayor biodegradación final de los TPH, a los 200 días.

Los HAP de dos y tres anillos fueron ampliamente degradados durante los primeros 45 días, con la misma tasa de degradación en todos los tratamientos. La biodegradación de benzo(a)antraceno y criseno (4 anillos) fue significativamente mayor en el tratamiento sin nutrientes y, finalmente, no se observó una biodegradación significativa de HAP de 5 o más anillos en ninguno de los tratamientos (**figura 4**).

Para analizar el efecto de los diferentes tratamientos de biorremediación en las poblaciones microbianas heterótrofas y degradadoras de HAP, se llevó a cabo una enumeración mediante el método del número más probable (NMP) (**figura 5**).

Figura 4

perfiles cromatográficos de la fracción TPH del suelo contaminado con creosota a los 0 y 200 días de incubación en los microcosmos 1M (suelo no tratado), 2M (bioestimulación sin nutrientes) y 4M (bioestimulación con nutrientes)



IS: estándar interno **Acft:** acenafteno **Fe:** fenantreno **Fo:** fluoranteno **B(a)A:** benzo(a)antraceno
SIS: estándar interno surrogate **Fl:** fluoreno **A:** antraceno **Py:** pireno **Cry:** criseno

En los tratamientos de bioestimulación con adición de nutrientes ambas poblaciones aumentaron entre 2 y 3 órdenes de magnitud en los primeros 21 días. Sin embargo, en la bioestimulación sin adición de nutrientes las poblaciones microbianas incrementaron de forma más gradual, con un aumento de 1 a 2 órdenes de magnitud. Pero lo que nos pareció más sorprendente es que en este tratamiento la proporción degradadora de HAP, con respecto a la heterótrofa total, alcanzó valores superiores al 50% durante el periodo comprendido entre los 90 y los 200 días, con un máximo del 100% en el día 135. Sin embargo, en el tratamiento con nutrientes no cambió la proporción de degradadores respecto a la que presentaba el suelo inicial y el no tratado, durante todo el periodo de incubación (12-24%).

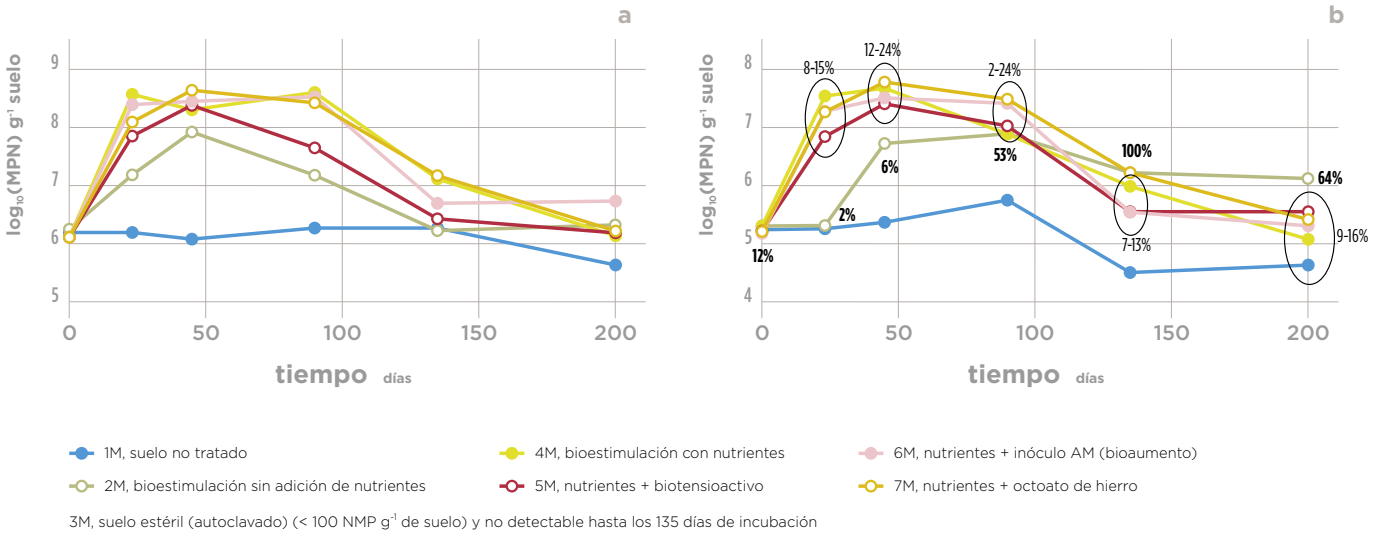
Estos resultados reforzaban los que habíamos obtenido en los descensos de los HAP diana y ponían de manifiesto que la no adición de nutrientes permitía el crecimiento de una población más lenta pero más especializada en la degradación de HAP de mayor tamaño molecular. Por el contrario, la adición de nutrientes inicial provocaba un aumento de una flora microbiana de rápido crecimiento pero no tan especializada.

Este resultado lo consideramos muy interesante y aporta una información muy novedosa al campo de la biorremediación de hidrocarburos de alto peso molecular. La adición inicial de nutrientes y a las proporciones que frecuentemente se recomiendan (C:N:P 100:10:4) es excesiva y debería hacerse en etapas mas tardías del proceso y de manera fraccionada.

Asimismo, a lo largo del proceso de biorremediación, se estudió la toxicidad aguda mediante el ensayo Microtox (*Vibrio Fisheri*). El lixiviado inicial del suelo, analizado por Microtox, presentó una EC_{50} inicial del 20% (20g de suelo en 100 ml de lixiviado), que se mantuvo constante en el suelo no tratado a lo largo de los 200 días de incubación (**figura 6**). Sin embargo, en los tratamientos de bioestimulación (con y sin nutrientes) la toxicidad disminuyó de forma significativa, al aumentar 3-3,5 veces la EC_{50} durante los primeros 45 días. Durante el periodo 90-200 días la toxicidad disminuyó ligeramente respecto al día 45, alcanzándose una EC_{50} del 76-80% en ambos tratamientos. Es importante destacar la correlación lineal que existió entre la disminución de la concentración de TPH y la EC_{50} observada en los lixiviados, en ambos tratamientos de bioestimulación.

Figura 5
evolución de las poblaciones microbianas heterótrofas (a) y degradadoras de HAP (b) a lo largo de los 200 días de incubación en los 7 tratamientos de biorremediación en microcosmos

Las áreas con círculo indican el porcentaje de degradadores de HAP para los tratamientos 4M-7M (en el tratamiento 2M los porcentajes se muestran en negrita)



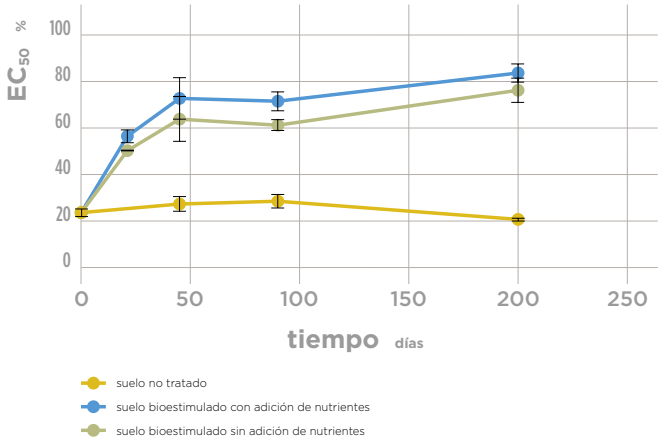
ENSAYO PILOTO

Para caracterizar el emplazamiento y definir y acotar el nivel y la profundidad de la contaminación, se realizaron una serie de sondeos a distintas profundidades. Se realizaron siete sondeos, tres de ellos a 2 metros de profundidad y los cuatro restantes a 1 metro. Para la realización de los sondeos y obtención de muestras de suelo se utilizó la maquinaria de perforación Geoprobe 4220. Los sondeos se realizaron a 1 ó 2 m y se tomaron muestras cada 50 cm para su análisis (**tabla 3**). La obtención, envasado, conservación, registro y transporte de muestras se realizó según las directrices de las principales normas existentes tales como EPA SW–846 y ASTM 4687.

Los dos primeros sondeos corresponden a zonas muy próximas a las instalaciones donde se realiza el proceso de creosotado.

Figura 6
evolución de la toxicidad (EC_{50}) de lixiviados de suelo en microcosmos, analizada por Microtox a lo largo de los 200 días de incubación

Las barras en cada punto representan la desviación estándar (n=3)



El tercer sondeo corresponde a una zona de acopio de troncos creosotados, y del cuarto hasta el séptimo a una segunda zona de acopio.

Desde un punto de vista geológico, los materiales encontrados en los terrenos alrededor de la fábrica están formados por

Tabla 3 Características de los sondeos realizados el 24 de enero

Sondeo	Profundidad	Materiales	Muestras para analizar
S-1	2 m	0-2 m: limos	S-1 0-0,5 m S-1 0,5-1 m S-1 1-1,5 m S-1 1,5-2 m
S-2	2 m	0-2 m: limos	S-2 0-0,5 m
S-3	2 m	0-0,5 m: limos-gravas 0,5-2 m: arenas-grava	S-3 0-0,5 m
S-4	1 m	0-1 m: limos-grava	S-4 0-0,5 m S-4 0,5-1 m
S-5	1 m	0-1 m: limos-grava	S-5 0-0,5 m
S-6	1 m	0-1 m: limos-grava 1-2 m: argilitas	S-6 0-0,5 m
S-7	1 m	0-0,7 m: limos-gravas	S-7 0-0,5 m

Tabla 4 Concentraciones de TPH y HAP (mg · kg^{-1} suelo) en los diferentes sondeos realizados en febrero de 2006 en las muestras de suelos contaminados por creosota

	S1 0-0,5	S1 0,5-1	S1 1-1,5	S1 1,5-2	S2 0-0,5	S3 0-0,5*	S4 0-0,5	S4 0,5-1	S5 0-0,5	S6 0-0,5	S7 0-0,5
TPH Gravimetría	160,0	118,0	120,0	110,0	820,0	209,0	560,0	100,0	210,0	125,0	840,0
TPH (GC-FID) patró tph creosota	**62,3**	39,6	35,0	35,2	**454,6**	**96,1**	**533,0**	47,1	**107,2**	**58,5**	**420,2**
TPH (GC-FID) patró 16EPA	**63,6**	34,4	33,2	33,5	**449,1**	**101,3**	**536,5**	46,7	**113,6**	34,5	**410,9**
Total 16EPA	4,3	1,6	1,9	1,5	**228,0**	48,9	**208,3**	4,5	38,3	16,4	**202,2**
Naftaleno	0,0	0,0	0,0	0,0	0,0	0,0	0,5	0,0	0,0	0,2	0,0
Acenaftileno	0,1	0,0	0,0	0,0	2,2	0,2	2,6	0,1	0,2	0,2	1,3
Acenafteno	0,1	0,0	0,1	0,1	1,0	0,3	18,3	0,2	0,3	0,0	1,2
Fluoreno	0,2	0,2	0,2	0,2	1,4	0,9	43,9	0,5	0,4	0,2	1,6
Fenantreno	0,3	0,3	0,2	0,3	2,5	0,9	46,3	1,0	0,6	0,5	2,0
Antraceno	0,3	0,2	0,3	0,2	5,0	1,4	6,3	0,2	1,1	0,9	3,2
Fluoranteno	0,6	0,2	0,2	0,2	9,3	3,6	25,4	0,7	2,0	1,5	6,7
Pireno	0,6	0,4	0,3	0,3	13,1	2,9	15,7	0,6	1,8	1,3	7,9
B(a)Antraceno	0,3	0,2	0,2	0,1	**28,9**	**4,6**	**9,1**	0,3	**3,4**	1,4	**23,6**
Criseno	0,4	0,2	0,0	0,1	44,7	8,4	10,1	0,2	6,9	2,1	48,4
B(b)Fluoranteno	0,2	0,0	0,2	0,0	**35,3**	0,9	**7,1**	0,2	0,6	1,6	**36,1**
B(k)Fluoranteno	0,2	0,0	0,0	0,0	29,3	11,8	7,1	0,1	9,6	**1,4**	**26,3**
B(a)Pireno	0,2	0,0	0,2	0,1	**22,3**	**5,0**	**5,1**	0,0	**4,2**	**1,4**	**20,5**
Indeno-Pireno	0,1	0,0	0,0	0,0	**13,0**	**2,4**	**3,9**	0,0	**2,2**	**0,9**	**9,9**
Dibenzo-Antraceno	0,0	0,0	0,0	0,0	**5,9**	**1,0**	0,0	0,0	**0,9**	0,4	**5,2**
Benzo-perileno	0,4	0,0	0,2	0,0	13,9	4,8	6,9	0,3	4,2	2,3	8,2
Índice de biodegradación	**S1 0-0,5**	**S1 0,5-1**	**S1 1-1,5**	**S1 1,5-2**	**S2 0-0,5**	**S3 0-0,5**	**S4 0-0,5**	**S4 0,5-1**	**S5 0-0,5**	**S6 0-0,5**	**S7 0-0,5**
Relación (Naft-Pireno)/BaA-Bperileno	1,2	3,5	1,6	4,5	**0,2**	**0,3**	**3,2**	3,2	0,2	0,4	**0,1**

* Única muestra con un valor > 50 mg/kg de TPH por Eurofin-Analytico.
En negrita valores superiores a los NGR de uso industrial.
En rojo valores superiores a los NGR de uso urbano.
Los valores más bajos del índice de biodegradación indican que la muestra está degradada (S2-05, S3-0,5 y S7-0,5).
Los valores más altos del índice de biodegradación indican que la muestra está menos degradada (S4 0,05).

Se estableció un índice de biodegradación que nos permitió establecer el grado de envejecimiento de la contaminación de la muestra.

limos, gravas y bajos niveles de margas y limolitas. En cambio, los terrenos destinados al almacenaje de los postes creosotados están formados por gravas y bolos de terrazas aluviales. Hidrogeológicamente se puede indicar que no existen formaciones porosas y permeables que configuren un acuífero productivo. En cuanto a hidrología superficial, cabe destacar que los terrenos se encuentran ubicados entre 100 y 500 metros del cauce del río más próximo.

Se determinó la contaminación por hidrocarburos totales del petróleo (TPH) y por los 16 hidrocarburos aromáticos (HAP) considerados por la Agencia Americana del Medio Ambiente (EPA). El valor de TPH se determinó por tres metodologías diferentes: gravimetría, cromatografía de gases con detector FID utilizando un patrón de creosota y cromatografía de gases con detector FID utilizando un patrón de los 16 HAP de la EPA. Por otro lado, el valor de los 16 HAP diana se determinó por cromatografía de gases – FID utilizando curvas patrón para cada uno de los hidrocarburos. De esta forma se definió la afección del suelo y su distribución en profundidad.

Teniendo en cuenta los trabajos realizados con anterioridad por nuestro grupo, se estableció un índice de biodegradación que nos permitió establecer el grado de envejecimiento de la contaminación de la muestra. El índice se basa en el cociente entre la concentración de los HAP de 2, 3 y 4 anillos (naftaleno, acenaftileno, acenafteno, fluoreno, fenantreno, antraceno, fluoranteno y pireno) y los de 4, 5 y 6 (B(a)antraceno, criseno,

Figura 7
determinación de la concentración de oxígeno y dióxido de carbono en el sistema

B(b)fluoranteno, B(k)fluoranteno, B(a)pireno, indeno-pireno, dibenzo-antraceno y benzoperileno), es decir, entre los más biodegradables y los más recalcitrantes.

Índice de biodegradabilidad = (Naftaleno – Pireno) / (B(a)A-Bperileno)

El suelo contaminado de forma reciente presentó un índice de 5,7, y el suelo biorremediado con el tratamiento más efectivo lo presentó de 0,7. Por lo tanto, los valores cercanos a 5,7 corresponden a suelos poco degradados y contaminados de forma reciente, y valores próximos a 0,7 corresponden a suelos degradados, envejecidos y enriquecidos en los HAP más recalcitrantes.

Todos los valores correspondientes tanto a la concentración de hidrocarburos en cada muestra y su correspondiente profundidad, junto a los valores del índice de biodegradación, se pueden observar en la **tabla 4**.

En el primer sondeo, donde se llegó hasta dos metros de profundidad, no se halló contaminación por encima de los valores establecidos a ningún nivel. El segundo sondeo presentó una contaminación de unas 450 ppm, con 5 HAP con concentraciones por encima de los NGR industriales. El índice de biodegradación fue de 0,2 y eso indicó que se estaba ante un suelo degradado y, por lo tanto, rico en los HAP más pesados. En el tercer y quinto sondeo los TPH duplicaron los NGR, pero sólo dos de los HAP superaron los valores establecidos. El cuarto sondeo llegó hasta 1 metro, pero sólo entre 0 y 0,5 metros presentó contaminación por encima de los NGR. Los TPH estuvieron alrededor de 530 ppm y el índice de biodegradación fue de 3,2, por lo tanto, era un suelo con una contaminación mucho más reciente. En este sondeo es importante destacar que

SE DECIDIÓ LLEVAR A CABO UNA EXPERIENCIA PILOTO MEDIANTE LA TECNOLOGÍA DE UNA BIOPILA DINÁMICA.

el índice de biodegradación entre 0,5 y 1 metros también fue de 3,2. En el sexto sondeo los valores de TPH y de HAP individuales están por debajo de los NGR. Por último, en el séptimo sondeo los valores de TPH fueron de 415 ppm. Su índice de biodegradabilidad mostró que se estaba delante de un suelo envejecido y enriquecido en HAP pesados. 5 HAP estaban por encima de los NGR industriales.

Resumiendo, se pueden dividir los 7 sondeos en 3 zonas: zonas no contaminadas (S1 y S6), zonas con baja contaminación (S3 y S5) y zonas con un elevado grado de contaminación (S2, S4 y S7).

A la luz de estos resultados se decidió llevar a cabo una experiencia piloto mediante la tecnología de una biopila dinámica. El suelo escogido fue de la segunda zona de almacenaje, cerca de donde los sondeos habían señalado una mayor contaminación. Se procedió a la extracción del suelo con una pala excavadora que posteriormente era depositado en un camión remolque y trasladado a la zona escogida para la construcción de la biopila cercana a la zona de creosotado.

Tabla 5 Concentración de O_2 y CO_2 en profundidad y superficie en los distintos días de mantenimiento de la biopila

VOLTEO Y RIEGO DE LA BIOPILA	O_2-Sup.1	CO_2-Sup.1	O_2-Sup.2	CO_2-Sup.2	O_2-Prof.1	CO_2-Prof.1	O_2-Prof.2	CO_2-Prof.2
19-06-06	-	-	-	-	-	-	-	-
26-06-06	18	1,4	14,6	4,4	12	6,1	10,3	9,6
05-07-06	16,2	3	16,6	2,3	9,2	10,7	11,7	8,5
18-07-06	17,3	1,6	16,8	2,6	12,6	8,1	7,4	12,7
27-07-06	16,5	2,3	17,6	1,4	11,8	8,7	8,1	11,6
08-08-06	15,4	4,6	16,2	3,2	9,3	10,8	8,8	11,4
29-08-06	16,8	2,4	17,1	2,8	5,6	14,9	6,9	13,8
18-09-06	15,6	4,2	14,9	5,8	7,7	12,6	6,3	13,7
05-10-06	17,4	2,7	18,6	1,8	9,4	10,1	7,2	13,3
30-10-06	17,8	2,5	16,9	3,1	6,8	14	9,1	10,5
17-11-06	16,5	3,3	18,4	2,1	10,2	9,7	8,5	11,2
04-12-06	17,3	2,8	16,7	3,9	9,8	10,4	8,3	12

Figura 8
panorámica de la biopila con la tela protectora

Tabla 6 Poblaciones heterótrofas totales y degradadoras de HAP (NMP/g suelo) durante los 180 días de biorremediación

	0 días	70 días	105 días	180 días
Heterótrofos totales	$1,52 \pm 0,57\ 10^6$	$2,75 \pm 1,5\ 10^7$	$5,59 \pm 0,4\ 10^7$	$3,89 \pm 0,85\ 10^7$
Degradadores de HAP	$1,84 \pm 1,6\ 10^6$	$1,63 \pm 1,4\ 10^6$	$4,06 \pm 2,4\ 10^6$	$1,01 \pm 0,27\ 10^6$

A medida que se iba construyendo la biopila, se fueron colocando una serie de tubos de PVC flexibles para poder determinar la concentración de oxígeno en zonas profundas y así poder determinar la frecuencia de volteo (**figura 7**). Se determinó la concentración de O_2 y CO_2 de manera superficial (20-25 cm de la superficie) y también en el interior del sistema. Los resultados se pueden observar en la **tabla 5**.

También se procedió al riego para llegar a una humedad óptima del 60% y posteriormente se cubrió con una tela (**figura 8**). Durante todo el proceso se llevaron a cabo determinaciones de oxígeno, anhídrido carbónico (parámetro de indicación de actividad microbiana) y de humedad para que siguieran siendo óptimos, tanto a nivel de superficie como del interior de la biopila. Se observó que a partir de los 15-20 cm de la superficie, el suelo preservaba una buena humedad. Para mantener estos niveles óptimos se procedió al volteo y riego de la biopila, en intervalos de tiempo no superiores a las tres semanas. En una misma fecha se realizaban dos volteos y dos riegos para conseguir una óptima homogeneización del sistema.

HAY QUE DESTACAR LA GRAN HOMOGENEIDAD DE LAS MUESTRAS, QUE REFORZÓ EL VALOR DE LOS RESULTADOS.

Para controlar la evolución tanto de la biodegradación de los hidrocarburos como de las poblaciones microbianas, se recogieron muestras a los 0, 30, 70 y 105 días. Se determinaron las poblaciones microbianas heterótrofas y degradadoras de HAP así como la concentración de TPH, HAP y los porcentajes de biodegradación.

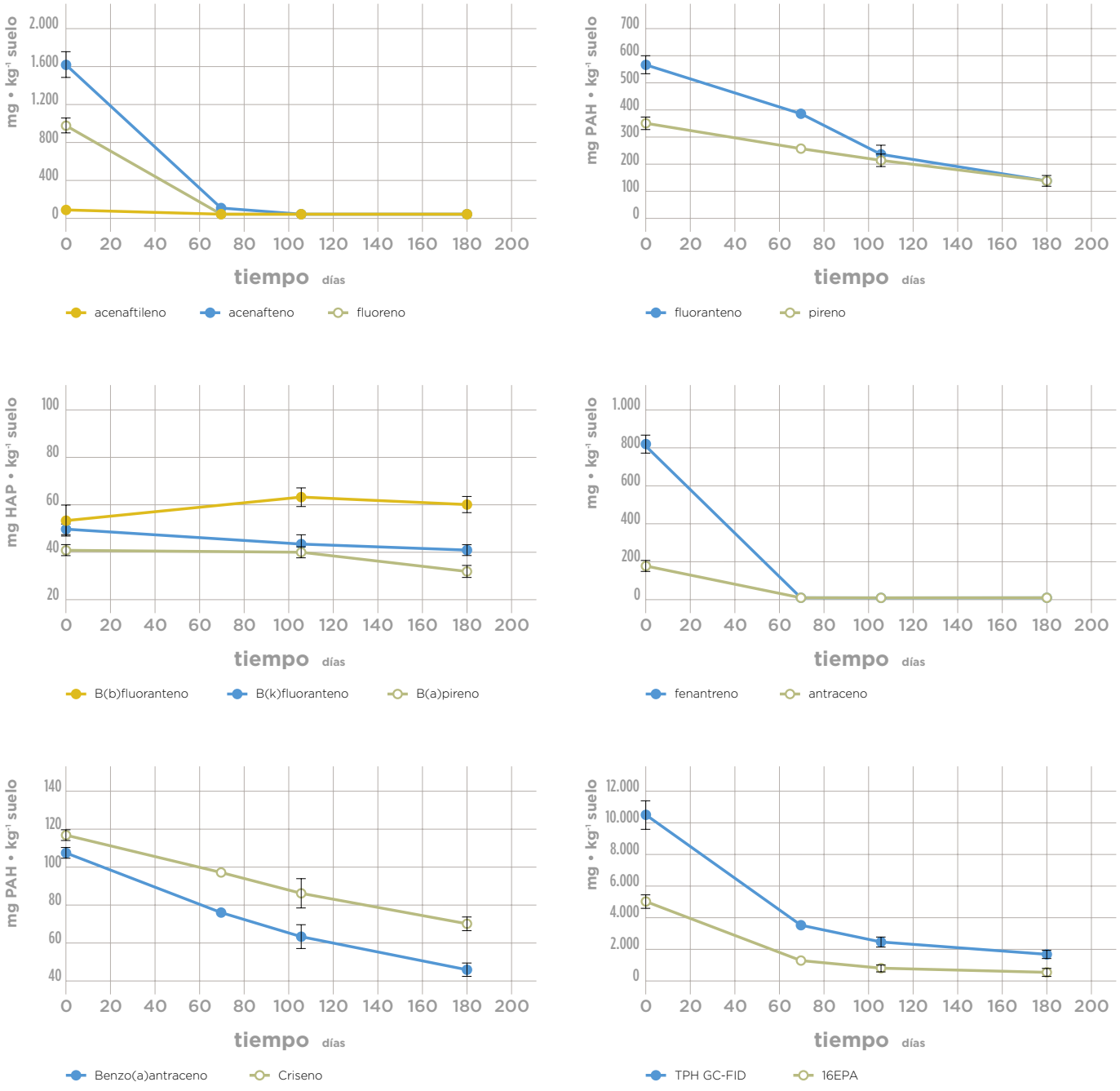
Tabla 7 Porcentajes de biodegradación (%) respecto al día 0

	70 días	105 días	180 días
Naftaleno	100	100	100
Acenaftileno	86	89	95
Acenafteno	94	99	99
Fluoreno	99	99	100
Fenantreno	98	99	99
Antraceno	91	92	96
Fluoranteno	33	58	76
Pireno	28	38	61
B(a)antraceno	31	40	56
Criseno	19	26	48
B(b)fluoranteno	0	-8	2
B(k)fluoranteno	23	15	18
B(a)pireno	31	3	23
Indeno-pireno	21	-16	43
Dibenzoantraceno	8	-21	55
Benzoperileno	-7	-15	44
Total HAP	77	82	89
TPH (gravimetría)	61	67	73
TPH (GC-FID)	83	92	85

En las **tablas 6** y **7** se pueden observar los resultados tanto de los recuentos por NMP de las poblaciones heterótrofas y degradadoras de HAP como el porcentaje de biodegradación de los 16 HAP de la EPA y los TPH totales, respecto al día 0, a lo largo de los 180 días que duró el ensayo de biorremediación.

El control para mantener los niveles de oxígeno y de humedad en valores próximos a los óptimos permitió que el proceso de biodegradación llegara a valores elevados. Al final del tratamiento, el porcentaje de degradación de los TPH llegó al 85%, y por lo que a los 16 HAP de la EPA se refiere, se consiguió un 89% de degradación respecto al día 0. Es importante destacar la gran homogeneidad de las muestras, ya que esto reforzó el valor de los resultados. También hay que tener en cuenta que al final del proceso las poblaciones microbianas implicadas en la degradación de hidrocarburos todavía eran elevadas, por lo

Figura 9
cinéticas de degradación de los HAP individuales y de los TPH totales, así como de los 16 HAP de la EPA en conjunto



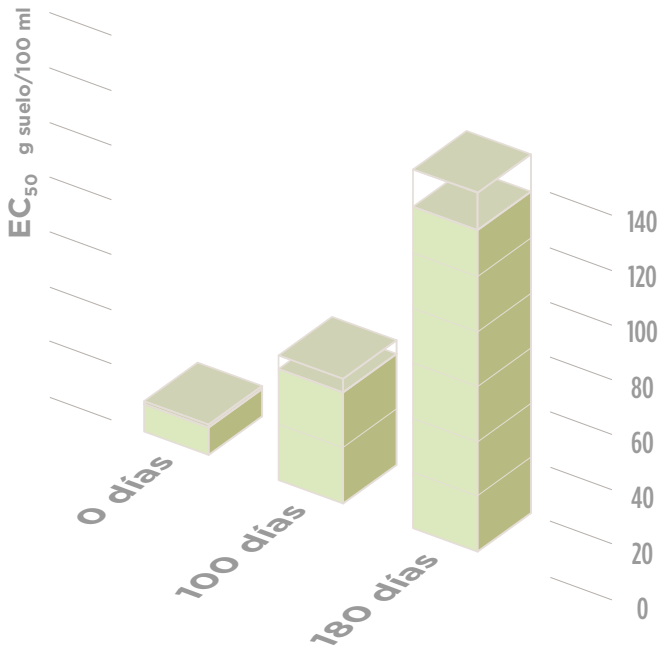
que el proceso biodegradativo todavía estaba activo. Este comportamiento hace pensar que la biodegradación todavía podía continuar.

Observando las cinéticas de eliminación de cada uno de los HAP diana (**figura 9**), se pueden prever distintos comportamientos. Los HAP de tres anillos (fenantreno y antraceno) se degradaron totalmente. Los HAP de 4 anillos (fluoranteno, pireno, criseno y benzo(a)antraceno) se continuarían degradando hasta que dejaran de estar disponibles. Por último, los HAP de 5 anillos prácticamente no se degradaron y únicamente el benzo(a)pireno pasados 105 días presentó una ligera disminución.

En relación con los HAP de 5 anillos cuyas concentraciones al final del proceso suelen presentar valores que superan los NGR establecidos, se deberían tener en cuenta dos comportamientos de estos compuestos. En primer lugar, en las curvas de calibración de estos HAP de elevado peso molecular, que permiten relacionar área con concentración, las pendientes son muy ligeras, por lo que pequeñas variaciones de área pueden dar valores muy distintos de concentración. La solución sería procesar un gran número de muestras y hacer una media. Sin embargo, esto encarecería los análisis.

Una segunda consideración es que estos HAP de elevado peso molecular tienen una biodisponibilidad muy baja debido a pro-

Figura 10
evolución de la EC50 a lo largo del proceso de biorremediación en la biopila dinámica (Microtox de lixiviados del suelo)



cesos de adsorción a partículas del suelo y de absorción con la materia orgánica, y esta baja biodisponibilidad afecta también a su toxicidad.

Esta última consideración pudo ser confirmada con los ensayos de toxicidad aguda realizados. Los resultados obtenidos con el ensayo de Microtox (**figura 10**) nos indicaron que mientras inicialmente el suelo presentaba una toxicidad elevada, durante el proceso de biorremediación fue disminuyendo hasta poder ser considerado no tóxico.

A la vista de estos resultados, se puede evidenciar que los valores absolutos de las concentraciones de los HAP no pueden ser el único criterio para establecer el nivel de contaminación, puesto que factores como la biodisponibilidad y la toxicidad pueden ser importantes como parámetros complementarios a los NGR. ®

BIBLIOGRAFÍA

1. Alexander, M. "Biodegradation and Bioremediation" Segunda edición, Academic Press, Inc., San Diego (1999).

2. Van Hamme, J.D.; Singh, A. and Ward, O.P. 'Recent Advances in Petroleum Microbiology' *Microbiol. And Molec. Biol. Reviews* (2003) 67, pp 503-549.

3. Viñas, M.; Sabaté, J.; Grifoll, M. y Solanas, A.M. 'Ensayos de tratabilidad en la recuperación de suelos contaminados por la tecnología de la biorremediación' *Residuos Revista Técnica* (2001) 59, pp 78-82.

CAPÍTULO 3 / CHAPTER 3

Microbial populations related to PAH biodegradation in an aged biostimulated creosote-contaminated soil

Microbial populations related to PAH biodegradation in an aged biostimulated creosote-contaminated soil

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Un ensayo previo de biorremediación, en un suelo contaminado por creosota, mostró que la aireación del terreno y un contenido óptimo de humedad promovieron la completa degradación de los hidrocarburos aromáticos policíclicos (HAPs) de 3 anillos aromáticos, mientras que concentraciones residuales de HAPs de 4 anillos como el benzo(a)antraceno (B(a)A) y el criseno (Chry), permanecieron en el suelo.

Con el objetivo de explicar el estancamiento en la degradación de los HAPs de elevado peso molecular y analizar la población bacteriana responsable de su biodegradación, un nuevo ensayo, con análisis químicos y de microbiología molecular, fue llevado a cabo.

Usando una estrategia en "slurry", donde suelo contaminado se mezcló con medio mineral líquido con y sin suplemento adicional de B(a)A y Chry, se observó que el terreno contenía una potente comunidad bacteriana, capaz de degradar B(a)A y Chry hasta proporciones del 89% y 53%, respectivamente. Por otro lado, la falta de degradación de los mismos hidrocarburos en el suelo sin suplemento, permitió hipotetizar que la falta de biodisponibilidad no permitió un mayor nivel de biodegradación.

Los resultados obtenidos en los análisis cultivo dependientes e independientes permitió asociar a *Mycobacterium parmense, Pseudomonas mexicana* y al grupo Sphingobacteriales a la degradación de B(a)A y Chry en el suelo contaminado con creosota, en combinación con muchos otros microrganismos.

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ORIGINAL PAPER

Microbial populations related to PAH biodegradation in an aged biostimulated creosote-contaminated soil

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Abstract A previous bioremediation survey on a creosote-contaminated soil showed that aeration and optimal humidity promoted depletion of three-ringed polycyclic aromatic hydrocarbons (PAHs), but residual concentrations of four-ringed benzo(a)anthracene (B(a)A) and chrysene (Chry) remained. In order to explain the lack of further degradation of heavier PAHs such as four-ringed PAHs and to analyze the microbial population responsible for PAH biodegradation, a chemical and microbial molecular approach was used. Using a slurry incubation strategy, soil in liquid mineral medium with and without additional B(a)A and Chry was found to contain a powerful PAH-degrading microbial community that eliminated 89% and 53% of the added B(a)A and Chry, respectively. It is hypothesized that the lack of PAH bioavailability hampered their further biodegradation in the unspiked soil. According to the results of the culture-dependent and independent techniques *Mycobacterium parmense*, *Pseudomonas mexicana*, and Sphingobacterials group could control B(a)A and Chry degradation in combination with several microorganisms with secondary metabolic activity.

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Keywords Polycyclic aromatic hydrocarbons · Biodegradation · Bioavailability · Bioremediation · Pyrene · Chrysene · 16SrRNA · DGGE

Introduction

A major concern in the bioremediation of PAH-contaminated soils is the biodegradation of high-molecular-weight PAHs (HMW-PAHs), which have recalcitrant properties and mutagenic or carcinogenic effects (Farmer et al. 2003). While low-molecular-weight PAHs, composed of two and three aromatic rings, can be biodegraded under favorable conditions (Wilson and Jones 1993), PAHs with four or more rings offer greater resistance to microbial degradation (Alexander 1999; Sabaté et al. 2006) and may persist at residual concentrations that frequently exceed regulatory limits.

In a previous study we described the bioremediation of a real creosote-contaminated soil. The treatment lasted 200 days and was based on a range of biostimulation and bioaugmentation strategies (Viñas et al. 2005a, b). Given that the addition of nutrients seems to be a universal practice, with one exception all treatments were based on the addition of nitrogen and phosphorus at a C:N:P ratio of 300:10:1 Other biostimulation agents studied were a rhamnolipid produced by strain AT10 of *Pseudomonas aeruginosa*, used as a biosurfactant (Abalos et al.

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2004), ferric octoate, used as an iron source, and glucose, an easily assimilable substrate. In addition, the inoculation of a PAH-degrading consortium was assayed as a bioaugmentation strategy. The results showed that two- and three-ringed PAHs were fully removed in all treatments. The one exception was anthracene, which was 84% depleted. Fluoranthene and pyrene were 92% and 87% depleted, respectively. The extent of degradation (3–4 ringed PAH except benzo(a)anthracene (B(a)A) and crysene (Chry)) was the same in all treatments. Unexpectedly, B(a)A and Chry, which also have four benzene rings, were more degraded (72% and 62%, respectively) in the aerated treatment without nutrient amendments than in the fertilized treatment (43% and 39%, respectively). In addition, nutrient addition caused an important shift in the bacterial community. We hypothesised that nutrient addition enriched a fast-growing microbial population that degraded more easily biodegradable PAHs, while in the absence of nutrients, a slow-growing microbial population that was specialized in the degradation of more recalcitrant PAHs prevailed.

Here we describe an experimental strategy based on the incubation of the bioremediated soil in slurry with liquid mineral medium The soil, which can be considered as an aged soil in which the more easily biodegradable PAHs are depleted, was incubated with and without additional B(a)A and Chry. Our aim was to analyse the microbial population involved in the degradation of high-molecular-weight PAHs (HMW-PAHs) in the creosote-contaminated soil, and to attempt to explain the lack of further degradation of these two PAHs in soil.

Materials and methods

Chemicals

Benzo(a)anthracene, chrysene, and o-terphenyl were purchased from Aldrich Chemical Co., Milwaukee, Wisconsin. Solvents were purchased from Scharlab S.L., Barcelona. Solvents and other chemicals and reagents were of the highest purity available. 16 EPA PAH standard solution (10 ng μl^{-1} in cyclohexane) was purchased from Dr. Ehrenstorfer-Schäfers (Augsburg, Germany). PAH standards for quantification in GC-FID.

Soil material

We combined a sample of creosote-contaminated soil that had been previously bioremediated with aeration and an optimal humidity. The concentrations of the most predominant PAHs in the contaminated soil at the beginning and at the end of the bioremediation process are shown in Table 1.

PAH spiked slurries

In the present study two sets of slurries (spiked and unspiked) were incubated in conditions of horizontal agitation at 25°C in 250 ml flasks covered with sterile aluminium foil, and protected from light, for 30 days. The slurries contained 100 mg of aged soil (Table 1) resuspended in 50 ml of 10 times-diluted mineral medium BMTM (Hareland et al. 1975). The diluted BMTM contained (per liter): $K_2HPO_4 \cdot 3H_2O$, 0.425 g; $NaH_2PO_4 \cdot H_2O$, 0.10 g; NH_4Cl, 0.20 g; $MgSO_4 \cdot 7H_2O$, 0.020 g; $FeSO_4 \cdot 7H_2O$, 0.0012 g; $MnSO_4 \cdot H_2O$, 0.0003 g; $ZnSO_4 \cdot 7H_2O$, 0.0003 g; $CoCl_2 \cdot 6H_2O$, 0.0001 g; nitrilotriacetic acid, 0.012 g. The medium was sterilized by autoclaving at 121°C for 20 min.

The spiked slurries contained 2 mg of B(a)A and 1 mg of Chry. To avoid toxicity, B(a)A and Chry

Table 1 PAH concentration of soil submitted to a bioremediation

PAH compound	Initial concentration ($\mu g \cdot g^{-1}$ of soil)	Final concentration ($\mu g \cdot g^{-1}$ of soil)
Phenanthrene	465.5	15.8
Anthracene	114.3	13.6
3-Methyl-phenanthrene	71.5	n.d.[a]
2-Metyhylphenanthrene	77.7	n.d.
Methylanthracene	25.1	n.d.
9-Methyl-pehanthrene or 4-Cyclopentaphenanthrene	131.1	4.4
1-Methylphenanthrene	40.0	n.d.
Fluoranthene	693.1	54.7
Pyrene	386.9	52.8
Benzo(a)anthracene	108.3	32.1
Chrysene	144.4	58.1
Total	2257.9	231.5

[a] Not detected

were firstly added as a dichloromethane suspension (1 ml) to sterilized empty Erlenmeyer flasks. Dichloromethane was allowed to evaporate under sterile conditions into a laminar-flow cabinet. After that, 50 ml of sterile mineral medium was amended and an additional ultrasonic-bath step of spiked flasks was applied to detach PAH crystals from glass. Finally, 100 mg of aged soil was amended to all treatments. Each treatment was carried out in triplicate for chemical and microbial analysis. Abiotic controls with the sterilized soil were incubated in the same conditions.

PAH analysis

The samples were liquid–liquid extracted with 5 × 10 ml of dichloromethane. Orthoterphenyl dissolved in acetone was added to each slurry, as a surrogate internal standard. The total organic extracts (TOE) obtained were dried over Na_2SO_4 and concentrated in a rotary evaporator to dryness To obtain the total petroleum hydrocarbon (TPH) fraction, TOE was resuspended in dichloromethane and cleaned up by column chromatography using the EPA 3611 method (US Environmental Agency). Both TPH and PAH concentrations were analyzed by gas chromatography with flame-ionization detection (GC-FID) using a Trace 2000 gas chromatograph (Thermo Quest, Milan, Italy) fitted with a DB-5 (30 m × 25 mm i.d. × 0.25 μm film) capillary column (J&W Scientific Products Gmbh, Köln, Germany). The column temperature was held at 50°C for 1 min, ramped to 320°C at 7°C min^{-1} and held for 10 min.

Monitoring of heterotrophic and hydrocarbon-degrading microbial populations

Soil bacterial counts were performed using a miniaturized most probable number (MPN) method in 96-well microtiter plates, with eight replicate wells per dilution (Wrenn and Venosa 1996). Total heterotrophs were counted in tryptone soy broth and aromatic hydrocarbon-degraders were counted in mineral medium (Wrenn and Venosa 1996) containing a mixture of phenanthrene (0.5 $g \cdot l^{-1}$), fluorene, anthracene, and dibenzothiophene (each at a final concentration of 0.05 $g \cdot l^{-1}$). Aged soil was used as the starting point (day 0). MPN Plates were incubated at room temperature (25°C ± 2°C) for 30 days. Positive wells were detected by turbidity (heterotrophs) and the presence of coloration (brownish/yellow) for PAH degraders.

DNA extraction

Samples for DNA extraction were collected from slurry cultures and the highest positive dilutions from microtiter plates, placed in sterile Eppendorff tubes and stored at −20°C prior to analysis. To ascertain the repeatability of the DNA extraction process and PCR protocols, a set of replicates was analyzed by denaturing gradient gel electrophoresis (DGGE). This showed a high degree of repeatability of the sampling and molecular protocols (DNA extraction). Thus, a 20 ml sample of each slurry culture was centrifuged at 14,000*g* for 10 min and the pellets were extracted by a bead beating protocol using the Power Soil DNA extraction kit (MoBio Laboratories, Solano Beach, CA, USA), following the manufacturer's instructions. A further purification step with Clean DNA Wizard kit (Promega, WI, USA) was necessary to avoid PCR inhibition. To obtain DNA from microtiter plates, a composite sample of 1.6 ml containing 200 μl of each replicate ($n = 8$) belonging to the last dilution with eight positives was centrifuged and treated as described above.

Polymerase chain reaction

The V3–V5 hypervariable regions of the 16S rRNA gene were amplified using primers F341-GC and R907 (Yu and Morrison 2004). The primer F341-GC included a GC clamp at the 5′ end (5′-CGCCCGCCGC GC CCCGCGCCCGTCCCGCCGCCCCCGCCCG-3′). All PCR reactions were performed on a Mastercycler personal thermocycler (Eppendorff, Hamburg, Germany). Fifty microliters of PCR mixture contained 2.5 U Takara Ex Taq DNA Polymerase (Takara Bio, Otsu, Shiga, Japan), 25 mM TAPS (pH 9.3), 50 mM KCl, 2 mM $MgCl_2$, 200 μM of each deoxynucleoside triphosphate, 0.5 μM of each primer, and 100 ng of template DNA quantified by means of Low DNA Mass Ladder (Gibco BRL, Rockville, MD). After 9 min of initial denaturation at 95°C, a touchdown thermal profile protocol was performed and the annealing temperature was decreased by 1°C per cycle from 65 to 55°C, at which temperature 20 additional cycles were carried out.

Amplification was carried out with 1 min of denaturation at 94°C, 1 min of primer annealing and 1.5 min of primer extension at 72°C, followed by 10 min of final primer extension.

Denaturing gradient gel electrophoresis

Approximately 400 ng of purified PCR product was loaded onto a 6% (wt/vol) polyacrylamide gel, 0.75 mm thick (to obtain better resolution), with denaturing gradients ranging from 50% to 70% (100% denaturant contains 7 M urea and 40% formamide). Low DNA Mass Ladder (Gibco BRL, Rockville, MD, USA) was used for quantification. DGGE was performed in 1× TAE buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, pH 7.4) using a DGGE-2001 System (CBS Scientific Company, Del Mar, CA, USA) at 100 V and 60°C for 16 h.

The gels were stained for 45 min in 1× TAE buffer containing SybrGold (Molecular probes, Inc., Eugene, OR, USA), then scanned using a Bio-Rad molecular imager FX Pro Plus multi-imaging system (Bio-Rad Laboratories, Hercules, CA, USA) in DNA stain gel mode for SybrGold at medium sample intensity. Images of DGGE gels were digitalized and DGGE bands were processed using Quantity-one version 4.1 image analysis software (Bio-Rad Laboratories) and corrected manually.

Sequencing and phylogenetic analysis of DGGE bands

Predominant DGGE bands were excised with a sterile razor blade, under blue light using a Visi-Blue Converter Plate (UVP, Upland, CA, USA), resuspended in 50 µl sterilized MilliQ water and stored at 4°C overnight. An aliquot of supernatant (2 µl) was used to reamplify the DGGE bands with primers F341, without the GC clamp, and R907, under the same conditions. The reamplified bands were purified using a Wizard SV Gel and PCR Clean-Up System (Promega, WI, USA). The ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit version 3.1 (Applied Biosystems) was used for sequencing cleaned PCR amplicons.

Reamplified bands that were impossible to sequence with this method were cloned into the pGEM-T Easy vector (Promega, WI, USA), and transformed into competent *E. coli* DH5α. The recombinant clones were amplified with primers SP6 and T7, cleaned up and sequenced using primers R907 and F341, as explained above.

Sequences were edited and assembled using version 4.8.7 of the BioEdit software (Hall 1999), inspected for the presence of ambiguous base assignments and subjected to the Check Chimera program of the Ribosomal Database Project (Maidak et al. 2000). The sequences were then submitted to BLAST and RDP search using alignment tool comparison software.

Statistical analysis

Data were subjected to analysis of variance (ANOVA) using the Statgraphics Plus package (version 5.1; Statistical Graphics Corp., Manguistics Inc., United States). Duncan's multiple-range test of means, with a significance level of 0.05, was applied to the results to determine their statistical significance.

Nucleotide accession numbers

The 35 nucleotide sequences identified in this study were deposited in the GenBank database under accession numbers EU512950 to EU512984.

Results and discussion

Degradation of B(a)anthracene and chrysene in the soil slurries

After 30 days of slurry incubation, the microbial population present in the bioremediated soil was able to degrade 89% and 53% of the added benzo(a)anthracene and chrysene (1639 and 947 µg/flask, respectively), but did not affect the residual concentration of these two PAHs in the slurry of the bioremediated soil (7.7 and 9.1 µg/flask, respectively, Fig. 1). This finding indicates that the aged soil contained a potent B(a)A and Chry-degrading community. The low concentrations of BaA and Chry in the slurries (0.064 mg/l and 0.116 mg/l, respectively), which were close to detection limit (0.1 mg/l), hindered peak integration. Indeed, after 30 days' incubation the concentrations were even higher than starting values but at the same low range below 1 mg/ml (0.55 ± 0.11 for BaA and 0.46 ± 0.001 for Chry).

As the incubation was carried out with agitation, the lack of degradation of B(a)A and Chry in the

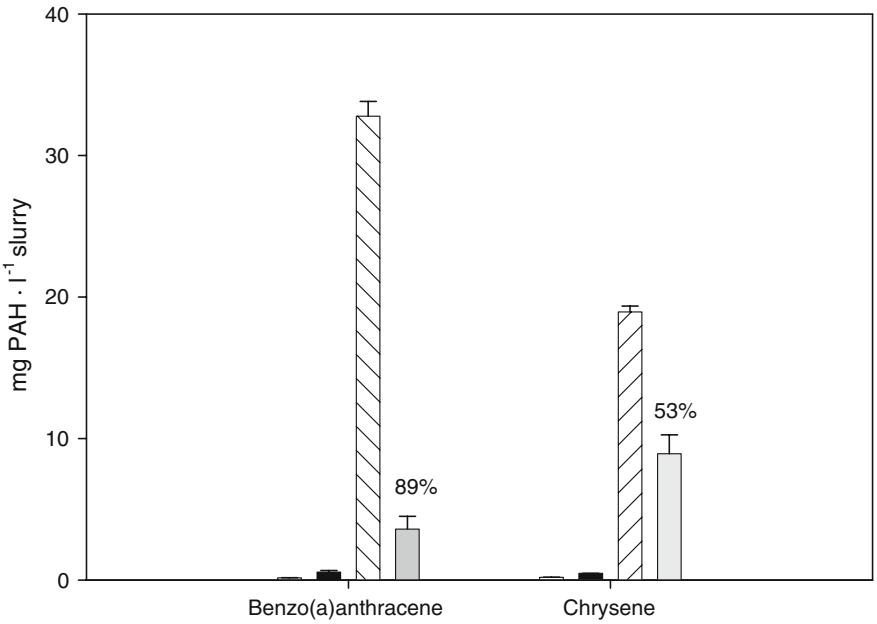

Fig. 1 Benzo(a)anthracene and chrysene concentration at 0 and 30 days, in creosote-contaminated soil with and without supplementation of these two PAHs. White bars represent aged soil; Black bars represent unspiked aged soil after 30 days; Dashed bars represent spiked aged soil at starting point. Gray bars represent spiked aged soil after 30 days of incubation. Percentages show the biodegradation of BaA and Chry after 30 days

unspiked microcosms reflects a high level of sequestration, with the subsequent lack of bioavailability to the microbial population. The residual concentrations of B(a)A and Chry in the soil after application of biostimulation were 32.1 and 58.1 μg/g soil, respectively (Table 1), both above the majority of standard legal limits. Since toxicity and bioavailability correlate strongly, (Alexander 1995), sequestration of PAHs should be considered when establishing of the concentrations below which a soil is to be deemed non-decontaminated.

Monitoring of heterotrophic and hydrocarbon-degrading microbial populations

Low molecular-weight PAHs, namely phenanthrene, fluorene, anthracene, and dibenzothiophene, which are normally used to enumerate the PAH-degraders (Wrenn and Venosa 1996), were replaced by B(a)A and Chry. Nevertheless, no growth was observed, possibly due to the excessively low solubility of these substrates or to the absence of other PAHs, which may be required for cometabolism. We thus decided to use the traditional PAH mixture. The hetrotrophic population and PAH degraders increased up to 3 and 2 magnitude orders, respectively, after 30 days of incubation, reaching populations of 10^5 and 10^4 MPN/ml, respectively. Surprisingly, no significant differences were found between heterotrophic and PAH degraders in either set of slurries ($P > 0.05$) (Table 2). While in the spiked slurries a total of 2586 μg/flask of B(a)A and Chry was depleted, in the non-spiked slurries (from which the residual B(a)A and Chry were not removed) a maximum of 23.1 μg/flask of a mixture of PAHs (Table 1) was consumed. This result has two possible explanations.

Table 2 MPN of heterotrophic and PAH-degrading population at 0 day and at 30 days of slurry incubation with and without supplementation of B(a)anthracene and chrysene

	Heterotrophs (MPN/ml)	PAH degraders (MPN/ml)
Soil 0 day	$5.89 \pm 3.12 \cdot 10^3$ (A)[a]	$7.78 \pm 4.41 \cdot 10^2$ (A)
Soil 30 days	$4.74 \pm 1.50 \cdot 10^5$ (B)	$3.39 \pm 0.71 \cdot 10^4$ (B)
Soil + BaA + Chry	$9.02 \pm 2.12 \cdot 10^5$ (B)	$4.31 \pm 4.00 \cdot 10^4$ (B)

Data are presented as the mean value ± SD (n = 3)

[a] Different letters in brackets in the same column (hetrotrophs or PAH degraders) indicate significant differences between treatments ($P < 0.05$)

Firstly, the depletion of B(a)A and Chry in the spiked slurries may have been due to co-metabolic oxidation rather than the use of these compounds as growth substrates. Alternatively, the possible obligate B(a)A and Chry-degraders present in the spiked slurries may not have been detected by the medium used.

DGGE analysis

To analyze the microbial population initially present in the soil and its response to the presence of high amounts of B(a)A and Chry, two DGGE analyses were carried out. In one we measured the total DNA in the slurries with and without the spiked PAHs. In the other we measured DNA from the more diluted wells of the microtiters used to enumerate the PAH degraders in both types of slurry.

Total DNA

At the end of the experiment, four additional bands (B2, B5, B6, B7) appeared in the total DNA profile of the spiked slurry in comparison to the unspiked one (lane 2 and 3, Fig. 2). However, after purification, band 5 turned out to be six different co-eluting sequences (B5a, B5b, B5c, B5d, B5e, B5f) and band B8 contained two sequences (B8a and B8b). No signal was detected in the lane corresponding to the aged soil (lane 1, Fig. 2).

Band B2 corresponded to *Sphingobacterials*. Bands in B5 corresponded mainly to Sphingomonaceae (Table 3), except B5d, which corresponded to an uncultured Burkolderiaceae and was coincident with Band B17. Band B6 was a chimera and Band B7 corresponded to *Azohydromonas australica* (Group Burkholderials), a nitrogen-fixing bacterium (previously *Alcaligenes latus*) (Xie and Yokota 2005). Given that the soil was not fertilized, this nitrogen fixing bacterium would have a role in the supply of nitrogen. Band B8a presented high similarity (99%) to *Methylibium petroleiphilum,* which has been described as a methyl *tert*-butyl ether-degrading methylotroph bacterium that can also use several monoaromatic hydrocarbons.

Some bands were shared by the two profiles (B9 = B21, B10 = B22, B11 = B23, B12 = B24), and these belonged to *Skermanella* sp. These strictly aerobic bacteria are isolated from airborne particulate matter enriched in PAHs (Weon et al. 2007), but their capacity to degrade hydrocarbons has not been reported.

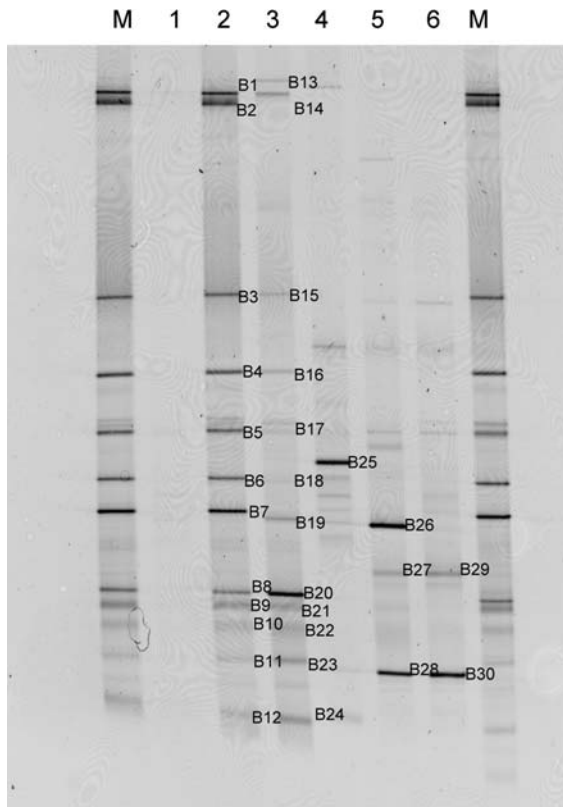

Fig. 2 Denaturing gradient gel electrophoresis profiles of PCR-amplified 16S rRNA gene fragments (V3–V5 regions) of total community DNA. Lane 1: aged soil; Lane 2: Soil + BaA + Chry (slurry 30 days): Lane 3: Soil (slurry 30 days). Lane 4: PAH-degraders MPN plates starting aged soil. Lane 5: PAH degraders (MPN Plates) spiked slurry (30 days). Lane 6: PAH degraders (MPN plates) unspiked slurry (30 day). Lane M: Marker which contains the same DNA sample as Lane 2

DGGE from the wells of the microtiters used to enumerate the PAH degraders

The DGGE profiles of the PAH-degrading populations obtained from microtiter plates from the slurries with and without additional B(a)A and Chry corresponded to lane 5 and lane 6 respectively (Fig. 2). The appearance of many fewer bands than those corresponding to the total DNA it would be indicative of a strong selectivity caused by the liquid mineral medium used in the enumeration of PAH degraders.

Band B26 corresponded to an uncultured Comamonadaceae and Band B27 corresponded to

Table 3 Properties of DGGE bands: designations and accession numbers for the band sequences and levels of similarity to related organisms

Band	Length (bp)	Accession no.	Closest organism in GenBank database (accession no.)	% similarity[a]	Phylogenetic group[b]
B1 = B14	542	EU512950	Uncultured Bacteroidetes bacterium (AY758564)	98.2%	*Sphingobacteriales* (CFB group)
B2	579	EU512951	Uncultured Bacteroidetes bacterium (AY921801)	96%	*Sphingobacteriales* (CFB group)
B3 = B15	446	EU512952	Uncultured Arizona's soil bacterium (AF507716)	94.6%	*Sphingobacteriales* (CFB group)
B4 = B16	565	EU512953	Uncultured tallgrass prairie soil bacterium (AY957901)	96.8%	Unclassified *proteobacterium* (β)
B5a	436	EU512954	*Sphingomonadaceae* bacterium KF16 (AB269802)	97.9%	*Sphingomonadaceae* (α)
B5b	462	EU512955	Uncultured tallgrass prairie soil bacterium clone FFCH2236 (EU134542)	94.4%	Unclassified *proteobacterium* (δ)
B5c	460	EU512956	Unidentified Rizosphere's Maize bacterium clone 39c (AY669100)	96.2%	Unclassified *Proteobacterium* (β)
B5d = B17	460	EU512957	Uncultured *Burkholderiaceae* bacterium clone GASP-WC2S2_B05 (EF074975)	97.8%	*Burkholderiaceae* (β)
B5e	435	EU512958	Uncultured *proteobacterium* clone Amb_16S_1274 (AF505720)	93.4%	Unclassified *proteobacterium* (γ)
B5f	436	EU512959	Uncultured *Sphingomonas* sp. clone AUVE_04G07 from Australian Vertisoil (EF651167)	95.4%	*Sphingomonadaceae* (α)
B7	586	EU512960	*Azohydromonas australica* (AB188124)	99%	*Alcaligenaceae* (β)
B8a	434	EU512961	*Methylibium petroleiphilum* PM1 (CP000555)	98.9%	*Burkholderiaceae* (β)
B8b = B20	403	EU512962	Uncultured *alpha proteobacterium* from TCE-contaminated site (AY133099)	99.8%	*Rhodospirillaceae* (α)
B9 = B21	371	EU512963	*Skermanella* sp. (DQ672568)	100%	*Rhodospirillaceae* (α)
B10 = B22	402	EU512964	*Skermanella* sp. (DQ672568)	100%	*Rhodospirillaceae* (α)
B11 = B23	449	EU512965	*Skermanella* sp. (DQ672568)	99.8%	*Rhodospirillaceae* (α)
B12 = B24	547	EU512966	*Skermanella* clone (EF651077)	99.5%	*Rhodospirillaceae* (α)
B13	480	EU512967	Uncultured bacterium from California's grassland (EF516948)	97.9%	*Sphingobacteriales* (CFB group)
B14 = B1	473	EU512968	Uncultured Bacteroidetes bacterium (AY758564)	99.6%	*Sphingobacteriales* (CFB group)
B15 = B3	437	EU512969	Uncultured Arizona's soil bacterium (AF507716)	94%	CFB group
B16 = B4	482	EU512970	Uncultured tallgrass prairie soil bacterium (AY957901)	96.5%	Unclassified *proteobacterium* (β)
B17 = B5d	477	EU512971	Uncultured *Burkholderiaceae* bacterium clone GASP-WC2S2_B05 (EF074975)	96.2%	*Burkholderiaceae* (β)
B18	403	EU512972	Uncultured bacterium clone 18 (DQ413077)	97.5%	*Hyphomicrobiaceae*(α)
B19	383	EU512973	*Nitrosospira* sp. Nsp2 (AY123802)	96.9%	*Nitrosomonadaceae* (β)
B20 = B8b	484	EU512974	Uncultured *alpha proteobacterium* from TCE-contaminated site (AY133099)	99.6%	*Rhodospirillaceae* (α)
B21 = B9	456	EU512975	*Skermanella* sp. (DQ672568)	97.1%	*Rhodospirillaceae* (α)
B22 = B10	546	EU512976	*Skermanella* sp. (DQ672568)	99.6%	*Rhodospirillaceae* (α)
B23 = B11	407	EU512977	*Skermanella* sp. (DQ672568)	98.8%	*Rhodospirillaceae* (α)
B24 = B12	454	EU512978	*Skermanella* clone (EF651077)	99.5%	*Rhodospirillaceae* (α)
B25	542	EU512979	*Sphingobium herbicidivorans* strain FL (EF065102)	100%	*Sphingomonadaceae* (α)

Table 3 continued

Band	Length (bp)	Accession no.	Closest organism in GenBank database (accession no.)	% similarity[a]	Phylogenetic group[b]
B26	569	EU512980	Uncultured *beta proteobacterium* clone IRD18H05 from two temperate rivers (AY947979)	99.1%	*Comamonadaceae* (β)
B27 = B29	355	EU512981	*Pseudoxanthomonas mexicana* (EU119264)	96.9%	*Xanthomonadaceae* (γ)
B28 = B30	555	EU512982	*Mycobacterium parmense* (AF466821)	99.6%	*Mycobacteriaceae*
B29 = B27	576	EU512983	*Pseudoxanthomonas mexicana* (EU119264)	97.9%	*Xanthomonadaceae* (γ)
B30 = B30	549	EU512984	*Mycobacterium parmense* (AF466821)	99.6%	*Mycobacteriaceae*

[a] Sequences were matched with the closest relative from the Genbank database

[b] Sequences were matched with the closest relative from the Ribosomal Database Project (Maidak et al. 2000). α, β, and γ represent Alpha and Gammaproteobacteria, respectively

Pseudoxanthomonas mexicana isolated from an anaerobic digester (Thierry et al. 2004). Band B28 corresponded to *Mycobacterium parmense* (99.6% similarity). Although this species was isolated from a cervical lymph node (Fanti et al. 2004), several species of *Mycobacterium* that are difficult to classify have a high capacity to degrade PAHs (Turenne et al. 2001; Walter et al. 1991, Miller et al. 2007). Taking into account that Bands B29 and B30 were coincident with Bands B27 and B28 we conclude that both microorganisms could play a role in the biodegradation of the residual PAHs of the bioremediated soil. The microorganism corresponding to the uncultured *Comamonadaceae*, which was only present in the spiked slurry (Band B26), may have a role in the biodegradation of Ba(a)A and Chry. Surprisingly, none of these bands appeared in the total community DNA. This absence is difficult to explain, but it could be attributable to the fact that the mixed cultures in the soil had a more complex microbial population than the microtiters, which may have affected the PCR amplification.

We conclude that *Mycobacterium parmense* and *Pseudomonas mexicana* may contribute to the degradation of both 3- and 4-ringed PAHs, in which Sphingobacterials of the CFB group could also have a role. This is consistent with previous results obtained with the same creosote-contaminated soil (Viñas et al. 2005a, b). In connection with other identified microorganisms, supply of nitrogen could be attributed to *Azohydromonas australica*, and other secondary metabolic activities could be attributed to Skermanella sp. and *Methylibium petroleiphilum.*

The use of the PAH-spiked slurry approach coupled with molecular ecology may help us to understand biodegradation and microbial aspects encountered in aged hydrocarbon-polluted environments.

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References

Abalos A, Viñas M, Sabaté J, Manresa MA, Solanas AM (2004) Enhanced biodegradation of casablanca crude oil by a microbial consortium in presence of a rhamnolipid produced by *Pseudomonas aeruginosa* AT10. Biodegradation 15:249–260. doi:10.1023/B:BIOD.0000042915.28757.fb

Alexander M (1995) How toxic are toxic chemicals in soil? Environ Sci Technol 29:2713–2717. doi:10.1021/es00011a003

Alexander M (1999) Biodegradation and bioremediation. Academic Press, San Diego

Fanti F, Tortoli E, Hall L, Roberts GD, Kroppenstedt RM, Dodi I, Conti S, Polonelli L, Chezzi C (2004) *Mycobacterium parmense* sp. nov. Int J Syst Evol Microbiol 54:1123–1127. doi:10.1099/ijs.0.02760-0

Farmer PB, Singh R, Kaur B, Sram RJ, Binkova B, Kalina I, Popov TA, Garte S, Taioli E, Gabelova A, Cebulska-Wasilewska A (2003) Molecular epidemiology studies of carcinogenic environmental pollutants: effects of polycyclic aromatic hydrocarbons (PAHs) in environmental pollution on exogenous and oxidative DNA damage. Mutat Res 544:397–402. doi:10.1016/j.mrrev.2003.09.002

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41:95–98

Hareland W, Crawford RL, Chapman PJ, Dagley S (1975) Metabolic function and propierties of 4-hydroxyphenylacetic acid 1-hydroxylase from *Pseudomonas acidovorans*. J Bacteriol 121:272–285

Maidak BL, Cole JR, Lilburn TG, Parker CT Jr, Saxman PR, Stredwick JM, Garrity GM, Li B, Olsen GJ, Pramanik S, Schmidt TM, Tiedje JM (2000) The RDP (Ribosomal Database Project) continues. Nucleic Acids Res 28:173–174. doi:10.1093/nar/28.1.173

Miller CD, Child R, Hughes JE, Benscai M, Der JP, Sims RC, Anderson AJ (2007) Diversity of soil mycobacterium isolates from three sites that degrade polycyclic aromatic hydrocarbobns. J Appl Microbiol 102:1612–1624. doi:10.1111/j.1365-2672.2006.03202.x

Sabaté J, Viñas M, Solanas AM (2006) Bioavailability assessment and environmental fate of PAHs in biostimulated creosote-contaminated soil. Chemosphere 63:1648–1659. doi:10.1016/j.chemosphere.2005.10.020

Thierry S, Macarie H, Iizuka T, Geissdoerfer W, Assih EA, Spanevello M, Verhe F, Thomas P, Fudou R, Monroy O, Labat M, Ouattara AS (2004) *Pseudoxanthomonas mexicana* sp. nov. and *Pseudoxanthomonas japonensis* sp. nov., isolated from diverse environments, and emended descriptions of the genus *Pseudoxanthomonas* Finkmann et al. 2000 and of its type species. Int J Syst Evol Microbiol 54:2245–2255. doi:10.1099/ijs.0.02810-0

Turenne CY, Tschetter L, Wolfe J, Kabani A (2001) Necessity of quality-controlled 16S rRNA gene sequence databases: identifying nontuberculous *Mycobacterium* species. J Clin Microbiol 39:3638–3648. doi:10.1128/JCM.39.10.3638-3648.2001

Viñas M, Sabaté J, Solanas AM (2005a) Culture-dependent and -independent approaches establish the complexity of a PAH-degrading microbial consortium. Can J Microbiol 51:897–909. doi:10.1139/w05-090

Viñas M, Sabaté J, Espuny MJ, Solanas AM (2005b) Bacterial community dynamics and PAHs degradation during bioremediation of heavily creosote-contaminated soil. Appl Environ Microbiol 71:7008–7018. doi:10.1128/AEM.71.11.7008-7018.2005

Walter U, Beyer M, Klein J, Rehm HJ (1991) Degradation of pyrene by *Rhodococcus* sp. UW1. Appl Microbiol Biotechnol 34:671–676. doi:10.1007/BF00167921

Weon HY, Kim BY, Hong SB, Joa HJ, Nam SS, Lee KH, Kwon SW (2007) *Skermanella aerolata* p. nov., isolated from air, emended description of the genus *Skermanella*. Int J Syst Evol Microbiol 57:1539–1542. doi:10.1099/ijs.0.64676-0

Wilson SC, Jones KC (1993) Bioremediation of PAHs contaminated soils. Environ Pollut 81:229–249. doi:10.1016/0269-7491(93)90206-4 (Review)

Wrenn BA, Venosa AD (1996) Selective enumeration of aromatic and aliphatic hydrocarbon-degrading bacteria by a most-probable-number procedure. Can J Microbiol 42:252–258

Xie CH, Yokota A (2005) Reclassification of *Alcaligenes latus* strains IAM 12599T and IAM 12664 and *Pseudomonas saccharophila* as *Azohydromonas lata* gen. nov., comb. Nov., *Azohydromonas australica* sp. nov. and *Pelomonas saccharopila* gen. nov., comb. Nov., respectively. Int J Syst Evol Microbiol 55:2419–2425. doi:10.1099/ijs.0.63733-0

Yu Z, Morrison M (2004) Comparisons of different hypervariable regions of rrs genes for use in fingerprinting of microbial communities by PCR-denaturing gradient gel electrophoresis. Appl Environ Microbiol 70:4800–4806. doi:10.1128/AEM.70.8.4800-4806.2004

CAPÍTULO 4 / CHAPTER 4

Fungal/bacterial interactions throughout bioremediation assays in an aged creosote polluted soil

Fungal/bacterial interactions throughout bioremediation assays in an aged creosote polluted soil

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La utilización de inóculos fúngicos exógenos, en suelos industriales contaminados por hidrocarburos aromáticos policíclicos de elevado peso molecular, se presenta, de forma habitual, como una alternativa atractiva para degradar las fracciones menos disponibles. Sin embargo, se conoce poco como el bioaumento fúngico afecta a las poblaciones autóctonas del suelo.

El objetivo del presente estudio fue tratar de profundizar en como el bioaumento fúngico afecta a las poblaciones microbianas nativas, tanto bacterianas como fúngicas, en un suelo contaminado por creosota previamente tratado y, por lo tanto, enriquecido en la fracción más recalcitrante de hidrocarburos aromáticos policíclicos (HAPs), a través de una serie de bioensayos en "slurry" que abarcaron diferentes estrategias, tanto de bioestimulación como de bioaumento.

Curiosamente, la capacidad de degradación del hongo de podredumbre blanca *Trametes versicolor*, inoculado en los microcosmos, se vió negativamente afectada por la microbiota nativa del suelo. Por otro lado, estas mismas poblaciones fueron capaces de conseguir los niveles más altos de degradación de HAPs de cuatro anillos aromáticos, bajo condiciones limitantes debido al bajo contenido en carbono del medio. Los géneros bacterianos *Chryseobacterium, Pusillimonas* y *Sphingobium*, junto con el género fúngico *Fusarium*, pueden tener un papel relevante en los procesos de biodegradación de HAPs de elevado peso molecular observados en el suelo contaminado.

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Abstract

Utilization of exogenous fungal inoculums on industrially polluted soils affected by high molecular weight polycyclic aromatic hydrocarbons (HMW-PAHs) is often presented as an attractive alternative to manage the lowest biodegradable chemical fractions. However, little is known about how fungal bioaugmentation affects autochthonous soil microbial communities.

The aim of this study was to gain insight into how fungal bioaugmentation assays affect both PAH biodegradation and autochthonous microbial populations in a former biotreated aged-creosote polluted soil impacted by HMW-PAHs, through a set of slurry bioassays encompassing different biostimulation and bioaugmentation strategies. Interestingly, the degradation capability of inoculated white-rot fungus *Trametes versicolor* was negatively affected by active autochthonous soil microbiota, while the highest 4-ring PAH biodegradation levels were achieved by authochthonous microbial populations under carbon-limiting conditions. The ribotypes closely related to eubacterial genera *Chryseobacterium, Pusillimonas* and *Sphingobium*, concomitant with the fungal genus *Fusarium*, could be important in HMW-PAH biodegradation processes in polluted soil.

Keywords: Polycyclic aromatic hydrocarbons; ·creosote; *Trametes versicolor;* · 16SrRNA; ITS;· fungal-bacterial interactions

1. Introduction

As a consequence of the widespread presence of polycyclic aromatic hydrocarbons (PAHs) in soils, mainly due to the increase in industrial activity over the last decade, their remediation through the field application of biological treatments has been growing, both for its low environmental impact as well as for its relatively low cost compared with other technologies (Singh and Tripathi, 2007).

It is well know that autochthonous microbial populations can remove PAHs from polluted soils. Furthermore, it is widely described that soil characteristics such as moisture content, aeration condition and nutrients, among others, can affect removal rates (Chaîneau et al., 2003). In these cases, biostimulation technology would be recommended rather than natural attenuation. However, on aged historically contaminated soils, where bioavailability of the most recalcitrant compounds, such as high molecular weight polycyclic aromatic hydrocarbons (HMW-PAHs), may be extremely low, or in those soils where there is no active microbial population capable of degrading PAH compounds, biostimulation success may be greatly restricted (Chung andAlexander, 1999). In difficult cases such as these, amelioration of bioavailability of contaminants and microbial or,in particular, fungal bioaugmentation can yield better biodegradation results (Juhasz and Naidu, 2000).

A wide variety of fungi have been shown to metabolize PAHs. Among them, white-rot basidiomycetes are one of the most important groups used in soil bioremediation treatments due to their enzymatic system, which includes intracellular cytochrome P450 and extracellular lignin peroxidase, manganese peroxidase and laccase. As a consequence, some have the ability to cleave to the aromatic rings and mineralize the PAHs (Harms et al., 2011).

One of the major obstacles to the implementation of field-scale mycoremediation is that most of the laboratory-scale investigations have been carried out using artificially (spiked or sterilized) polluted soils. For this reason it is important to increase the number of studies using non-sterile soils from real polluted sites.

In a recent work (Llado et al., 2009), in order to ascertain the reasons for lack of further degradation of HMW-PAHs on a previously bioremediated creosote contaminated soil, and to analyse the microbial population related to PAH degradation, a slurry incubation with a liquid mineral medium strategy was assessed. In this case, it was concluded that bioavailability was a key factor in the lack of degradation of 4-ring PAHs, and that the slurry approach, coupled with molecular ecology techniques, was a suitable method to increase biodegradation and to better understand the chemical and microbial aspects of aged hydrocarbon polluted sites submitted to a bioremediation process. However, the same strategy by means of slurry experiments, when conducted with 5-ring PAHs, failed to enhance biodegradation rates.

This unsuccessful attempt led us to undertake a similar slurry method, optimizing the fungal bioaugmentation strategy with a view to enhancing biodegradation of the residual PAHs remaining in soil. The white-rot fungus *Trametes versicolor* was chosen because its laccase has been widely described as optimal for degrading HMW-PAHs (Collins et al., 1996). Taking into account that interactions with native soil microflora may lead to failure in a bioremediation process by white-rot fungi (Singh and Tripathi, 2007), the study of such interactions becomes essential in order to improve mycoremediation technologies.

The aim of this work was to study PAH-degrading capability and the evolution of autochthonous soil microbial communities after fungal bioaugmentation with a white-rot fungus such as *T. versicolor* as an alternative for degrading HMW-PAHs

remaining in the soil after a dynamic biopile, for which the degradation kinetics were very close to their theoretical limit (Sabaté et al., 2006; Viñas et al., 2005).

2. Materials and methods

2.1. Chemicals

Phenanthrene, fluorene, anthracene, dibenzotiophene, benzo(*a*)anthracene, chrysene, benzo(*k*)fluoranthene, benzo(*a*)pyrene, ergosterol, 7-dehidrocolesterol, o-terphenyl and methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate were purchased from Sigma-Aldrich, Spain. Solvents were purchased from Scharlab S.L., Barcelona. Solvents, chemicals and reagents were of the highest purity available. PAH standards for gas chromatography (GC-FID) analyses were obtained from Dr. Ehrenstorfer GmbH, Germany.

2.2. Soil

A composite sample of an aged creosote-contaminated soil that was previously bioremediated by biostimulation in a pilot scale biopile as elsewhere described (Realp et al., 2008). One month after finishing the biopile monitoring, a composite sample of the soil (20kg) was obtained and sieved (<6mm) and stored at 4ºC until use.

2.3. Fungal inoculum for bioaugmentation

Trametes versicolor (ATCC#42530) was utilized as fungal inoculum for bioaugmentation purposes. The fungus was maintained on 2% malt agar slants at 25 °C until use. Subcultures were routinely made as elsewhere described (Borràs et al., 2010).A mycelial suspension of *T. versicolor* was obtained by inoculation of four 1 cm diameter plugs, from the fungus growing zone on 2% malt agar, in 150 mL of 2% (w/v)

malt extract medium in a 500 mL Erlenmeyer flask. This was incubated at 25 °C at constant horizontal rotary agitation (135 rpm,*r*= 25 mm). After 4–5 days a dense mycelial mass was formed, that was separated from the culture medium, resuspended in an equal volume of a sterile saline solution (0.8% (w/v) NaCl) and then disrupted with an Ultra-Turrax macerator (IKA Labortechnik, Germany) homogenizer. The resulting mycelial suspension was stored at 4 °C until use.

2.*4. PAH-degrading capability of T. versicolor*

The PAH-degrading capability of *T. versicolor* was assessed in liquid malt extract glucose medium (MEG) in 250 mL cotton-stoppered Erlenmeyer flasks, sheltered from light, for 10 days at 25ºC and 200rpm in an orbital shaker. Each flask contained 50 mL of MEG medium (Novotny et al., 2000), whereas a mycelia suspension was utilized to inoculate the experiments (5% v/v). A mixture of six PAHs (phenanthrene, anthracene, benzo(*a*)anthracene, chrysene, benzo(*k*)fluoranthene and benzo(*a*)pyrene) was utilized for degradation experiments in the MEG medium, with each PAH at a final concentration of 50mg $\cdot$ L^{-1}. Triplicate flasks were sacrificed at the beginning of the experiment and after 10 days of incubation to quantify PAH degradation by means of gas chromatography with a flame ionization detector (GC-FID) analysis. An abiotic control inoculated with 5% (v/v) of autoclaved mycelia suspension was performed to control abiotic loses of PAHs after the incubation period.

2.*5. Soil slurry experiments*

In the present study, six sets of slurry experiments, designated as 1S to 6S, were undertaken (Table 1). Fungal-bacterial interactions were studied by means of ergosterol quantification, a culture-dependent technique such as the Most Probable Number

(MPN), and culture-independent techniques such as Denaturing Gradient Gel Electrophoresis (DGGE) and quantitative real time polymerase chain reaction (qPCR). As glucose is necessary for the survival of *T. versicolor*, a mineral medium was used as control to analyse the glucose effect on soil communities and on levels of PAH degradation.

Cotton-stoppered flasks (100 mL) were incubated under horizontal agitation at 25ºC covered with sterile aluminium foil, and protected from light, for 30 days at 50rpm in order to minimize myceliar rupture due to friction with soil particles. Each slurry contained 5 g of aged creosote polluted soil suspended in 20 mL of MEG medium or mineral medium (BMTM) (Hareland et al., 1975), depending on the treatment.

Each treatment was undertaken in triplicate for PAHs, ergosterol and molecular ecology analyses. Abiotic controls with the sterilized soil were incubated in the same conditions. In those treatments where fungal bioaugmentation by *T. versicolor* was assayed, the fungus was inoculated with mycelia suspension (5% v/v). Slurry flasks were incubated without agitation for the first seven days, and under agitation from the eighth to the 30th day. All analyses were carried out on sampling days 0, 15 and 30.

2.5.1. PAH extraction and quantification

The soil samples from slurry flasks were filtered (Whatman nº3) and air-dried for16 hours at room temperature before being extracted by an overnight (O/N) Soxhlet extraction with 200 mL of acetone:dichloromethane (1:1) mixture. Ortho-terphenyl was added to each sample as a surrogate internal standard before each chemical extraction. Previously, the discarded liquid medium was also extracted, confirming that did not contain detectable concentrations of PAHs (detection limit of 0,1 µg PAH· mL^{-1} slurry). The total organic extracts (TOE) obtained were dried over Na_2SO_4 and concentrated in

a rotary evaporator to dryness. To obtain the total petroleum hydrocarbon (TPH) fraction, TOE was resuspended in dichloromethane and cleaned up by column chromatography using the EPA 3611 method (US Environmental Agency). PAHs concentrations were analyzed by gas chromatography with flame-ionization detection (GC-FID) using a Trace 2000 gas chromatograph (Thermo Quest, Milan, Italy) fitted with a DB-5 (30 m x 25 mm i.d. x 0.25 μm film) capillary column (J&W Scientific Products Gmbh, Köln, Germany). The column temperature was held at 50 ºC for 1 min, ramped to 320 ºC at 7 ºC · min^{-1} and held for 10 min. The concentrations of the most predominant PAHs in the contaminated soil at the beginning and at the end of the slurry set-up, as well as initial and final concentration values of the previous dynamic biopile are shown in Table 2.

2.5.2. Ergosterol extraction and quantification

Fungal growth was estimated by means of ergosterol method. Soil slurries were previously homogenized with an Ultra-Turrax macerator before being extracted with n-hexane/10% KOH in methanol (1:4) according to Davis & Lamar, 1992. 7-dihidrocolesterol dissolved in dichloromethane was added to each sample as a surrogate internal standard. The samples were different from those where PAHs biodegradation was measured. Due to experimental problems, no ergosterol extraction was carried out with the treatment 6S.

Finally, samples were analyzed in an Agilent technologies 6890N gas chromatograph coupled to an Agilent technologies 5975 inert mass spectrometer. The GC chromatographs were equipped with DB5 (30 m x 25 mm i.d. x 0.25 μm film) capillary columns. The column temperature was held at 50ºC for 1 min and then increased 320ºC at 7ºC · min^{-1}. This final temperature being held for 10 min. Injector

and detector temperatures were set at 290ºC and 320ºC respectively. The samples were injected in splitless mode using helium as carrier gas, at a flow rate of 1.1mL · min^{-1}.

2.5.3. Heterotrophic and hydrocarbon-degrading microbial populations quantification

Soil bacterial counts were performed using a miniaturized most probable number (MPN) method in 96-well microtiter plates, with eight replicate wells per dilution (Wrenn & Venosa, 1996). Total heterotrophs were counted in tryptone soy broth (TSB) and aromatic hydrocarbon-degraders were counted in mineral medium BMTM (Abalos et al., 2004) containing a mixture of phenanthrene (0.5 g · L^{-1}), fluorene, anthracene, and dibenzothiophene (each at a final concentration of 0.05 g · L^{-1}). Methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate was added as a fungicide at 20 mg · kg^{-1} for avoiding fungal growth in the MPN plates.

2.5.4. DGGE molecular profiling

Samples for DNA extraction were collected both from slurry homogenized cultures, and the highest positive PAH degrading dilutions from microtiter plates and placed in sterile Eppendorff tubes stored at –20 ºC prior to analysis. Thus, a sample of 2 mL of each slurry culture was centrifuged at 14,000 x *g* for 10 minutes and the pellets were extracted by a bead beating protocol by using the Power Soil DNA extraction kit (MoBio Laboratories, Solano Beach, CA, USA), according to the manufacturer's instructions. To obtain DNA from PAH-degrading populations from microtiter plates, a composite sample of 1.6 mL containing 200µL of each replicate (n=8) belonging to the last dilution with eight positives was centrifuged and treated as described above.

Two primer sets were used to selectively amplify bacterial and fungal rDNA fragments. Universal eubacterial forward F341GC and reverse R907 primers were used to amplify the hypervariable V3–V5 region from the 16S rRNA gene, as previously reported (Yu & Morrison, 2004). The fungal first internal transcriber spacer (ITS1) from the ribosomal DNA was amplified with the primer pair ITS5 and ITS2 (White et al., 1990).The forward primer ITS5 and F341 contained the GC clamp (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGG-3'). All PCR reactions were performed on a Mastercycler personal thermocycler (Eppendorff, Hamburg, Germany). 25 microliters of PCR mixture contained 1.25 U Takara ExTaq DNA Polymerase (Takara Bio, Otsu, Shiga, Japan), 12.5 mM dNTPs, 0.25 µM of each primer and 100ngofDNA.

The obtained PCR amplicons were loaded in two 8% (w/v) polyacrylamide gels with a chemical denaturing gradient ranging from 40% to 60% (100% denaturant contained 7 M urea and 40% formamide (w/v)) and electrophoretically resolved in a DGGE-4001 equipment (CBS Scientific Company, Del Mar, CA, USA). Electrophoresis were carried out at 60ºC and at 100 V for 16 h in a 1×TAE buffer solution (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, pH 7.4).The DGGE gels were stained for 45 min in 1×TAE buffer solution containing SybrGold^{TM}(Molecular Probes, Inc., Eugene, OR, USA) and then scanned under blue light by means of a blue converter plate (UV Products Ltd, Cambridge, UK). Images of DGGE gels were digitalized and DGGE bands were processed using the Gene Tools software v. 4.0 (SynGene Synoptics) and manually corrected. Predominant DGGE bands were excised with a sterile filter tip, poured in 50 µL molecular grade water and stored at 4ºC overnight. A 1:50 dilution of the supernatants was subsequently reamplified by PCR as described previously and sequenced by using R907 and ITS2 primers, for eubacterial and fungal sequences, respectively. Sequencing was accomplished using the ABI Prism

Big Dye Terminator Cycle-Sequencing Reaction Kit v. 3.1 and an ABI 3700 DNA sequencer (both Perkin–Elmer Applied Biosystems, Waltham, MA,USA), according to the manufacturer's instructions. Sequences were edited using the BioEdit software package v. 7.0.9 (Ibis Biosciences, Carlsbad, CA, USA) and inspected for the presence of ambiguous base assignments and subjected to the Chimera check with Bellerophon version 3 (Huber et al., 2004). The sequences were then aligned with the NCBI genomic database using the BLAST search alignment tool.

The nucleotide sequences identified in this study were deposited in the GenBank database under accession numbers JQ079783-JQ079808 and JQ079809-JQ079815, for 16S and ITS sequences, respectively.

2.5.5. Quantitative PCR assay

Gene copy numbers of eubacterial 16S rRNA and fungal ITS1 rRNA fragments were quantified with the quantitative real time PCR (qPCR). Each sample was analyzed in triplicate by means of three independent DNA extracts. The analysis was carried out by using Brilliant II SYBRGreen®qPCR Master Mix (Stratagene) in qPCR equipment MX3000-P (Stratagene, La Jolla, CA) operated with the following protocol: 10 min at 95°C,followed by 40 cycles of denaturation at 95°C for 30 s, annealing for 30 s at 50ºC and 55ºC (for 16S rRNA gene and region ITS1 from rRNA, respectively), extension at 72°C for 45 s and fluorescence measurement at 80°C. The specificity of PCR amplification was determined by observations on melting curve and gel electrophoresis profile. Melting curve analysis to detect the presence of primer dimers was performed after the final extension step by increasing the temperature from 55 to 95°C with a heating rate of 0.05°C per cycle. Each reaction was performed in a 25 µL volume containing 2 µL of DNA template (approx 100 ng of DNA), 200 nM of each primer,

12.5 µL of the ready reaction mix and 30 nM of ROX reference dye. The specific primer pairs used for the eubacterial and fungal populations were 519F–907R and ITS5–ITS2 (all primers were purified by means of HPLC). The standard curves were performed with the following reference genes: 16S rRNA gene from *Desulfovibrio vulgaris* subsp. vulgaris ATCC 29579, inserted in a TOPO TA vector (Invitrogen, Belgium); and an ITS1 gene fragment obtained from a single DGGE band (GenBank accession number JN982550) cloned onto the pGEM plasmid vector using pGEM-T Easy Vector System I (Promega, Madison, WI, USA). All reference genes were quantified by Quant-iT™ PicoGreen®dsDNA Reagent using MX3000P (Stratagene, La Jolla, CA) as a detector system. Ten-fold serial dilutions of known copy numbers of the plasmid DNA in the range from 10^1 to 10^8 copies were subjected to a qPCR assay in duplicate to generate both standard curves. The qPCR efficiencies of amplification were greater than 96%; the Pearson correlation coefficients (R) of the standard curves were between 0.997 and 0.994; and the slopes were between -3,353 and -3,416 for *16S rRNA and ITS rRNA*, respectively. All results were processed by means of MxPro™ QPCR Software (Agilent Technologies, USA).

2.5.6. Statistical analysis

Multiple pairwise comparisons were performed by the Tukey test with a significance level of 0.05. Eubacterial DGGE data was also subjected to principal component analysis (PCA). For both ANOVA and PCA analyses, the Statgraphics Plus package (version 5.1; Statistical Graphics Corp., Manguistics Inc., United States) was used.

3. Results and discussion

3.1. Assessment ofHMW-PAH biodegradation capability of T. versicolor

T. versicolor was capable of degrading all the assayed 3-5 ringed-PAHs (Fig. 1), with degradation percentages varying from 50% for benzo(a)pyrene to 95% for phenantrene, while growing in liquid MEG medium for 10 days. These results confirmed the capability of this white-rot fungus to degrade PAHs and other xenobiotics in liquid medium, as has been reported previously by other research groups. For exemple, Vyas et al. (1994) described an anthracene degradation of 60% below 21 days of treatment and a depletion of PCBs was described by Ruiz-Aguilar et al. (2002). The high degradation rates of 5-ring PAHs in just 10 days of incubation are worthy of note.

3.2. PAH removal in soil slurry

Table 2 reports final HMW-PAH concentrations in all biostimulation and bioaugmentation treatments, compared to the initial soil contamination levels. The highest 4-ring PAH degradation was achieved by the native microbiota while growing in carbon limiting conditions. However, depletion of the 5-ring PAHs was only enhanced by *T. versicolor* bioaugmentation, while the biostimulation strategy failed to enhance biodegradation of these highly dangerous pollutants, independently of the carbon medium content.

After 30 days of treatment, the autochthonous microbial population was able to achieve degradation values of between 51% and 54% for fluoranthene and pyrene, and between 26% and 28% for benzo(a)anthrance and chrysene, only when incubation was performed in BMTM medium (6S), as shown in Table 2. However, there was no degradation of the 5-ring PAHs within 30 days.

It should be noted that the carbon rich MEG medium promoted lower biodegradation of the 4-ringed PAHs by the autochthonous microbial population (3S treatment, Table 1). This phenomenon was also reported in a previous study focused on

bioremediation of a mineral oil-contaminated soil (Sabaté et al., 2004), and might be promoted by utilization of glucose and other carbon sources such as malt extract, as a preferred source of carbon and energy by autochthonous degrading populations; this causes a depletion of biodegradation kinetics in the short-term throughout biostimulation experiments. The lower 4-ringed PAH biodegradation levels achieved by autochthonous microbiota with MEG media (3S) were not significantly enhanced by the fungal bioaugmentation treatment when inoculated onto the non-sterile soil (1S), although a seven-days pre-incubation was previously performed in order to obtain higher allochthonous biomass values and to reduce the glucose concentration of the medium before soil addition (Rodriguez-Rodriguez et al., 2010).

By contrast, when autoclaved soil was utilized (2S), fungal bioaugmentation could significantly promote fluoranthene and pyrene biodegradation, achieving biodegradation levels of 29% and 31%, respectively. Therefore, the 5-ringed PAH degradation was significantly better than in the other treatments, but with lower percentages (15% of degradation). These facts suggest that more incubation time would probably lead to a higher biodegradation of 5-ring PAHs in treatment 2S and that the autochthonous microbial populations, bacterial or fungal, may be producing an antagonistic effect on *T. versicolor* in treatment 1S. However, benzo*(a)*anthracene and chrysene degradation was not enhanced in comparison to treatments 1S and 3S when no active autochthonous microbial populations were present. Other studies described low percentages of degradation for HMW-PAHs for soil slurry systems where a fungal inoculation was performed(Zappi et al., 1996), and a *T. versicolor* preference has even been described for degradation of certain HMW-PAHs such as Benzo*(a)*pyrene, to the detriment of others such as chrysene during soil bioremediation (Borràs et al., 2010).

Contrarily, the capacity of *T. versicolor* to degrade a mixture of 3-5-ringed PAHs in liquid culture (Fig. 1) was not maintained when the aged creosote polluted soil was added in the slurry experiments, probably due to antagonistic effects encountered in the natural soil microbiota and limited bioavailability of PAHs due to their adsorption to mineral surfaces and organic matter of soil particles (Bosma et al., 1997).

3.3. Microbial community characterization throughout soil slurry experiments

3.3.1.Mycelial growth on biostimulation and bioaugmentation treatments

To evaluate the feasibility of *T.versicolor* in the slurry system, it is necessary to confirm the growth and biological activity of the fungus. Ergosterol concentration was monitored as a biomass indicator due to its importance in the membrane of the fungal cells (Fig. 2A). The initial ergosterol values of those treatments where *T. versicolor* was inoculated (1S, 2S and 5S) were higher than those where no bioaugmentation was performed (3S), due to the fungal seven-day pre-incubation step. During the 30 days of incubation, a slightly decrease of the *T. versicolor* ergosterol concentration was observed while growing without the polluted soil (5S). Autochthonous fungal soil microbiota (3S) suffered only a small increase during the first 15 days, but still remained at low concentrations (6.4 ± 2.2 mg $\cdot kg^{-1}$) in comparison to the other bioaugmentated treatments where concentrations were one magnitude order higher.

Moreover, in the bioaugmentation treatments (1S and 2S) there was an increase of ergosterol at 15 days of incubation that was independent of authocthonous microbial population activity. Furthermore, the ability to generate new *T. versicolor* biomass in the autoclaved soil (2S treatment) would indicate both that the polluted soil did not have a toxic effect on *T. versicolor* in the short-term, and that soil slurry agitation was not a drawback for fungal growth. In contrast, ergosterol concentration dropped in both

bioaugmentation treatments (1S and 2S) after 30 days of incubation, reaching similar ergosterol levels to those described for *T. versicolor* without soil amendment (5S), but higher than obtained without allochthonous fungal inoculation (3S).

It is noteworthy that fungal bioaugmentation in non-sterile soil suffered a greater decrease in the concentration of ergosterol after 30 days of incubation than in autoclaved soil, again suggesting certain antagonistic effects of the autochthonous microbial populations on *T. versicolor.*

3.3.2.Effect of augmentation and carbon availability on cultivable bacteria

The evolution of heterotrophic microbial populations and PAH degraders during the 30 days of incubation is illustrated in Figure 2B. The heterotrophic population from the polluted soil increased by almost 2.5 orders of magnitude during the first 15 days in those treatments where MEG medium was used (1S and 3S), whereas on BMTM only a one order of magnitude increase was observed. Nevertheless, in biostimulated soil with mineral medium (treatment 6S), the hydrocarbon-degrading microbial populations showed higher levels, reaching 10^7 MPN $\cdot$ g^{-1}, as well as the higher ratio of PAH degraders (65-81%) in respect to the total heterotrophic population. This is in accordance with the fact that the autochthonous soil microbial population growing on this mineral medium was able to achieve the higher 4-ring PAH degradation values observed among all the treatments. This is an important point to be highlighted, and is concomitant with our previous results (Viñas et al., 2005). The presence in the soil of easily assimilable substrates, such as those present in the MEG medium, might favour those fast-growing microorganisms to the detriment of the hydrocarbon degrading microbial population, which encompasses organisms with slower growth-rate. Therefore, when a mineral medium such as BMTM is utilized, the level of heterotrophic

microbial populations was lower, but was also enriched with the specialized PAH-degrading microorganisms, which are able to achieve the higher values and ratios. This is an interesting point to be taken into account for further field-scale soil bioremediation when implemented at later stages enriched in HMW-PAHs. The fact that heterotrophic populations grew similarly on MEG medium with or without fungal bioaugmentation suggested that the bacterial population grew antagonistically with the fungus according to fungal biomass analyses. Furthermore, Gramss et al. (1999) describes how heterotrophic populations can present different reactions, from growth to a major reduction in their presence in the medium, depending on the fungus used in the assays.

3.3.3. Fungal/bacterial ratio quantified by qPCR

Most studies have used this methodology to quantify specific bacterial degradative genes, but qPCR can also be used to quantify the whole microbial population in soils of interest using 16SrRNA and internal transcribed spacer (ITS) primers, for both bacterial and fungal communities respectively.

The results obtained showed that, in those treatments where *T. versicolor* was previously inoculated (1S, 2S and 5S), approximately 10^9ITS gene copies were quantified by qPCR (Fig. 3A), whereas approximately 10^6gene copies were found in those where only the native microbial community was present (3S and 6S), according to ergosterol quantification. On the other hand, the initial amount of 16S in soil was between 10^7 and 10^8 gene copies, depending on the treatment (Fig. 3B). As a consequence, the fungal/bacterial ratio (Fig. 3C) was three-fold higher in those flasks where *T. versicolor* was inoculated (1S), compared to those treatments where only the autochthonous population was present (3S and 6S).

Moreover, where native populations grew with an easily assimilable carbon source but without the *T. versicolor* bioaugmentation (3S), the number of ITS copies suffered a three-fold increase during the first 15 days of incubation, probably due to the large amount of glucose present in the medium, producing a two-fold increase of the fungal/bacterial ratio; in the presence of the white-rot fungus (1S) however, the growth of heterotrophic bacteria, combined with a slightly but statistically significant loss of ITS gene copies, produced a reduction by two orders of magnitude of the ratio; this was concomitant to that described in sections*3.2*,*3.3.1* and *3.3.2* as representing an antagonistic effect of an active bacterial autochthonous population against *T. versicolor*. This process was accentuated at 30 days of incubation (Fig. 3B).

However, during the last 15 days, the number of ITS gene copies remained stable where *T. versicolor* was not present (3S), but a one-fold increase was observed in the fungal/bacterial ratio due to the significant reduction in the 16S gene copies, returning to initial levels. The maintenance of fungal populations for long-term incubation periods could be explained on the basis that fungal populations may still survive using the reserves of glycogen produced (Rúa et al., 1993).

Very remarkable is the fact that this high ITS increment of the fungal autochthonous soil population growing in rich carbon conditions (3S), observed during the first 15 days of incubation, was not determined when membrane ergosterol was quantified, contrary to the large mycelial growth visually observed in the flasks. The results showed that is difficult to correlate fungal biomass and ergosterol content in complex matrices such as a soil. To date, very few studies have been conducted comparing these two techniques (Pilgard et al., 2009). Therefore, further research is needed in order to understand which methods, or combination of them, can more faithfully reveal the evolution of fungal biomass in real systems. In this sense, qPCR

seems to be a sensitive technique that should be further investigated in other studies. However, although qPCR avoids the bias inherent in culture-based methods, remaining uncertainties, such as amplification of the DNA of dead cells, might mask the real dynamics and need to be taken into account during data analyses (Tay et al., 2001).Also, significant differences between *T. versicolor* when growing with and without the autochthonous bacterial population (1S and 2S) were revealed by the qPCR results, while the ergosterol concentration showed a very similar evolution for both treatments during the first 15 days of incubation, with a significant increase of fungal biomass. Interestingly, higher fungal biomass levels, detected by both techniques at the end of the incubation in treatment 2S, may be related to the slightly higher PAH degradation observed when the soil had previously been sterilized.

Finally, it is important to highlight that qPCR results in treatment 6S surprisingly indicated the existence of an active native fungal population. This community, dominated by the Ascomycota phylum (Table 3), could be playing a role in the high 4-ring PAH degradation that was detected.

3.3.4.Effect of bioaugmentation and carbon availability on microbial community structure

3.3.4.1. Eubacterial community analysis

To analyze the eubacterial population initially present in the soil and its response to the different treatments, a DGGE analysis with DNA from two different origins was performed (Fig. 4). The total DNA present in the slurries was compared to the DNA obtained from the more diluted wells used to enumerate the PAH degraders, in non-sterile treatments, in order to better understand the metabolic functions of the microbial community. A total of twenty-seven bands were sequenced (Table 4).

The DGGE band B1, corresponding to the genus *Bacillus,* was very prevalent in the initial soil. However, after addition of soil to the liquid mediums, the diversity profile shifted dramatically, increasing in both MEG and BMTM, but differently according to the carbon content. The shift produced by the presence of either glucose or the presence of the white-rot fungus was different when compared to the shift produced by only activating the soil with water and mineral nutrients (6S).

Surprisingly, the *T. versicolor* inoculation and growth in the MEG medium did not noticeably change the detectable biodiversity for treatment 1S compared to the 3S treatment (Fig. 5). The same behaviour was also observed from DGGE profiles of PAH-degrading populations, where almost all the sequenced bands corresponding to the 1S treatment were shared by the DGGE profiles corresponding to 3S.

Among all genotypes (Table 3), band B5 (identical on the basis of DNA sequence to B12; B13; B17; B18; B19; B22), very similar to the *Chryseobacterium* genus, seems to be playing an important role, since it was the only band that was also found in the slurry profiles at days 15 and 30 of incubation. Chryseobacteria, belonging to the Bacteroidetes phylum, are ubiquitous bacteria that have been previously related to hydrocarbon-polluted sites (Martinez-Pascual et al., 2010). *Chryseobacterium sp.* was also found in treatment 6S, on the fifteenth day, coinciding with the better degradation rates of 4-ring PAHs achieved, suggesting that this genus can be related to the degradation of the more bioavailable fraction of the HMW-PAHs present in the soil. Nevertheless, *Pusillimonas* sp. (B10) and *Sphingobium* sp. (B11) could also be playing an important role in the degradation of 4-ring PAHs observed during the first 15 days of treatment.

It is of note that slurry set-up conditions produced a huge shift in PAH-degrading microbial populations when BMTM was used, compared to the initial soil,

probably because hydrocarbons, in addition to N and P sources, became more available to PAH degraders. However, independently of the treatment carried out, Proteobacteria phyla constituted practically all the detectable biodiversity, with α and β families the most represented in the DGGE profiles.

3.3.4.2. Fungal communityanalysis

With the aim of studying the autochthonous fungal population of the creosote polluted soil and the effect on it of bioaugmentation of the allochthonous fungus *T. versicolor*, as well as the use of different culture media during a slurry bioremediation process, a DGGE analysis of the ITS region was carried out (Fig. 6). To date, no similar studies have been found in the literature which have provided an exploration of autochthonous fungal diversity by means of a DNA-based approach in historically polluted sites throughout a bioremediation process.

With respect to the effect of the medium (MEG or BMTM) on indigenous fungal soil populations, it can be observed in the DGGE profiles that *Fusarium solani* was the only fungus found in those treatments where MEG or BMTM medium was used, in spite of the presence of *T. versicolor*. On the other hand, other sequences closely related to fungal genera such as *Peziza* sp. and *Chromelosporium* sp. harboured in creosote polluted soils were not detected from the DGGE profiles at the expense of *F. solani* and *T. versicolor* during the slurry incubation. In contrast, *F. solani* was not one of the most important fungi in the initial soil. This fact would suggest that the MEG medium probably promotes the growth of *F. solani* versus microorganisms that are more acclimated to the initial polluted soil conditions, probably because of faster growth kinetics (Wu et al., 2010). Moreover, *Fusarium spp*.were also detected after 30 days of

biostimulation with BMTM as mineral media, which could indicate that this genus is playing a role throughout the biodegradation process in the polluted soil.

The ability of *Fusarium spp*. to degrade HMW-PAHs has been demonstrated elsewhere (Chulalaksananukul et al., 2006). Furthermore, Wu et al., 2010 described how the metabolic pathways of non-white-rot fungi are in part similar to those of white-rot fungi, and also that laccase is involved in the transformation of PAHs. In the present study, the presence of *F. solani* in treatments 1S and 3S cannot be related to an improvement in HMW-PAH degradation. Further research is needed in order to explain this fact.

Finally, it is important to point out that fungal diversity seemed to be higher on treatment 6S after 30 days of incubation, probably due to the lack of glucose and *T. versicolor* in the BMTM medium. Unfortunately, no DNA signal was found on the lane corresponding to the fifteenth day of incubation, where the presence of certain fungal species could be associated with higher biodegradation rates of fluoranthene and pyrene. However, four bands were sequenced after 30 days, all belonging to the Ascomycota phylum.

The presence of two fungal species previously related to HMW-PAH degradation, *F. solani* and *F. oxysporum* (Silvaa et al., 2009), as well as bacterial community changes during this incubation period, suggests that low PAH concentrations, at the end of the incubation period, and its strong adsorption to the clay soil particles might enhance the lack of HMW-PAH bioavailability.

4. Conclusions

A slurry incubation strategy was a feasible assay in order to enhance our knowledge of fungal-bacterial interactions in real historically polluted soils.

The native microbial populations in soil were able to degrade 4-ring PAHs at high rates, but only under carbon limiting conditions. MPN-DGGE compared to soil DGGE allows the postulation of PAH-degrading members.

The microbial genera *Chryseobacterium, Pusillimonas, Sphingobium* and *Fusarium* could be playing an important role in HMW-PAH depletion.

T. versicolor bioaugmentation was not able to improve autochthonous HMW-HAP degrading capabilities in non-sterile slurries, due to an antagonistic effect of the autochthonous populations.

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References

Abalos, A., Viñas, M., Sabaté, J., Manresa, M.A., Solanas, A.M., 2004. Enhanced biodegradation of Casablanca crude oil by a microbial consortium in presence of a rhamnolipid produced by *Pseudomonas aeruginosa* AT10. Biodegradation15(4), 249-260.

Borràs, E., Caminal, G., Sarrà, M., Novotný, C., 2010. Effect of soil bacteria on the ability of polycyclic aromatic hydrocarbons (PAHs) removal by *Trametes versicolor* and *Irpex lacteus* from contaminated soil. Soil Biol.Biochem.42(12), 2087-2093.

Bosma, T.N.P., Middeldorp, P.J.M., Schraa, G., Zehnder, A.J.B., 1997. Mass transfer limitations of biotransformation: quantifying bioavailability. Environ.Sci.Technol.31, 248–252.

Chaîneau, C.H., Yepremian, C., Vidalie, J.F., Ducreux, J., Ballerini, D., 2003. Bioremediation of a crude oil-polluted soil: biodegradation, leaching and toxicity assessments. Water AirSoil Poll.144(1-4), 419-440.

Chung, N., Alexander, M., 1999. Effect of concentration on sequestration and bioavailability of two polycyclic aromatic hydrocarbons. Environ.Sci.Technol. 33(20), 3605–3608.

Collins, P.J., Kotterman, M., Field, J.A., Dobson, A., 1996. Oxidation of anthracene and benzo[a]pyrene by laccases from *Trametes versicolor*. Appl. Environ. Microbiol.62(12), 4563-4567.

Chulalaksananukul, S., Gadd, G.M., Sangvanich, P., Sihanonth, P., Piapukiew, J., Vangnai, A.S., 2006. Biodegradation of benzo(a)pyrene by a newly isolated *Fusarium* sp. FEMS Microbiol. Lett.262(1), 99-106.

Davis, M.W., Lamar, R.T., 1992. Evaluation of methods to extract ergosterol for quantitation of soil fungal biomass. Soil Biol. Biochem. 24(3), 189-198.

Gramss, G., Voigt, K.D., Kirsche, B., 1999. Degradation of polycyclic aromatic hydrocarbons with three to seven aromatic rings by higher fungi in sterile and unsterile soils. Biodegradation10(1), 51-62.

Hareland, W.A., Crawford, R.L., Chapman, P.J., Dagley, S., 1975. Metabolic function and properties of 4-hydroxyphenylacetic acid 1-hydroxylase from *Pseudomonas acidovorans*. J. Bacteriol.121(1), 272-285.

Harms, H., Schlosser, D., Wick, L.Y., 2011. Untapped potential: exploiting fungi in bioremediation of hazardous chemicals. Nat. Rev. Microbiol.9(3), 177-192.

Huber, T., Faulkner, G., Hugenholtz, P., 2004. Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. Bioinformatics.20, 2317-2319.

Juhasz, A.L., Naidu R., 2000. Bioremediation of high molecular weight polycyclic aromatic hydrocarbons: a review of the microbial degradation of benzo[a]pyrene. Int. Biodeter. Biodegr.45(1-2), 57-88.

Llado, S., Jimenez, N., Vinas, M., Solanas, A.M., 2009. Microbial populations related to PAH biodegradation in an aged biostimulated creosote-contaminated soil. Biodegradation.20(5), 593-601.

Martinez-Pascual, E., Jimenez, N., Vidal-Gavilan, G., Vinas, M., Solanas, A.M., 2010. Chemical and microbial community analysis during aerobic biostimulation assays of non-sulfonated alkyl-benzene-contaminated groundwater. Appl. Microbiol. Biotechnol.88(4), 985-995.

Novotny, C., Erbanova, P., Cajthaml, T., Rothschild, N., Dosoretz, C., Sasek, V., 2000. *Irpex lacteus*, a white rot fungus applicable to water and soil bioremediation. Appl. Microbiol. Biotechnol.54(6), 850-853.

Pilgard, A., Alfredsen, G., Børja. I., Björdal, C., 2009. Durability and fungal colonisation patterns in wood samples after six years in soil contact evaluated with qPCR, microscopy, TGA, chitin- and ergosterol assays, in: The International Research Group on Wood Protection, 40th annual meeting.IRG/WP 09- 20402. Beijing, China.

Realp, E., Domenech, J.A., Martinez-García, R., Restrepo, C., Lladó, S., Viñas, M., Solanas, A.M., 2008. Ensayo piloto de biorremediación por la tecnología de la biopila dinámica para la descontaminación de suelos contaminados por creosotas provenientes de las actividades dedicadas a la preparación de la madera. Residuos: Revista Técnica102, 38-49.

Rodriguez-Rodriguez, C.E., Marco-Urrea, E., Caminal, G., 2010. Degradation of naproxen and carbamazepine in spiked sludge by slurry and solid-phase Trametes versicolor systems. Bioresour. Technol.101(7), 2259-2266.

Rúa, J., Busto, F., Arriaga, D.D., Soler, J., 1993. Glycogen in *Phycomyces blakesleeanus*: influence of growth conditions and nutrient limitation. Microbiology139(2), 217-222.

Ruiz-Aguilar, G.M.L., Fernandez-Sanchez, J.M., Rodriguez-Vazquez, R., Poggi-Varaldo, H., 2002. Degradation by white-rot fungi of high concentrations of PCB extracted from a contaminated soil. Adv. Environ. Res.6, 559-568.

Sabaté, J., Viñas, M., Solanas, A.M., 2004. Laboratory-scale bioremediation experiments on hydrocarbon-contaminated soils. Int. Biodeter. Biodegr. 54, 19-25.

Sabaté, J., Viñas, M., Solanas, A.M., 2006. Bioavailability assessment and environmental fate of polycyclic aromatic hydrocarbons in biostimulated creosote-contaminated soil. Chemosphere.63(10), 1648-1659.

Silvaa, I.S., Grossmanb, M., Durranta, L.R., 2009. Degradation of polycyclic aromatic hydrocarbons (2–7 rings) under microaerobic and very-low-oxygen conditions by soil fungi. Int. Biodeter. Biodegr.63(2), 224-229.

Singh, S.N., Tripathi, R.D., 2007. Environmental bioremediation technologies. Springer, New York.

Tay, S.T., Hemond, F.H., Krumholz, L.R., Cavanaugh, C.M., Polz, M.F., 2001. Population dynamics of two toluene degrading bacterial species in a contaminated stream. Microb. Ecol.41(2), 124-131.

Viñas, M., Sabate, J., Espuny, M.J., Solanas, A.M., 2005. Bacterial community dynamics and polycyclic aromatic hydrocarbon degradation during bioremediation of heavily creosote-contaminated soil. Appl. Environ. Microbiol.71(11), 7008-7018.

Vyas, B.R.M., Bakowski, S., Šašek, V., Matucha, M., 1994. Degradation of anthracene by selected white rot fungi. FEMS Microbiol.Lett.14, 65-70.

Wang, Q., Garrity, G.M., Tiedje, J.M., Cole, J.R., 2007. Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl. Environ. Microbiol.73(16),5261-5267.

White, T.J., Bruns, T.D., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, in: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J.(Eds.), PCR Protocols: A Guide to Methods and Applications. Academic Press, New York, pp. 315-322.

Wrenn, B.A., Venosa, A.D., 1996. Selective enumeration of aromatic and aliphatic hydrocarbon degrading bacteria by a most-probable-number procedure. Can. J. Microbiol.42(3), 252-258.

Wu, Y.R., Luo, Z.H., Vrijmoed, L.L., 2010. Biodegradation of anthracene and benz[a]anthracene by two *Fusarium solani* strains isolated from mangrove sediments. Bioresour. Technol.101(24), 9666-9672.

Yergeau, E., Arbour, M., Brousseau, R., Juck, D., Lawrence, J.R., Masson, L., Whyte, L.G., Greer, C.W., 2009. Microarray and real-time PCR analyses of the responses of high-arctic soil bacteria to hydrocarbon pollution and bioremediation treatments. Appl. Environ. Microbiol.75(19), 6258-6267.

Yu, Z., Morrison, M., 2004. Comparisons of different hypervariable regions of RSS genes for use in fingerprinting of microbial communities by PCR-denaturing gradient gel electrophoresis. Appl. Environ. Microbiol.70, 4800-4806.

Zappi, M.E., Rogers, B.A., Teeter, C.L., Gunninson, D., Bajpai, R., 1996. Bioslurry treatment of a soil contaminated with low concentrations of total petroleum hydrocarbons. J. Hazard. Mater. 46, 1-12.

Figure Captions

Fig. 1. PAH concentrations in synthetic PAH biodegradation experiments with *T. versicolor* in MEG medium. 0 days (black); 10 days abiotic control (grey); 10 days (dark grey).Same lowercase letters indicate lack of statistically significant difference within treatments for each PAH compound ($P \geq 0.05$). Values represent the means of three independent experiments and error bars indicate the standard deviations.

Fig. 2. (A) Ergosterol concentration at 0 days (black); 15 days (grey); 30 days (dark grey). **(B)** Heterotrophic (black) and PAH-degrading population (grey) in soil slurry treatments over the course of 30 days of incubation.Same lowercase letters indicate lack of statistically significant difference within the same treatment at different incubation times ($P \geq 0.05$). Same uppercase letters indicate lack of statistically significant difference between treatments at same incubation time ($P \geq 0.05$).The percentage (%) of the heterotrophic population that was represented by the PAH-degrading population is also indicated (B). Values represent the means of three independent experiments and error bars indicate the standard deviations.

Fig. 3.16SrRNA gene copies quantified by qPCR **(A)**, ITS region gene copies quantified by qPCR **(B),** and fungi/bacteria ratio between ITS and 16S gene copies **(C)** in soil slurry treatments over the course of 30 days of incubation.0 days (black); 15 days (grey); 30 days (dark grey).Same lowercase letters indicate lack of statistically significant difference within the same treatment at different incubation times ($P \geq 0.05$). Same uppercase letters indicate lack of statistically significant difference between treatments at same incubation time ($P \geq 0.05$). Values represent the means of three independent experiments and error bars indicate the standard deviations.

Fig. 4. Denaturing Gradient Gel Electrophoresis profiles of PCR-amplified *16SrRNA* gene fragments (V3-V5 regions) of slurry communities and MPN plates. Numbers are disposed at the left side of the corresponding band.

Fig. 5. Principal component analysis of Denaturing Gradient Gel Electrophoresis profiles (see Fig. 4).

Fig. 6. Denaturing Gradient Gel Electrophoresis profiles of PCR-amplified ITS region fragments (ITS1) of slurry communities. Numbers are disposed at the left side of the corresponding band.

Table 1: Description of the soil treatment strategies performed.

Code	Treatment	Description
1S	MEG + Soil + Fungal bioaugmentation	5g of soil[a] in 20 mL of malt extract glucose medium with a seven-day pre-grown mycelium of *T. versicolor*, inoculated at 5% (*v/v).*
2S	MEG + Autoclaved Soil + Fungal bioaugmentation	5g of autoclaved soil[b] in 20 mL of malt extract glucose medium with a seven-day pre-grown mycelium of *T. versicolor*, inoculated at 5% *(v/v).*
3S	MEG + Soil	5g of soil[a] in 20 mL of malt extract glucose medium.
4S	MEG + Autoclaved Soil	5g of autoclaved soil[b] in 20 mL of malt extract glucose medium.
5S	MEG + Fungal bioaugmentation	20 mL of malt extract glucose medium with a seven-day pre-grown mycelium of *T. versicolor*, inoculated at 5% *(v/v).*
6S	BMTM + Soil	5g of soil[a] in 20 mL of mineral medium BMTM

[a]Soil was previously ground in order to avoid very large particles that could damage the mycelium.
[b]Soil was previously ground and then autoclaved three times at 121ºC for 21min on consecutive days in an attempt to eliminate the growth of sporulated microorganisms.

Table 2: PAH residual concentration of treated soil.

Compound	Initial biopile (mg · kg^{-1} of soil)	Final biopile (mg · kg^{-1} of soil)	Slurry 0 days (mg · kg^{-1} of soil)	1S (30d) (mg · kg^{-1} of soil)	2S (30d) (mg · kg^{-1} of soil)	3S (30d) (mg · kg^{-1} of soil)	4S (30d) (mg · kg^{-1} of soil)	6S (30d) (mg · kg^{-1} of soil)
Phenanthrene	814±62	n.d.[a]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Anthracene	183±13	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Fluoranthene	562±39	133±12	70.3±1.0	58.4±2.9C[c]	50.4±3.1B	60.7±3.4C	69.9±1.2D	33.2±0.7A
Pyrene	343±22	134±7	75.7±1.6	58.2±3.2C	52.3±2.2B	60.0±4.6C	77.2±3.6D	36.7±1.8A
Benzo(*a*)anthracene	106±3	47±3	32.5±0.2	33.0±3.2B	30.8±2.0B	35.8±1.7B	34.2±0.8B	24.0±2.9A
Chrysene	117±3	61±27	48.8±0.9	51.6±0.9B	46.3±2.4B	51.2±0.3B	51.6±2.8B	35.3±4.8A
Benzo(*b*+*k*)fluoranthene	105±6	94±13	72.7±2.5	65.9±2.7B	60.9±0.6A	67.6±0.4B	71.5±1.7C	70.2±1.1C
Benzo(*a*)pyrene	40±1	31±2	30.2±0.2	29.6±0.2B	25.6±1.2A	29.6±0.1B	29.8±1.1B	29.1±1.2B
Total PAHs[b]	**2270**	**500**	**330**	**296**	**266**	**305**	**334**	**228**

[a]Not detected.
[b]Total PAHs determined from the total target PAHs analysed.
[c]Data are the mean ± standard deviation of three independent experiments. Statistical pairwise multiple comparisons of homogenous data was carried out by the Tukey test: column means followed by the same uppercase letters were not significantly different ($P \geq 0.05$).

Table 3: Properties of ITS DGGE bands: designations and accession numbers for the band sequences and levels of similarity to related organisms.

Band	1S	3S	5S	6S	Length (bp)	Closest organism in GenBank database (accession no.)	% similarity[a]	Phylogenetic group
ITS B1[b]	x		x		208	*Trametes versicolor*FP1022316sp (JN164984)	100%	*Polyporaceae*(Basidiomycota)
ITS B2		x			201	*Peziza pseudoviolacea* 16504 (JF908564)	97%	*Pezizaceae*(Ascomycota)
ITS B3		x			164	*Chromelosporium* sp. CID601 (EF89890)	96%	*Pezizaceae*(Ascomycota)
ITS B6[c]	x	x		x	171	*Fusarium solani* isolate 177 (JN232143)	100%	*Nectriaceae*(Ascomycota)
ITS B39				x	152	*Scedosporium prolificans* strain 776497 (GU594770)	90%	*Microascaceae* (Ascomycota)
ITS B41				x	161	*Fusarium oxysporum* isolate 1 (JN558555)	93%	*Nectriaceae*(Ascomycota)
ITS B42				x	161	*Cosmopora* sp. strain GJS96186 (JN995635)	100%	*Nectriaceae*(Ascomycota)

[a]Sequences were aligned against the GenBank database with the BLAST search alignment tool.
[b]Band ITS: B1=B4=B9=B16=B17=B18=B19=B20=B26=B37
[c]Band ITS: B5=B6=B7=B8=B10=B11=B12=B13=B14=B15=B21=B22=B23=B24=B25=B27=B28=B29=B30=B31=B32=B33=B34=B35=B36=B40

Table 4: Properties of 16S DGGE bands: designations and accession numbers for the band sequences and levels of similarity to related organisms

Band	Band detection[a]								Length (bp)	Closest organism in GenBank database (accession no.)	% similarity[b]	Phylogenetic group[c]
	0d	0dMPN	S1	S1MPN	S3	S3MPN	S6	S6MPN				
16S B1	X	-	-	-	-	-	-	-	505	*Bacillus niacin* strain Y2S4 (EU221374)	99%	*Bacillaceae (*Firmicutes)
16S B2	-	X	-	-	-	-	-	-	400	*Bordetella sp.* (EU082149)	100%	*Burkholderiaceae* (β)
16S B3	-	X	-	-	-	-	-	-	389	*Rhizobium sp.* NJUST18 (JN106368)	99%	*Rhizobiaceae* (α)
16S B4	-	-	-	X	-	-	-	-	419	*Sphingomonas sp.* FI301 (GQ829498)	100%	*Sphingomonadaceae* (α)
16S B5[d]	-	-	x	x	x	x	-	-	439	*Chryseobacterium sp.* LKL10 (HQ331141)	100%	*Flavobacteriaceae* (CFB group)
16S B6	-	-	-	-	x	-	-	-	381	*Enterobacter pulveris* strain E443 (EF614996)	95%	*Enterobacteriaceae* (γ)
16S B8	-	-	-	-	-	X	-	-	445	*Herbaspirillum sp.* Os45 (HQ728575)	93%	*Oxalobacteriaceae* (β)
16S B9	-	-	-	-	-	x	-	-	441	*Sphingomonas sp.* FO416 (GQ849286)	98%	*Sphingomonadaceae* (α)
16S B10	-	-	-	-	-	-	-	X	483	*Pusillimonas sp.* PB3-7B (FJ948170)	100%	*Alcaligenaceae* (β)
16S B11	-	-	-	-	-	-	-	X	422	*Sphingobium sp.* F2 (EF534725)	99%	*Sphingomonadaceae* (α)
16S B14	-	-	-	X	-	-	-	-	477	*Achromobacter sp.* HPABA02 (HQ257212)	98%	*Alcaligenaceae* (β)
16S B15	-	-	-	X	-	-	-	-	450	*Sphingomonas sp.* MPSS (GQ214027)	98%	*Sphingomonadaceae* (α)
16S B16	-	-	-	X	-	x	-	x	449	Uncultured bacterium clone EBL50 (GU591539)	97%	-
16S B20	-	-	-	-	X	-	-	-	250	*Enterobacter sp.* DG10 (JN208201)	94%	*Enterobacteriaceae* (γ)
16S B21	-	-	-	-	X	-	-	-	437	Uncultured bacterium DGGE band B4 (JF729189)	100%	-
16S B23	-	-	-	-	-	X	-	-	400	*Achromobacter xylosoxidans* strain E1 (JN590249)	100%	*Alcaligenaceae* (β)
16S B24	-	-	-	-	-	X	-	-	446	*Novosphingobium sp.* MN38 (JN082747)	99%	*Sphingomonadaceae* (α)
16S B25	-	-	-	-	-	X	-	-	405	*Azospirillum oryzae* strain KNUC9025 (JF505959)	99%	*Rhodospirillaceae* (α)

[a]Band detection (+) above 1% of relative intensity.
[b]Sequences were aligned against the GenBank database with the BLAST search alignment tool.
[c]Phylogenetic groups were defined by using the Ribosomal Data Project (RDP) Naive Bayesian Classifier (Wang et al., 2007). Family is represented.α, β, γ represent α-proteobacteria, β-proteobacteriaand γ-proteobacteria, respectively.
[d]Band16S: B5=B7=B12=B13=B17=B18=B19=B22; B16=B26=B27.

Figure 1

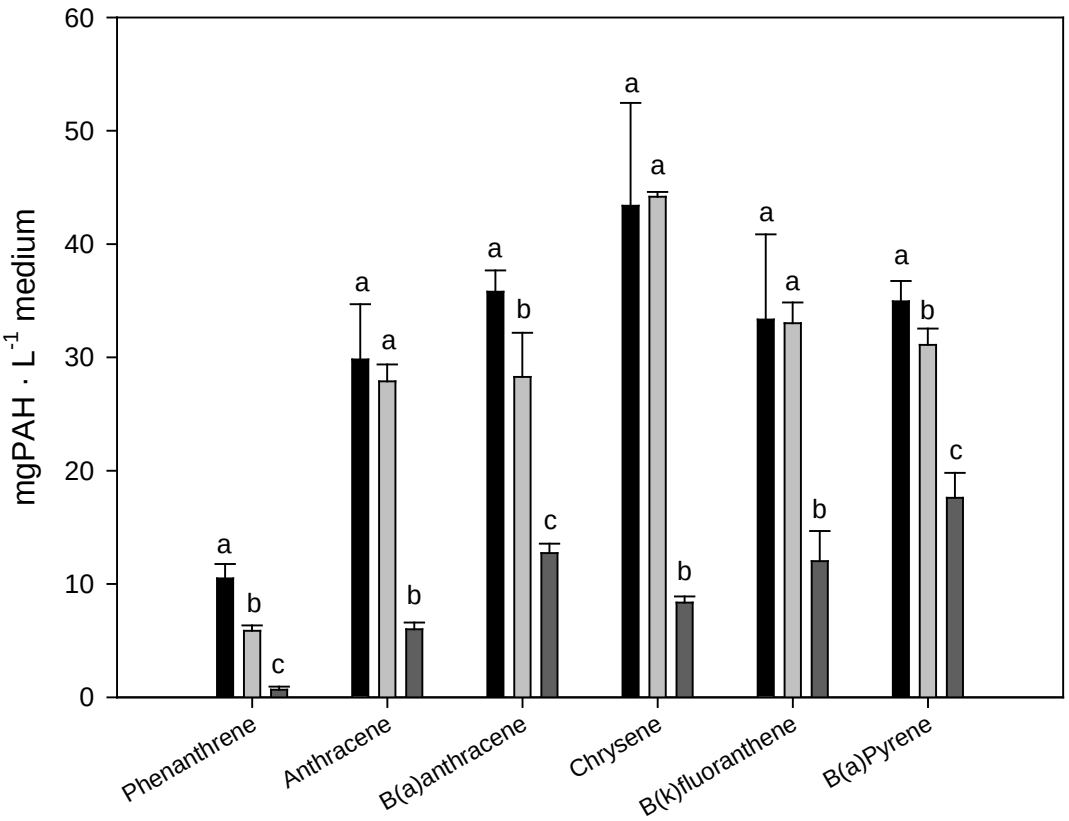

60
50
40
30
20
10
0
mgPAH · L-1 medium
a
b
c
a
a
b
a
a
b
c
a
a
b
a
a
b
a
b
c
Phenanthrene
Anthracene
B(a)anthracene
Chrysene
B(k)fluoranthene
B(a)Pyrene

Figure 2

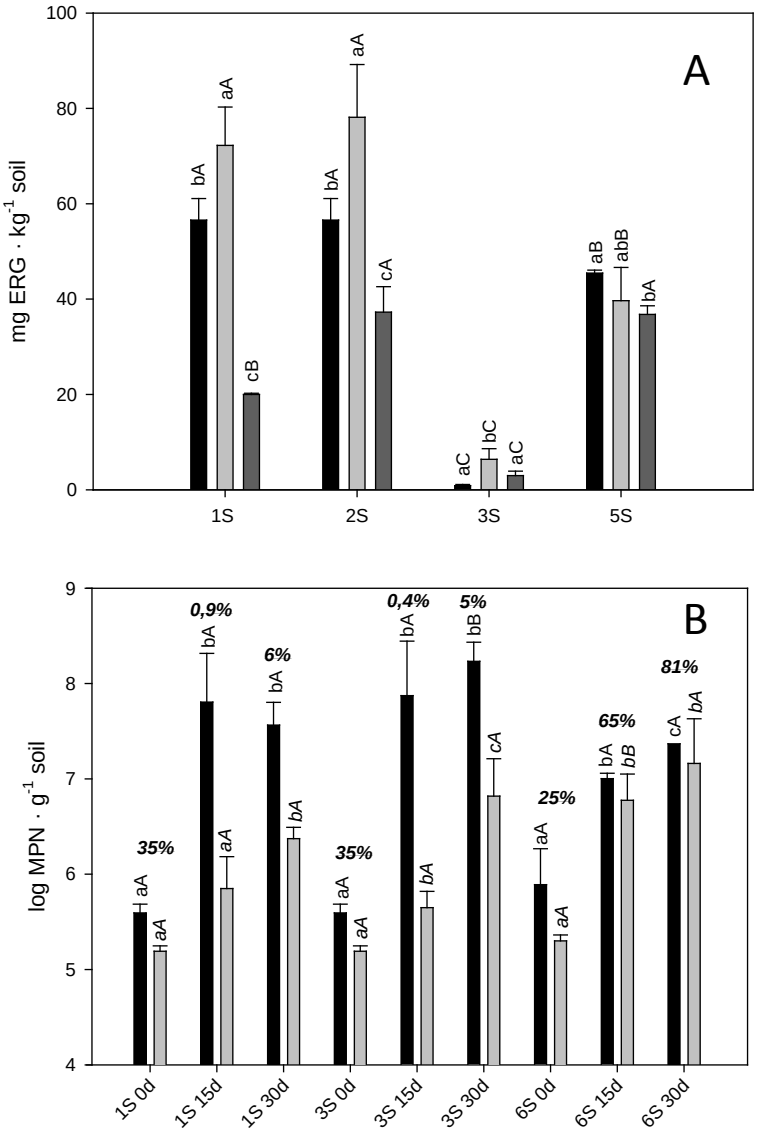
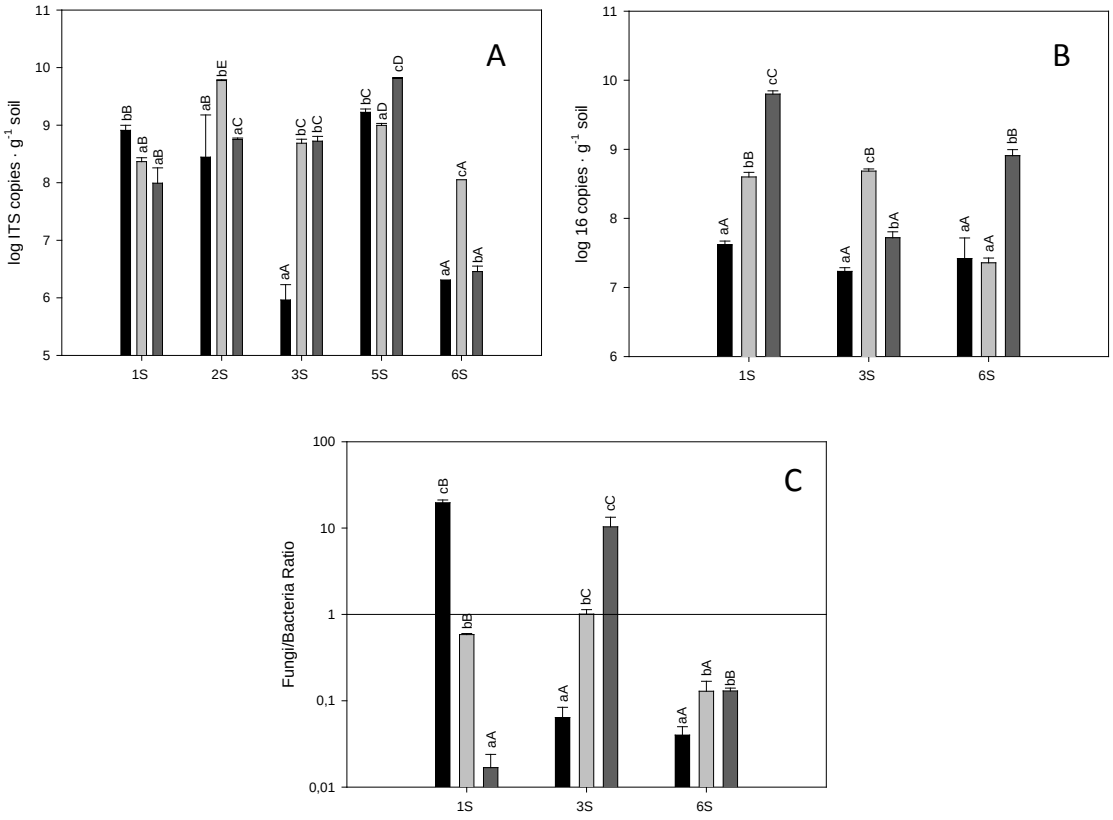
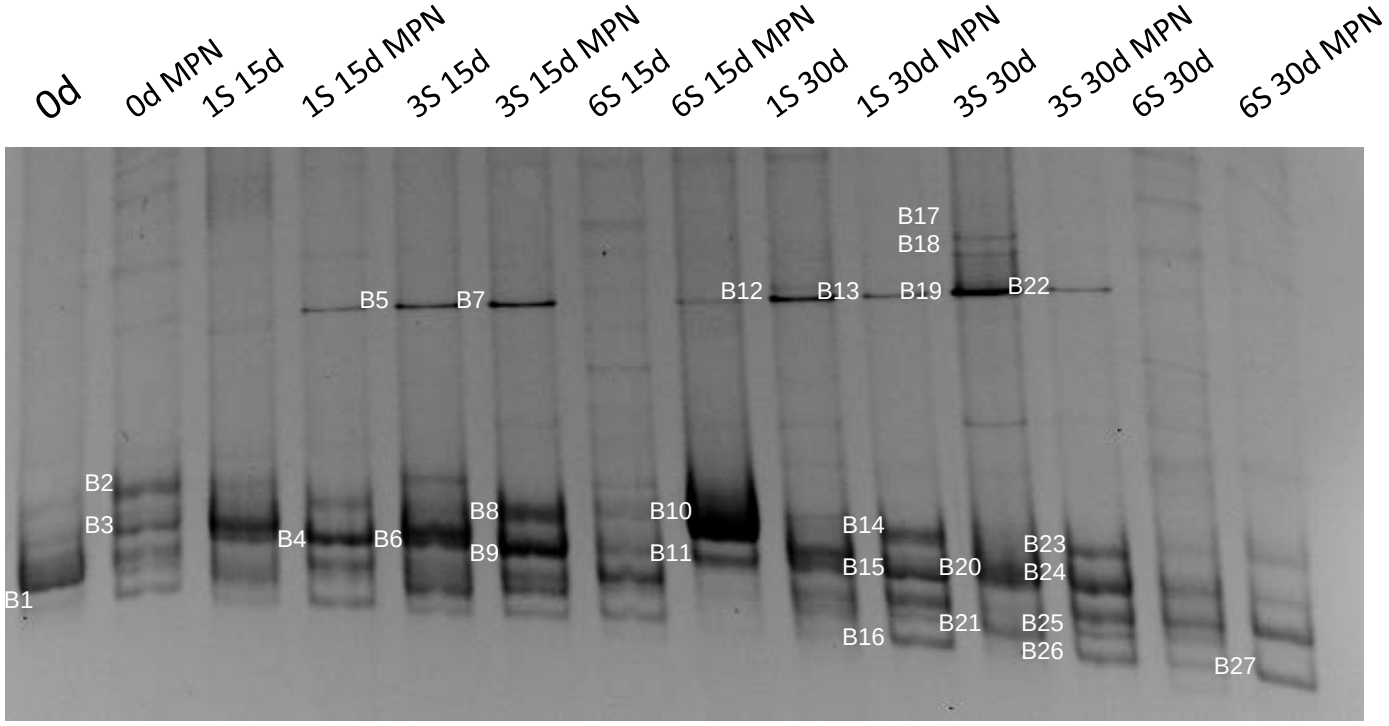

A
mg ERG · kg-1 soil
100
80
60
40
20
0
bA
aA
cB
bA
aA
cA
aC
bC
aC
aB
abB
bA
1S
2S
3S
5S
B
log MPN · g-1 soil
9
8
7
6
5
4
35%
0,9%
6%
35%
0,4%
5%
25%
65%
81%
1S 0d
1S 15d
1S 30d
3S 0d
3S 15d
3S 30d
6S 0d
6S 15d
6S 30d

Figure 3


A
log ITS copies · g-1 soil
11
10
9
8
7
6
5
bB
aB
aB
aB
bE
aC
aA
bC
bC
bC
aD
cD
aA
cA
bA
1S
2S
3S
5S
6S
B
log 16 copies · g-1 soil
11
10
9
8
7
6
aA
bB
cC
aA
cB
bA
aA
aA
bB
1S
3S
6S
C
Fungi/Bacteria Ratio
100
10
1
0,1
0,01
cB
bB
aA
aA
bC
cC
aA
bA
bB
1S
3S
6S

.

Figure 4


0d
0d MPN
1S 15d
1S 15d MPN
3S 15d
3S 15d MPN
6S 15d
6S 15d MPN
1S 30d
1S 30d MPN
3S 30d
3S 30d MPN
6S 30d
6S 30d MPN
B1
B2
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B4
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B21
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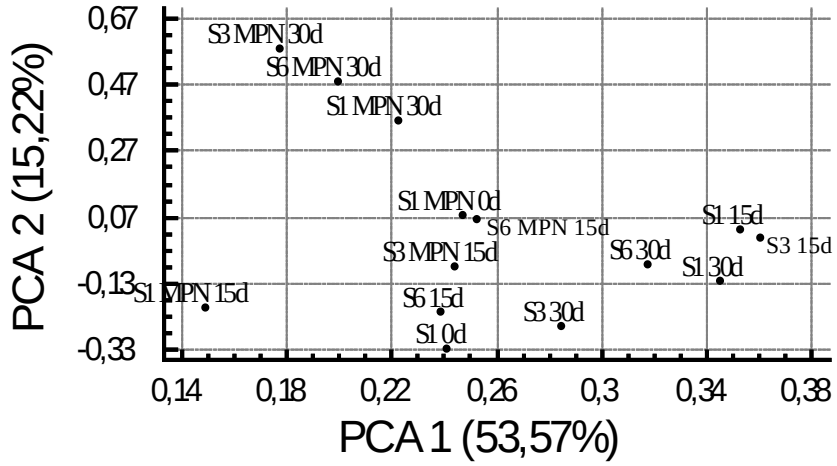
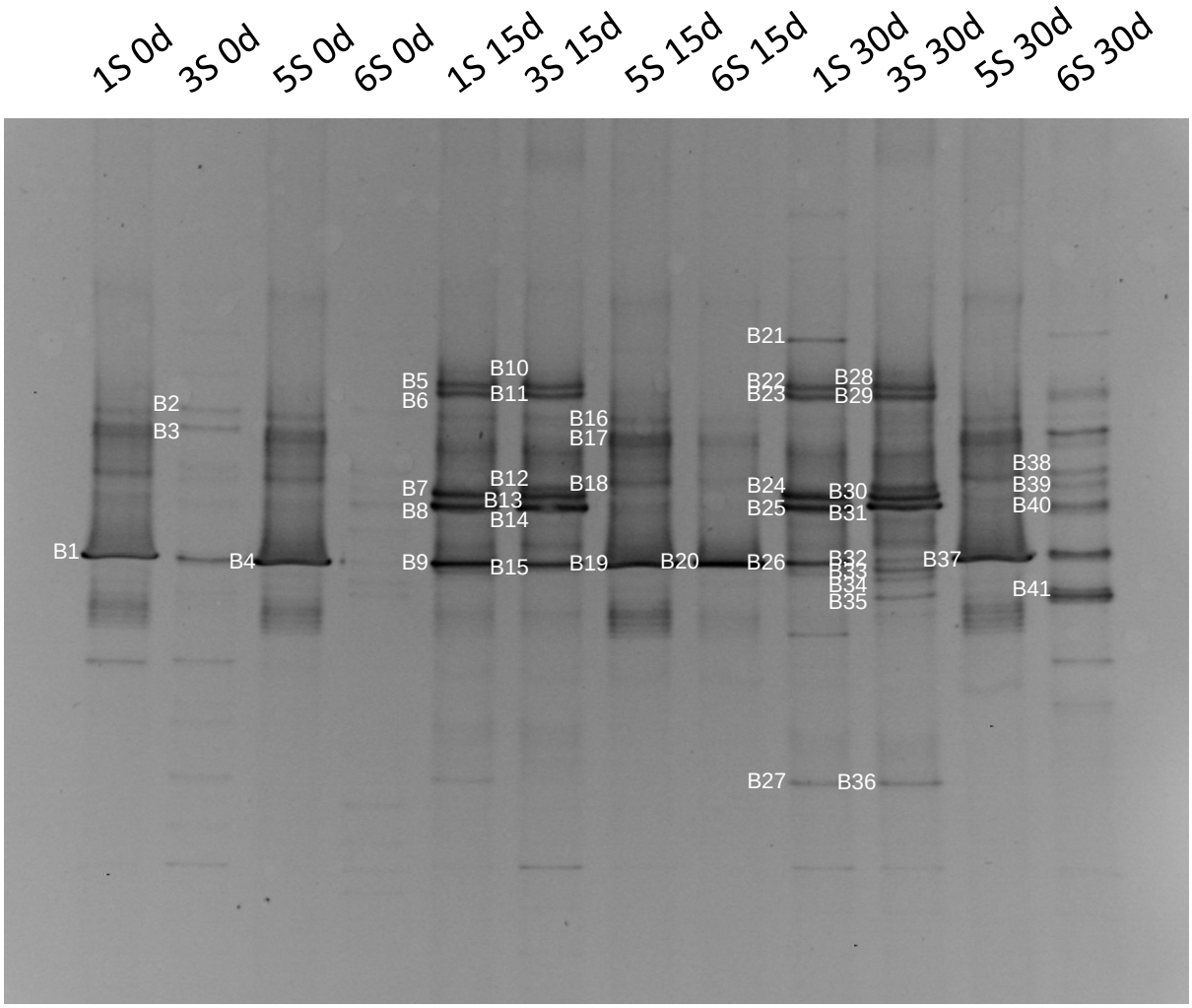
B23
B24
B25
B26
B27

Figure 5


0,67
0,47
0,27
0,07
-0,13
-0,33
PCA 2 (15,22%)
S3 MPN 30d
S6 MPN 30d
S1 MPN 30d
S1 MPN 0d
S6 MPN 15d
S1 15d
S3 15d
S3 MPN 15d
S6 30d
S1 30d
S1 MPN 15d
S6 15d
S3 30d
S1 0d
0,14
0,18
0,22
0,26
0,3
0,34
0,38
PCA 1 (53,57%)

.

Figure 6


1S 0d
3S 0d
5S 0d
6S 0d
1S 15d
3S 15d
5S 15d
6S 15d
1S 30d
3S 30d
5S 30d
6S 30d
B1
B2
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CAPÍTULO 5 / CHAPTER 5

Comparative assessment of bioremediation approaches to highly recalcitrant PAH degradation in a real industrial polluted soil

Comparative assessment of bioremediation approaches to highly recalcitrant PAH degradation in a real industrial polluted soil

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La elevada recalcitrancia, así como una baja disponibilidad debida a procesos de envejecimiento, pueden dificultar la bioremediaición de hidrocarburos aromáticos policíclicos de elevado peso molecular (HMW-PAHs) en suelos industriales contaminados.

Con el objetivo de reducir la fracción más recalcitrante de hidrocarburos totales del petróleo (TPH) y HMW-PAHs (2815 and 389 mg · kg^{-1}, respectivamente), remanentes en un suelo contaminado por creosota donde previamente se había realizado una biopila durante 180 días, diferentes tratamientos de bioestimulación (BS) de las poblaciones microbianas autóctonas con un sustrato ligninolítico (LS) o de bioaumento, con dos cepas diferentes de hongos de podredumbre blancs (WRF) (*i.e., Trametes versicolor* y *Lentinus tigrinus)*, fueron comparativamente ensayados, a escala de laboratorio. El impacto de iones manganeso y dos agentes movilizadores (MAs) (*i.e.*, aceite de soja y Brij 30) sobre la capacidad degradativa de los microcosmos biostimulados y biaumentados fue también comparada.

Después de 60 días, los mejores resultados de biodegradación (del 55% al 73% de degradación de TPH) se observaron cuando el suelo contaminado fue bioestimulado con LS, bajo condiciones de humedad y temperatura favorables al desarrollo de las poblaciones fúngicas autóctonas, mientras que la colonización del suelo por parte de las dos cepas de WRF fue claramente limitada por el gran crecimiento de las poblaciones autóctonas, tanto fúngicas como bacterianas.

Por otra parte, el efecto de añadir agentes movilizadores (MAs) fue despreciable en términos de degradación, excepto cuando el tratamiento BS-LS se combinó con la adición de Mn^{2+}. De hecho, el surfactante no iónico Brij 30 resultó ser tóxico para las

bacterias degradadoras de HMW-PAHs, teniendo como consecuencia una menor degradación de hidrocarburos de 4 anillos aromáticos.

Por lo tanto, el favorecimiento de las poblaciones fúngicas autóctonas, en suelos históricamente contaminados, mediante adición de algún sustrato ligninolítico puede promover la biodegradación de HMW-PAHs, incluso aquellos de 5 anillos aromáticos.

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Abstract

High recalcitrant characteristics and low bioavailability rates due to aging processes can hinder high molecular weight polycyclic aromatic hydrocarbons (HMW-PAHs) bioremediation in real industrial polluted soils.

With the aim of reducing the recalcitrant fraction of total petroleum hydrocarbons (TPH) and HMW-PAHs in creosote-contaminated soil (2815 and 389 mg $\cdot$ kg^{-1}, respectively) remaining after a 180-d treatment in a pilot-scale biopile, either biostimulation (BS) of indigenous microbial populations with a lignocellulosic substrate (LS) or fungal bioaugmentation with two strains of white-rot fungi (WRF) (*i.e.*, *Trametes versicolor* and *Lentinus tigrinus*) were comparatively tested. The impact of bivalent manganese ions and two mobilizing agents (MAs) (*i.e.*, Soybean Oil and Brij 30) on the degradation performances of biostimulated and bioaugmented microcosms was also compared.

After 60 d, the best biodegradation results (from 55% to 73% of TPH depletion) were achieved when the contaminated soil was biostimulated with LS under moisture and temperature conditions favoring development of indigenous mycobiota, whereas soil colonization by both WRF strains was clearly hampered by the outstanding autochthonous fungal and bacterial growth. Moreover, the effect of MAs supplementation was negligible in terms of biodegradation outcomes except when BS-LS treatment was concomitant with Mn^{2+} addition. Indeed, the nonionic surfactant Brij 30 was found to be toxic for the PAH-degrading bacteria, resulting in a decrease in 4-ring PAHs degradation. Therefore, a proper enhancement of fungal autochthonous populations in historically industrial polluted soils by means of LS amendment may promote the biodegradation of HMW-PAHs, even of those with five aromatic rings.

Keywords: Soil bioremediation, *Trametes versicolor*, *Lentinus tigrinus* bioaugmentation, mycoremediation, creosote, HMW-PAH, Lignin-modifying enzymes (LME)

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are pollutants of great concern, owing to their ubiquitous occurrence in the environment, recalcitrance to degradation, bioaccumulation potential and carcinogenic activity (Haritash and Kaushik, 2009). PAHs are produced during the incomplete combustion of organic matter and are present in petroleum-derived fuels and wood preservatives, such as creosote (Cerniglia, 1992). Creosote is a technical wood preservative preparation mainly composed of a mixture of PAHs (approx. 85%) (Rogers et al., 2007) and it has been extensively used worldwide by the wood industry, thus resulting in the pollution of areas proximal to sites of creosote manufacture and use (Mueller et al., 1989).

Bioremediation offers an environmentally friendly and cost-competitive approach for the clean-up of contaminated sites, although natural biodegradation may be slower than other technologies (Alexander, 1999). Unfortunately, on aged historically polluted soils, bioremediation may be strongly constrained by many limiting factors; among them, bioavailability of pollutants and degradation competence of the indigenous microbiota are of primary importance (Boopathy, 2002). With regard to the former, a rapid biodegradation of contaminants has been frequently observed in artificially spiked and recently polluted soils; this highlights the need of using actual industrial soils in order to transfer lab-scale experiments to the field-scale.

In a previous work, the application of a dynamic biopile as a remediation strategy for a creosote-contaminated soil led to an almost quantitative depletion of 3 ringed PAH fraction (98%) while some HMW-PAHs (4- and 5- ringed ones) were either partially or not degraded at all (Realp et al., 2008). These findings have been frequently reported in biostimulation approaches to PAH-contaminated soils and mainly ascribed to both low contaminant bioavailability and lack of catabolic abilities in the indigenous

soil microbiota (Fernandez-Luqueno et al., 2011; Llado et al., 2009; Reichenberg et al., 2010).

Bioaugmentation with white-rot fungi (WRF) might be a useful approach to overcome the aforementioned limitations associated with the use of biostimulation to PAH-contaminated soils. These organisms, in fact, are efficient degraders of a wide range of organopollutants sharing structural similarities to lignin due to the high non-specificity of their extracellular lignin-modifying enzymes (LMEs) (Pointing, 2001). The most characterized LMEs, namely manganese-peroxidase (MnP), lignin peroxidase (LiP) and laccase have been shown to oxidize PAHs under *in vitro* conditions (Majcherczyk et al., 1998; Eibes et al., 2006). Since these enzymes operate in the extracellular environment *via* the production of radical species and highly diffusible oxidants, such as manganic chelates, the degradation efficiency of WRF does not depend on contaminant uptake, which is the rate-limiting step for bacteria (Bamforth and Singleton, 2005). The use of WRF in soil, however, requires the concomitant addition of lignocellulosic materials in order to improve their competition ability with the resident microbiota and, in this respect, inocula formulation has been shown to be important (Leonardi et al., 2008; Zeddel et al., 1993).

One of the major determinants for the high PAH recalcitrance to biodegradation in soil derives from their high hydrophobicity and tendency to tightly adhere to organic soil colloids thus resulting in low bioavailability. In this specific respect, the use of mobilizing agents (MAs) to enhance mass transfer rate and bioavailability of PAHs has received considerable attention. Among them, nonionic polyoxyethylene-based MAs were found beneficial for the bioremediation of PAHs due to their low toxicity (Singh, 2006). In addition, plant oils, such as soybean oil, were found to be excellent alternatives to MAs, since, in addition to exhibiting molar solubility ratios towards

PAHs which were similar to those of conventional surfactants, they were cost-effective (Yap et al., 2010).

The main objective of this study was to comparatively evaluate the impact of single or combined supplements on both a biostimulation and a bioaugmentation approach to a PAH-contaminated soil in terms of biodegradation outcomes and evolution of the resident microbiota. The supplements under study included two MAs (*i.e.*, Brij 30 and soybean oil), wheat straw and bivalent manganese ions either alone or in combination. Regardless of the treatment typology, treatments were investigated under unsaturated solid-phase conditions due to their resemblance with those adopted at the field-scale. The selected matrix derived from a 180-d treatment in a pilot-scale biopile and, thus, was characterized by the presence of a highly recalcitrant PAH fraction. To perform the bioaugmentation approach, two allochthonous WRF strains, namely *Trametes versicolor* ATCC 42530 and *Lentinus tigrinus* CBS 577.79, were selected due to their previously reported PAH-degrading efficiencies and competition abilities with soil microbiota (Covino et al. 2010; Borrás et al., 2010).

2. Materials and methods

2.1. *Soil and materials*

The aged creosote-contaminated soil was collected from a pilot-scale biopile where it had undergone a remediation treatment for 180 d, in a wood preservative industrial facility near Barcelona (Spain), as described elsewhere (Realp et al., 2008). In particular, a composite sample (20 kg) was withdrawn, air-dried, sieved through a 6-mm screen and, finally, stored at 4 ºC until used. Its main properties were as follows: total petroleum hydrocarbons (TPH) content, 2815 mg · kg^{-1}; real acidity (in water), pH 7.5;

water holding capacity, 33.7%; total organic carbon, 3.5%; NO_3^-, 15 mg · kg^{-1}; NH_4^+, 1.6 mg kg^{-1}; texture: sand (32%); silt (28%) and clay (40%).

Ergosterol, 7-dehydrocolesterol, o-terphenyl and methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate and Brij 30 (dodecyl tetraethylene glycol ether) were purchased from Sigma-Aldrich, Spain. Soybean oil and manganese (II) sulfate ($MnSO_4$) were purchased from Sigma-Aldrich, Italy. Solvents were purchased from Scharlab S.L. (Barcelona, Spain). Solvents and other chemicals and reagents were of the highest purity available. PAH standards for quantification in GC-FID were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany).

2.2. Microrganisms

Trametes versicolor ATCC 42530 and *Lentinus tigrinus* CBS 577.79, obtained from the American Type Culture Collection (Manassas, USA) and the Centraalbureau voor Schimmelcultures (Utrecht, NL), respectively, were maintained and periodically sub-cultured on potato dextrose agar (Difco, Detroit, MI) slants. Fungal mycelial suspensions were prepared as previously described (D'Annibale et al., 2005).

2.3. Soil pre-treatment and inocula preparation

Previously sterilized (121 ºC for 15 min) MAs (either SO or Brij 30) were added to the soil by spraying and subsequent mixing to reach a final concentration of 4.5% (w/w). The soil thus pre-treated was kept for 6 d at 4ºC prior to its use (Leonardi et al., 2008).

On the other hand, lignocellulosic substrate (LS), made of a wheat straw/ wheat bran mixture (80: 20, w/w), and destined to the preparation of the fungal inoculum was transferred to 16 x 3.5 cm test-tubes (Covino et al., 2010). Then, its moisture content

was adjusted to 70% (w/w) with sterile deionized water prior to sterilization (121 ºC for 45 min). When required, $MnSO_4$ was added, as a source of bivalent manganese ions, at a rate of (20 mg kg^{-1}). Pre-colonized wheat (*Triticum aestivum*) seeds were used to inoculate the LS (5%, w/w) on those treatments were *Trametes versicolor* (TV) and *Lentinus tigrinus* (LT) were assayed. Fungal cultures were grown for 7 d at 28 ºC under stationary conditions and non-inoculated LS, to be used in biostimulation experiments, was incubated in parallel under the same conditions. Regardless of the inoculation, the LS mass amounted to 10% of the total dry weight for each microcosm.

2.4. Fungal treatment of contaminated soil

A layer of either MA-supplemented or bulk soil (25 g), the moisture content of which had been previously adjusted to 60% of its water holding capacity (w/w), was added to the test-tubes containing either inoculated or non-inoculated LS. The soil was previously amended by mixing with an additional 10% (w/w) of non-inoculated sterilized (121ºC for 45 min) LS. All microcosms were incubated at 28 ºC for 60 d in the dark and their moisture contents kept constant by periodic additions of sterile deionized water. All experiments were carried out in three parallel replicates under non-axenic conditions. Non-amended controls were prepared, incubated as above and from here onwards referred to as incubation controls. Subsequent analyses were carried out on the upper soil layer and the results were normalized by taking into account the dilution effect of the LS mixed with the polluted soil.

2.5. Extraction and analysis of organic contaminants

The soil samples were dried for 16 h at room temperature before undergoing an overnight Soxhlet extraction with 200 mL of an acetone:dichloromethane mixture (1:1,

v/v). Prior to the extraction, o-terphenyl dissolved in acetone was added to each sample as a surrogate internal standard. The total organic extracts (TOE) obtained were dried over Na_2SO_4 and concentrated under vacuum to dryness in a rotary evaporator. To obtain the total petroleum hydrocarbon (TPH) fraction, TOE was dissolved in dichloromethane and cleaned up by column chromatography using the EPA 3611B method (US Environmental Protection Agency). Both TPH and PAHs were analyzed by gas chromatography with flame-ionization detection (GC-FID) using a Trace 2000 gas chromatograph (Thermo Quest, Milan, Italy) fitted with a DB-5 (30 m x 25 mm i.d. x 0.25 mm film) capillary column (J&W Scientific Products Gmbh, Köln, Germany). The column temperature was held at 50 ºC for 1 min, ramped to 320 ºC at 7 ºC min^{-1} and, finally, isothermal at 320 °C for 10 min. TPH content was calculated from the total area compared with that of an aliphatic standard (AccuStandard, New Haven, USA) calibration curve. Individual PAH compounds were quantified with reference to commercially available pure standards **(**Dr. Ehrenstorfer GmbH, Germany).

2.6. Biochemical determinations

The content of ergosterol, a specific indicator of fungal growth, was determined in soil according to Davis and Lamar (1992). Prior to the extraction, 7-dihidrocolesterol, dissolved in dichloromethane, was added to each sample as a surrogate internal standard.

Finally, samples were analyzed in an Agilent technologies 6890N gas chromatograph coupled to an Agilent technologies 5975 inert mass spectrometer. The GC chromatographs were equipped with DB5 (30 m x 25 mm i.d. x 0.25 μm film) capillary columns. The column temperature program was as follows: isothermal at 50ºC

for 1 min, increase to 320 ºC at 7 ºC min^{-1} and, finally, isothermal at 320 ºC for 10 min. Injector and detector temperatures were set at 290 ºC and 320 ºC, respectively. Samples were injected in splitless mode using helium as the carrier gas at a flow rate of 1.1 ml min^{-1}.

Extracellular ligninolytic enzymes were extracted from soil samples and subsequently assayed as previously reported (D'Annibale et al., 2005).

2.7 Heterotrophic and hydrocarbon-degrading microbial populations counts

Soil bacterial counts were performed using a miniaturized most probable number (MPN) method in 96-well microtiter plates, with eight replicate wells per dilution (Wrenn and Venosa, 1996). Total heterotrophs were counted in tryptone soy broth and aromatic hydrocarbon-degraders were counted in mineral medium BMTM (Abalos et al., 2004) containing a mixture of phenanthrene (0.5 g · L^{-1}), fluorene, anthracene, and dibenzothiophene (each at a final concentration of 0.05 g · L^{-1}). Methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate was added as a fungicide at 20 mg · kg^{-1} when TSB and BMTM were prepared in order to avoid fungal growth in the MPN plates.

2.8. Quantitative PCR assay

Gene copy numbers of eubacterial 16S rRNA and fungal ITS1 rRNAfragments were quantified by quantitative real time PCR (qPCR). Each sample was analyzed in triplicate by means of three independent DNA extracts. The analysis was carried out by using Brilliant II SYBR®Green qPCR Master Mix (Stratagene) in a Real Time PCR System MX3000-P (Stratagene, La Jolla, CA) operated with the following protocol: 10 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing for 30 s

at 50 ºC and 55 ºC (for 16S rRNA and ITS1 rRNA, respectively), extension at 72 °C for 45 s and fluorescence measurement at 80 °C. The specificity of PCR amplification was determined by observations on melting curve and gel electrophoresis profile. Melting curve analysis to detect the presence of primer dimers was performed after the final extension step by increasing the temperature from 55 to 95 °C with a heating rate of 0.05 °C per cycle. Each reaction was performed in a 25 µL volume containing 2 µL of DNA template (approx. 100 ng of DNA), 200 nM of each primer, 12.5 µL of the ready reaction mix and 30 nM of ROX reference dye. The specific primer pairs used for the eubacterial and fungal populations were 519F–907R and ITS5–ITS2 (all primers were purified by HPLC). The standard curves were performed with the following reference genes: 16S rRNA gene from *Desulfovibrio vulgaris* subsp. vulgaris ATCC 29579, inserted in a TOPO TA vector (Invitrogen, Gent, Belgium); and an ITS1 gene fragment obtained from a single DGGE band (GenBank accession nr JN982550) cloned onto the pGEM plasmid vector using pGEM-T Easy Vector System I (Promega, Madison, WI, USA). All reference genes were quantified by Quant-iT™ PicoGreen ® dsDNA Reagent using MX3000P (Stratagene, La Jolla, CA) as the detector system. Ten-fold serial dilutions of known copy numbers of the plasmid DNA in the range from 10^1 to 10^8 copies were subjected to a qPCR assay in duplicate to generate both standard curves. The qPCR efficiencies of amplification were greater than 96%. The Pearson product moment correlation coefficients (R) of the standard curves for 16S rRNA and ITS rRNA,were 0.997 and 0.994, respectively, and the slopes were -3,353 and -3,416, respectively. All results were processed by means of the Agilent MxPro™ QPCR software.

2.9. Statistical analysis

Data were subjected to analysis of variance (ANOVA) followed by multiple pair-wise comparisons by the Fisher least significant difference (LSD) test. Data were also mean centred and unit variance-scaled (soft scaling) and then subjected to principal components analysis (PCA) by the use of the Statistica 8.0 software (StatSoft Tulsa, OK, USA). The possible presence of either moderate or strong outliers in observations was checked by the squared prediction errors of residuals and Hotelling (T2) of t-scores, respectively (MacGregor and Kourti, 1995). Variable power (VP), defined as the explained standard deviation was calculated by equation (1):

$$VP = 1 - \frac{SV_j}{SV_j^0} \qquad (1)$$

Where SV_j is the residual standard deviation of the jth variable and $SV_j°$, is its initial standard deviation, which, is equal to unity for all variables after soft-scaling.

3. Results

3.1. Impact of treatment on bacterial and fungal biota

The most evident effect on cultivable heterotrophic bacteria (CHB) was exerted by the presence of LS, which led to an invariably higher increase in their concentrations in biostimulated microcosms. Although high CHB densities were found in *T. versicolor*- and *L. tigrinus*-augmented microcosms amended with SO alone or combined with Mn^{2+} ions, they did not significantly differ from their respective incubation control where a high stimulatory effect of SO on CHB had been observed (Fig. 1A). This positive impact of LS and SO supplementation on CHB was confirmed by qPCR analyses of the *16S rRNA* gene (Fig. 1B). It is noteworthy that bioaugmentation treatments did not exert a depressive effect either on 16S rRNA gene copy number or on CHB; conversely, the

former parameter was found to be significantly higher in LS-amended microcosm colonized by *T. versicolor*.

A different behavior was observed with the cultivable hydrocarbon degrading bacteria (CHDB)from MPN assays. The high population density present in the initial contaminated soil ($2.3 \cdot 10^6$ MPN g^{-1} soil) was not significantly affected in incubation controls with the notable exception of those added with Brij 30 where a striking depressive effect was evident after 60 d incubation ($1.2 \cdot 10^3$ MPN g^{-1} soil) (Fig. 1C). The negative effect of this surfactant on CHDB was also confirmed in all biostimulated and *T. versicolor*-augmented microcosms, regardless of the presence or the absence of Mn^{2+} ions. Conversely, SO had a significantly stimulatory effect on CHDB, in both biostimulated and bioaugmented microcosms with respect to the respective incubation controls (Fig. 1C). Interestingly, the addition of LS significantly attenuated the aforementioned toxicity effects of Brij 30.

The initial soil, arising from a 180 d biopiling treatment exhibited a rather high CHDB/CHB ratio amounting to 20% and it decreased significantly in the non-amended control after 60 d incubation (6.6%); a dramatic decrease in this ratio was observed in incubation controls added with either SO or Brij 30 (0.73 and 0.01%, respectively) (Fig. 1D). In the presence of the former MA, in LS-amended microcosms inoculated with either *T. versicolor* or *L. tigrinus*, the CHDB/CHB ratios were higher than the respective incubation control (3.8 and 12%, respectively, *vs.* 0.73%); in the same bioaugmented microcosms, subjected to the concomitant SO and Mn^{2+} ions additions, the same ratio reached high values which were very close to that of the initial soil (Fig. 1D).

Fig. 2 shows that fungal growth, inferred from two independent methods, namely ergosterol quantitation and quantitative PCR analysis of the ITS region, was

mostly stimulated by the addition of LS as previously observed for bacteria. This can be desumed by comparing both parameters in incubation controls and respective microcosms, whether they be biostimulated or subjected to bioaugmentation with either *T. versicolor* or *L. tigrinus.* Conversely, no statistical significant differences were found between LS-amended microcosms, irrespective of the presence or the absence of the other additives, such as MAs and/or bivalent manganese ions. With this regard, it has to be highlighted that soil colonization by both *T. versicolor* and *L. tigrinus* mycelia was very fast in the early 15 days of incubation. Thereafter, exogenous fungi were strongly outcompeted by the resident fungal biota, the profuse growth of which was visually evident by the widespread presence of yellow/green spores in augmented microcosms. Thus, the lack of significant differences between bioaugmented and biostimulated microcosms might likely be due to a generalized boosting effect of LS on autochthonous fungi.

In all incubation controls, trace levels of extracellular laccase activity were found while Mn-peroxidase was not detected (Table 1). A slight increase in the former and the occurrence of the latter was observed in biostimulated microcosms; however, due to the large data variability, both activities therein observed did not significantly differ from incubation controls. For the same reason, only few bioaugmented microcosms were found to significantly differ from the respective incubation controls; with regard to laccase, in particular, in the majority of bioaugmented and Mn^{2+}-supplemented microcosms, laccase activity was significantly higher than in incubation controls. Best laccase activity (6.77 IU $\cdot$ g^{-1} soil) was found in *T. versicolor* microcosms undergoing simultaneous supplementation with LS, Mn^{2+} ions and Brij 30; regardless of the type of supplements, laccase activity was generally higher in *T. versicolor* than in *L. tigrinus* microcosms (Table 1).

In both bioaugmented microcosms, MnP activity appeared to be boosted by the presence of either Mn^{2+} ions or Brij 30; however, when the two additives were combined the stimulatory effect was markedly lower for both fungi.

3.2. Degradation of contaminants in soil

In the soil under study, derived from a previous 180-d-long biopiling treatment, the residual total petroleum hydrocarbon (TPH) and overall PAH contents amounted to 2815 and 389 mg · kg^{-1}, respectively (Table 2). Best TPH removals, ranging from 71% to 73%, were achieved in biostimulated microcosms supplemented with MAs and Mn^{2+} ions. In any case, regardless of the supplementation, the residual TPH contents in biostimulated microcosms were significantly lower than those found in respective incubation controls (Table 2).

Conversely, bioaugmented microcosms failed to significantly lead to a lower TPH contents than respective incubation controls with the only exceptions of Mn^{2+}-supplemented *L. tigrinus* microcosms; among them, the most effective was the microcosm concomitantly supplemented with SO and Mn^{2+} ions leading to a 61.2% TPH removal.

With regard to the PAH fraction, the soil at start mainly contained 4- and, to a lesser extent, 5-ring compounds. Among the former, the most abundant were fluoranthene (FLT) and pyrene (PYR) (83 and 84 mg · kg^{-1} soil, respectively) while the most representative 5-ring compounds included benzo[b]-fluoranthene (BbF) and benzo[k]fluoranthene (BkF) (57 and 38 mg kg^{-1} soil, respectively). It is noteworthy that, unlike that observed for TPH, all treatment typologies were able to yield significantly lower residual PAH contents than those in incubation controls.

The most susceptible PAH compounds to biodegradation were FLT and PYR, the removal extents of which ranged from 63 to 87% and 59 to 83%, respectively, as a function of the treatment typology. Brij 30 appeared to exert a negative impact on biodegradation of both FLT and PYR and this effect was also observed with other compounds such as benzo[a]anthracene (BaA) and chrysene (CHR). In *L. tigrinus* microcosms, the supplementation of Mn^{2+} ions alone exerted a positive effect on the degradation of FLT, PYR, BaA and CHR, the residual contents of which were significantly lower than with other supplements (Table 2). This stimulatory effect of Mn^{2+} ions, however, was not observed with 5-ring compounds in *L. tigrinus* microcosms; best removal efficiencies towards these compounds, namely benzo[a]pyrene, BbF and BkF were, instead, observed in biostimulated microcosms that underwent concomitant supplementation with Mn^{2+} and MAs (Table 2).

In order to obtain further insights into similarity of both treatments and variables and respective interplay, a multivariate approach, based on principal component analysis was used. To this aim, those variables (*i.e*., ergosterol, ITS, CHB and 16S rRNA gene copy number) which had not been shown to be significantly affected by both treatment typology and type of amendant were not recruited within the model. Around 72% of total variance was explained by the first two components (51.9 and 19.6%, respectively) and no moderate or strong outliers were detected by SPE analysis and Hotelling of scores, respectively (Fig. 3A). The similarity of behavior in response to treatments of the two variable couples BkF and BbF and FLT and PYR was confirmed by the nearly superimposed loadings while the remaining 5-ring compound BaP was clearly separated by both components (Fig. 3B). Incubation controls and biostimulated microcosms concomitantly added with MAs and Mn^{2+} ions were separated from the remaining treatments by the first component (Fig. 3A) and the most influential variables

were BaA, CHR, TPH, PYR, BkF and FLT residual contents as ranked by the respective vector lengths (Fig. 3B). The least influential variables on the PCA model were laccase and MnP activities and the CHDB/CHB ratio. Interestingly, variables CHR, PYR and FLT residual contents were located in the right lower quadrant corresponding to those treatments, namely ICSO and ICBR, which had been shown to be the least effective in the degradation of those compounds; the same MA-amended incubation controls that had been shown to yield the lowest ratios between cultivable hydrocarbon-degrading and total heterotrophic bacteria (CHDB/CHB) located in the diagonally opposed quadrant to those variables (Fig. 3). The Mn^{2+}-supplemented *L. tigrinus* microcosms in the presence and in the absence of SO that had been shown to lead to high MnP activities and CHDB/CHB ratios were located in the same quadrant of those variables. Finally, the high degradation abilities towards TPH, BaP and BkF of the biostimulated microcosms, concomitantly added with Mn^{2+} and MAs, were confirmed by the diagonally opposite position of their relative loadings and scores.

4. Discussion

Bioremediation of aged industrial contaminated soils is still a technical challenge. Two of the most important factors that conspire against biostimulation success in real historically PAH-polluted sites are the low bioavailability of pollutants due to high soil-PAH contact time, a phenomenon referred to as aging (Semple et al., 2003), and the lack of degradation efficiency in the indigenous microbial community (El Fantroussi and Agathos, 2005).

The creosote-contaminated soil selected in this study had previously undergone a 180-d-long biopiling treatment, which albeit being efficient, was unable to reduce a residual highly recalcitrant pollutant fraction upon extension of the incubation time.

The failure of the biopile system to proceed further was supposed to be due to either the scant ability of the resident microbiota to deplete high molecular mass PAHs or to their low bioavailability (Realp et al., 2008). Fungi are known to be efficient degraders of highly condensed PAHs and to be less affected by contaminant bioavailability than bacteria. This is due both to their filamentous growth mode, which enables them to reach pollutants, and to the presence of an extracellular enzyme degradation machinery, including copper oxidases and heme peroxidases, able to perform one-electron oxidation of PAHs (Majcherczyk et al., 1998; Eibes et al., 2006).

Thus, the aim of the present study was to assess whether the stimulation of the resident fungal biota or, alternatively, the inoculation of exogenous effective PAH-degrading fungi (Borràs et al., 2011; Covino et al., 2010), might be able to deplete the aforementioned residual contaminant fraction. Several studies report the ability of a variety of lignocellulosic materials to boost colonization by either resident or exogenously applied fungi in soil (Férnandez-Sánchez et al., 2001; Hultgren et al., 2009; Meysami and Baheri, 2003). Wheat straw was selected due both to its abundance in cell wall polysaccharides and to its wide commercial availability (Lu et al., 2009) and, in order to avoid artifacts, it was used in sterile form. The further possible impact of supplements, namely bivalent manganese ions and MAs, such as Brij 30 and soybean oil, was also assessed.

Wheat straw is one of the most commonly used LSs in bioaugmentation strategies (Lu et al., 2009). These supports can stimulate ligninolytic enzyme production, fungal growth and pollutant degradation (Walter et al., 2004). In the present study, LS addition to the soil exerted a notable biostimulation action which was evident on indigenous fungal growth (Fig. 2A-B) and, to a lesser extent, on CHB (Fig. 1A). Thus, the lignocellulosic mixture and incubation-controlled conditions supported the

development of a robust indigenous fungal population in the contaminated soil which had not been observed in the previous biopiling treatment (Realp et al., 2008) or in other lab-scale studies (Viñas et al., 2005).

Furthermore, Axtell et al. (2010) reported that the addition of lignocellulosic amendants able to promote both growth and activity of native populations markedly enhanced the degradation of residual toxic compounds in the soil. Our results confirmed these findings and showed that LS-promoted biostimulation led to better TPH and 5-ring PAHs than those obtained with effective PAH-degrading allochthnous fungal strains (Table 2). Indeed, as a consequence of the outstanding growth capabilities of indigenous soil mycobiota, the colonization of the upper (soil) layer by the WRF inoculants was clearly hindered after the early two weeks of the incubation. In this respect, marked antagonistic effects exerted by native soil populations towards augmented WRF had been frequently reported (Martens and Zadrazil, 1998; Canet et al., 2001; Magan et al. 2010).

The visually observed antagonistic effect of the native mycobiota towards both bioaugmented WRF was confirmed by both ergosterol and ITS qPCR data (Fig. 2A-B). In biostimulated microcosms, both number of copies of the ITS gene and the ergosterol concentrations were not significantly lower than those found in *T. versicolor*- or *L. tigrinus*-augmented ones; conversely, both parameters were significantly higher in biostimulated and bioaugmented microcosms than in incubation controls thus proving the stimulatory effect of LS towards indigenous fungi.

The presence of detectable laccase and MnP activities in biostimulated microcosms clearly showed that the indigenous microbiota included members able of producing both enzymes. In this respect, several yeasts, ascomycetes and other non-basidiomycete soil fungi are capable of producing laccase and other peroxidases (Silva

et al., 2009). Although ligninolytic enzymes activities in biostimulated microcosms were significantly lower than in bioaugmented ones, the extent of PAH biodegradation were higher in the former than in the latter. With this regard, it is known that non-ligninolytic fungi are able to transform and detoxify PAHs by means of intracellular enzymatic systems, such as the cytochrome P-450 monooxigenase/epoxide hydrolase system (Cerniglia, 1992). An additional reason might stem from the above reported strong competitive action mainly exerted by the indigenous mycobiota which led to a limited soil colonization by both *T. versicolor* and *L. tigrinus*.

The sole addition of Mn^{2+} did not exert relevant effects on the microcosms under study with the only exception of a stimulation of MnP production in bioaugmented ones. Although MnP has been shown to oxidize a variety of PAHs, the increase in its activity in the aforementioned microcosms did not lead to improved contaminant depletion; this was confirmed by PCA analysis showing that MnP activity was among the least influential variables. Conversely, combination of Mn^{2+} with MAs led to interesting effects. In particular, in biostimulated microcosms, 5-ring PAHs and TPH depletions were positively affected by the concomitant presence of MAs and Mn^{2+} ions, regardless of the MA type (Table 2). This fact was confirmed by the diagonally opposite position of PCA scores of these treatments with respect to the loadings plot of BbF, BkF and BaP residual contents (Fig. 3).

The very clear toxic effect of Brij 30 on the CHDB populations (Fig. 1C) was concomitant with lower 4-ring PAHs degradation in most of the microcosms where this surfactant had been added, suggesting that bacterial populations might be of paramount importance in the biodegradation of this relatively more accessible PAH fraction. This can be desumed by the absence of any significant inhibition of the fungal growth in Brij 30-containing microcosms (Fig. 2A-B). Interestingly, the inhibitory effect of Brij 30 on

4-ring PAHs degradation was also observed in incubation controls (Table 2). These findings confirm that the assessment of surfactant toxicity towards indigeous microbiota is a crucial step in the selection of appropriate MAs for bioremediation purposes (Zheng and Obbard, 2001).

It is noteworthy that such high depletion extent on 5-ring PAHs had not been previously observed with this soil (Vinas et al., 2005) and that they were obtained by relying on the stimulation of autochthonous fungal and bacterial populations.

Conversely, very curious is the case of the SO-amended incubation control, where the surfactant addition promoted unexpected levels of benzo(a)pyrene degradation and led to a stimulation of the bacterial heterotrophic population that was one order of magnitude higher than in the same soil with Brij 30 or with no MA addition. It is known that accessible carbon sources as SO, can enhance the cometabolism required for the bacterial benzo(a)pyrene degradation (Kanaly and Bartha, 2009) but is very interesting how specific this effect was in this case, because no other PAH was affected in the same way as benzo(a)pyrene; further research is needed to better ascertain this apparent SO-promoted and contaminant-specific co-metabolic mechanism.

5. Conclusions

The present study documented that a highly recalcitrant TPH and HMW-PAHs fraction, remaining in an actual creosote-polluted soil after a 180-d pilot-scale biopiling treatment, might be significantly degraded by a biostimulation approach based on LS addition. Degradation results might be further boosted by the presence of a concomitant mobilizing agent and Mn^{2+}.

In this respect, possible mycoaugmentation approaches, which strictly require the concomitant LS addition with fungal inoculants might fail due to the LS-promoted growth of indigenous fungal and bacterial populations as it was clearly observed in this study. Thus, the implementation of bioremediation technologies, based on exogenous inoculants, strictly require a lab-scale assessment of interactions between indigenous microbiota and the selected allochthonous species.

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References

Abalos A, Vinas M, Sabate J, Manresa MA, Solanas AM. Enhanced biodegradation of Casablanca crude oil by a microbial consortium in presence of a rhamnolipid produced by *Pseudomonas aeruginosa* AT10. Biodegradation 2004; 15: 249-60.

Alexander M. Biodegradation and bioremediation. Academic Press, London, 1999.

Axtell C, Johnston CG, Bumpus JA. Bioremediation of soil contaminated with explosives at the naval weapons station Yorktown. J Soil Contam 2010; 9: 537-548.

Bamforth SM, Singleton I. Bioremediation of polycyclic aromatic hydrocarbons: current knowledge and future directions. J Chem Technol Biotechnol 2005; 80: 723-736.

Boopathy R. Effect of food-grade surfactant on bioremediation of explosives-contaminated soil. J Hazard Mater 2002; 92: 103-114.

Borràs E, Caminal G, Sarrà M, Novotný C. Effect of soil bacteria on the ability of polycyclic aromatic hydrocarbons (PAHs) removal by *Trametes versicolor* and *Irpex lacteus* from contaminated soil. Soil Biol Biochem 2010; 42: 2087-2093.

Canet R, Birnstingl JG, Malcolm DG, Lopez-Real JM, Beck AJ. Biodegradation of polycyclic aromatic hydrocarbons (PAHs) by native microflora and combinations of white-rot fungi in a coal-tar contaminated soil. Bioresour Technol 2001; 76: 113-7.

Cerniglia CE. Biodegradation of polycyclic aromatic hydrocarbons. Biodegradation 1992; 3: 351-368.

Covino S, Cvancarova M, Muzikár M, Svobodová K, D'Annibale A, Petruccioli M, Federici F, Kresinová Z, Cajthaml T. An efficient PAH-degrading *Lentinus*

(*Panus*) *tigrinus* strain: Effect of inoculum formulation and pollutant bioavailability in solid matrices. J Hazard Mater 2010; 183: 669-676.

D'Annibale A, Ricci M, Leonardi V, Quaratino D, Mincione E, Petruccioli M. Degradation of aromatic hydrocarbons by white-rot fungi in a historically contaminated soil. Biotechnol Bioeng 2005; 90: 723-31.

Davis MW, Lamar RT. Evaluation of methods to extract ergosterol for quantitation of soil fungal biomass. Soil Biol Biochem 1992; 24: 189-198.

Eibes G, Cajthaml T, Moreira MT, Feijoo G, Lema JM. Enzymatic degradation of anthracene, dibenzothiophene and pyrene by manganese peroxidase in media containing acetone. Chemosphere 2006; 64:408–414.

El Fantroussi S, Agathos SN. Is bioaugmentation a feasible strategy for pollutant removal and site remediation? Cur Opin Microbiol 2005; 8: 268-275.

Fernandez-Luqueno F, Valenzuela-Encinas C, Marsch R, Martinez-Suarez C, Vazquez-Nunez E, Dendooven L. Microbial communities to mitigate contamination of PAHs in soil--possibilities and challenges: a review. Environ Sci Pollut Res Int 2011; 18: 12-30.

Haritash AK, Kaushik CP. Biodegradation aspects of Polycyclic Aromatic Hydrocarbons (PAHs): a review. J Hazard Mater 2009; 169: 1-15.

Hultgren J, Pizzul L, Castillo MP, Granhall U. Degradation of PAH in a creosote-contaminated Soil. A comparison between the effects of willows (*Salix viminalis*), wheat Straw and a nonionic Surfactant. Int J Phytorem 2009; 12: 54-66.

Kanaly RA, Bartha R. Cometabolic mineralization of benzo[a]pyrene caused by hydrocarbon additions to soil. Environ Toxicol Chem 2009; 18,2186-2190.

Leonardi V, Giubilei MA, Federici E, Spaccapelo R, Sasek V, Novotny C, Petruccioli M, D'Annibale A. Mobilizing agents enhance fungal degradation of polycyclic aromatic hydrocarbons and affect diversity of indigenous bacteria in soil. Biotechnol Bioeng 2008; 101: 273-85.

Lu Y, Yan L, Wang Y, Zhou S, Fu J, Zhang J. Biodegradation of phenolic compounds from coking wastewater by immobilized white rot fungus *Phanerochaete chrysosporium*. J Hazard Mater 2009; 165: 1091-7.

Llado S, Jimenez N, Vinas M, Solanas AM. Microbial populations related to PAH biodegradation in an aged biostimulated creosote-contaminated soil. Biodegradation 2009; 20: 593-601.

MacGregor JF, Kourti T. Statistical process control of multivariate processes. Contr Engin Pract 1995; 3: 403-414.

Magan N, Fragoeiro S, Bastos C. Environmental Factors and Bioremediation of Xenobiotics Using White Rot Fungi. Mycobiol 2010; 38: 238-248.

Martens R, Zadrazil F. Screening of white-rot fungi for their ability to mineralize polycyclic aromatic hydrocarbons in soil. Folia Microbiol (Praha) 1998; 43: 97-103.

Majcherczyk A, Johannes C, Hüttermann A. Oxidation of Polycyclic Aromatic Hydrocarbons (PAH) by laccase of *Trametes Versicolor*. Enzyme Microb Technol 1998; 22: 335–341.

Meysami P, Baheri H. Pre-screening of fungi and bulking agents for contaminated soil bioremediation. Adv Environ Res 2003; 7: 881-887.

Mueller JG, Chapman PJ, Pritchard PH. Action of a fluoranthene-utilizing bacterial community on polycyclic aromatic hydrocarbon components of creosote. Appl Environ Microbiol 1989; 55: 3085-90.

Pointing SB. Feasibility of bioremediation by white-rot fungi. Appl Microbiol Biotechnol 2001; 57: 20-33.
Realp E, Doménech JA, Martinez-García R, Restrepo C, Lladó S, Viñas M, Solanas AM. Ensayo piloto de biorremediación por la tecnología de la biopila dinámica para la descontaminación de suelos contaminados por creosotas provenientes de las actividades dedicadas a la preparación de la madera. Residuos: Revista técnica 2008; 102: 38-49.
Reichenberg F, Karlson UG, Gustafsson O, Long SM, Pritchard PH, Mayer P. Low accessibility and chemical activity of PAHs restrict bioremediation and risk of exposure in a manufactured gas plant soil. Environ Pollut 2010; 158: 1214-20.
Rogers SW, Ong SK, Moorman TB. Mineralization of PAHs in coal-tar impacted aquifer sediments and associated microbial community structure investigated with FISH. Chemosphere 2007; 69: 1563-73.
Semple KT, Morris AWJ, Paton GI. Bioavailability of hydrophobic organic contaminants in soils: fundamental concepts and techniques for analysis. Eur J Soil Sci 2003; 54: 809-819.
Silva IS, Grossman M, Durrant LR. Degradation of polycyclic aromatic hydrocarbons (2–7 rings) under microaerobic and very-low-oxygen conditions by soil fungi. Int Biodeter Biodegr 2009; 63: 224-229.
Singh H. Mycoremediation: Fungal Bioremediation. John Wiley and Sons, Inc. New Jersey, 2006.
Viñas M, Sabaté J, Espuny MJ, Solanas M. Bacterial Community Dynamics and Polycyclic Aromatic Hydrocarbon Degradation during Bioremediation of Heavily Creosote-Contaminated Soil. Appl Environ Microbiol 2005; 71: 7008-7018.
Walter M, Boul L, Chong R, Ford C. Growth substrate selection and biodegradation of PCP by New Zealand white-rot fungi. J Environ Manage 2004; 71: 361-9.
Wrenn BA, Venosa AD. Selective enumeration of aromatic and aliphatic hydrocarbon degrading bacteria by a most-probable-number procedure. Can J Microbiol 1996; 42: 252-8.
Yap CL, Gan S, Ng HK. Application of vegetable oils in the treatment of polycyclic aromatic hydrocarbons-contaminated soils. J Hazard Mater 2010; 177:28-41
Zeddel A, Majcherczyk A, Hüttermann A. Degradation of polychlorinated biphenyls by white−rot fungi *Pleurotus ostreatus* and *Trametes versicolor* in a solid state system. Toxicol Environ Chem 1993; 40:255-266.
Zheng Z, Obbard JP. Effect of non-ionic surfactants on elimination of polycyclic aromatic hydrocarbons (PAHs) in soil-slurry by Phanaerochaete chrysosporium. J Chem Technol Biotechnol 2001; 76: 423-429.

Figure captions

Fig. 1. Left graphics: Cultivable heterotrophic bacteria (CHB), expressed as MPN g^{-1} soil (A) and 16SrRNA gene copies quantified by qPCR (B) in all soil bioremediation treatments after 60 d of incubation at 28ºC. Right graphics: Cultivable PAHs-degrading specialized bacteria (CHDB), expressed as MPN g^{-1} soil (C) and CHDB/CHB percent

ratios (D) in all soil bioremediation treatments after 60 d of incubation at 28ºC. Multiple pair-wise comparisons were performed by the Fisher LSD test (P≤0.05). Same lowercase letters indicate that differences between microcosms (BS, TV, LT) within the same type of supplements were not significant. Same uppercase letters indicate lack of statistically significant difference within each biostimulation or bioaugmentation treatment in the presence of different supplements. The occurrence of significant differences between each soil treatment and its respective incubation control are represented by an asterisk.

Fig. 2. Ergosterol concentration (A) and ITS region gene copy number quantified by qPCR (B) in all soil bioremediation treatments after 60 d of incubation at 28ºC. Multiple pair-wise comparisons were performed by the Fisher LSD test (P≤0.05). Same lowercase letters indicate that differences between microcosms (BS, TV, LT) within the same type of supplements were not significant. Same uppercase letters indicate lack of statistically significant difference within each biostimulation or bioaugmentation treatment in the presence of different supplements. The occurrence of significant differences between each soil treatment and its respective incubation control are represented by an asterisk.

Fig. 3. Principal component analysis (PCA) of data showing scores (A) and variable loadings (B) plots. Percent variability explained by each principal component (PC) is shown in parentheses after each axis legend. The influence of variables in the model, expressed by the respective VP (Equation 1) was as follows: BaA, 0.963; CHR, 0.946; TPH, 0.928; PYR, 0.926; BkF, 0.911; FLT, 0.909; BbF, 0.852; BaP, 0758; CHDB, 0.581; LAC, 0.452; MnP, 0.204; CHDB/CSB, 0.155.

Table 1: Lignin-modifying enzyme activities in soil after 60 d of incubation at 28 ºC in all different bioremediation treatments.

Treatment	Laccase[a]	Mn-Peroxidase[a]
IC	0.12 ± 0.02	N.D.[b]
IC + SO	0.02	N.D.
IC + Br30	0.09 ± 0.00	N.D.
BS-LS	0.80 ± 0.23 aAB	0.11 ± 0.01 aAB
BS-LS + SO	0.60 ± 0.26 aAB	0.02 aA
BS-LS + Br30	0.22 ± 0.10 aA	0.03 aA
BS-LS + Mn^{2+}	1.02 ± 0.20 aB*	0.10 ± 0.01 aAB
BS-LS + SO + Mn^{2+}	0.40 ± 0.06 aAB	0.07 aAB
BS-LS + Br30 + Mn^{2+}	0.57 ± 0.08 aAB	0.26 ± 0.08 aB
TV-LS	2.22 ± 0.50 bAB*	0.71 ± 0.13 bA
TV-LS + SO	5.08 ± 0.63 bB*	0.78 ± 0.03 cA
TV-LS + Br30	0.68 ± 0.13 aA	2.49 ± 1.36 bB*
TV-LS + Mn^{2+}	2.07 ± 1.03 aAB*	2.27 ± 0.14 bB
TV-LS + SO + Mn^{2+}	1.97 ± 0.67 bAB*	2.69 ± 1.31 aB*
TV-LS + Br30 + Mn^{2+}	6.77 ± 1.80 bB*	0.34 ± 0.20 aA
LT-LS	0.93 ± 0.03 aAB	0.89 ± 0.51 bA
LT-LS + SO	0.40 ± 0.07 aA	0.12 bA
LT-LS + Br30	2.18 ± 1.12 aB	2.68 ± 0.42 bAB*
LT-LS + Mn^{2+}	1.95 ± 0.51 aB*	7.02 ± 3.43 cB*
LT-LS + SO + Mn^{2+}	0.40 ± 0.05 aAB	3.07 ± 1.48 aAB*
LT-LS + Br30 + Mn^{2+}	1.42 ± 0.21 aAB*	1.18 ± 0.66 bA

[a]All concentrations are expressed as IU $g^{-1}$of dry soil and data are the means of three independent experiments. Statistical multiple pair-wise comparison was carried out on column means by the Fisher LSD test ($P \leq 0.05$). Same lowercase letters indicate that differences between microcosms (BS, TV, LT) within the same amendment were not significant. Same uppercase letters indicate lack of statistically significant difference within each biostimulation or bioaugmentation treatment at different supplements. The occurrence of significant differences between each soil treatment and its respective incubation control is denoted by the presence of an asterisk; [b] not detected

Table 2: Initial concentrations of TPH and 4- and 5-ring PAHs in soil and residual concentrations observed after 60 d of incubation at 28 ºC in all different bioremediation treatments.

Treatment	TPH[a]	Fluor	Pyr	B(a)A	Chry	B(b)F	B(k)F	B(a)P
Initial Soil	2815±233	83±4	84±4	37±1	68±1	57±2	38±2	22±1
IC	1439±51	30±0.4	35±3	21±0.8	40±6	39±1	24±1	17±1
IC + SO	1395±30	43±2	48±3	19±0.1	47±3	38±1	24±0.3	9±0.3
IC + Br30	1515±179	61±3	70±2	28±0.4	55±3	38±2	25±0.7	14±0.3
BS-LS	1077±242 aB*	17±1 aA*	18±0.7 aA*	15±0.6 aB*	26±1 aAB*	29±3 aB*	18±1 aB*	15±0,7 aC
BS-LS + SO	1098±207 aB*	16±2 aA*	18±2 aA*	14±3 aB*	29±5 aB*	26±2 aAB*	16±2 aAB*	12±1 aB
BS-LS + Br30	1255±68 aB*	28±4 aB*	34±4 aB*	18±2 aC*	35±2 aC*	28±1 aAB*	19±1 aAB*	15±1 aC
BS-LS + Mn^{2+}	1106±26 aB*	13±1 abA*	15±2 abA*	12±0.5 aB*	23±0.4 aAB*	25±0,5 aAB*	16±0.4 aAB*	13±0.5 aB*
BS-LS + SO + Mn^{2+}	810±27 aA*	13±3 aA*	14±2 aA*	10±0.8 aA*	20±2 aAB*	18±1 aA*	11±0.7 aA*	8±0.2 aA
BS-LS + Br30 + Mn^{2+}	766±27 aA*	13±0.2 aA*	16±0.4 aA*	10±0.7 aA*	19±2 aA*	15±1 aA*	10±0.9 aA*	8.5±0.4 aA*
TV-LS	1545±153 bB	14±2 aA*	16±2 aA*	17±0.6 bAB*	30±1 bA*	35±2 bB*	23±1 bC*	19±2 aA
TV-LS + SO	1338±204 abA	14±2 aA*	17±3 aA*	17±2 aAB*	33±2 aAB*	32±1 aAB*	21±1 aB*	15±0.4 bA
TV-LS + Br30	1552±29 bB	31±4 aB*	36±5 aC*	21±0.9 aB*	39±0.8 abB*	32±2 bAB*	21±0.2 abB*	17±0.1 bA
TV-LS + Mn^{2+}	1417±155 bAB	16±3 bA*	17±3 bA*	16±2 bA*	29±4 aA*	32±4 bAB*	21±2 bB*	17±2 bA
TV-LS + SO + Mn^{2+}	1449±65 cAB	23±4 bAB*	24±4 bB*	16±1 bA*	31±2 cA*	27±2 bA*	18±0.5 bA*	17±4 bA
TV-LS + Br30 + Mn^{2+}	1436±60 cAB	28±3 bAB*	30±3 bB*	19±2 bB*	37±2 bAB*	31±0.8 bAB*	20±1 bB*	16±1 bA
LT-LS	1396±15 abAB	15±3 aAB*	17±2 aAB*	16±0.5 abB*	31±1 bAB*	32±1 abB*	21±1 bAB*	17±3 aB
LT-LS + SO	1467±170 bAB	20±7 aAB*	22±7 aAB*	16±3 aB*	33±8 aAB*	30±4 aAB*	20±3 aAB*	14±1 abA
LT-LS + Br30	1578±42 bB	32±5 aB*	35±5 aB*	21±1 aC*	43±3 bB*	31±2 bB*	21±1 bAB*	17±0.5 bB
LT-LS + Mn^{2+}	1147±53 aAB*	11±2 aA*	13±1 aA*	13±0.7 aA*	24±3 aA*	26±2 aAB*	18±0.1 aAB*	13±0.6 aA*
LT-LS + SO + Mn^{2+}	1093±71 bA*	16±4 abAB*	18±3 aAB*	13±2 bA*	25±4 bA*	24±2 bA*	15±3 bA*	11±0.9 aA
LT-LS + Br30 + Mn^{2+}	1260±2 bAB*	25±0.1 bAB *	31±3 bB*	19±0.4 bC*	37±0.2 bAB*	30±0.2 bB*	21±0.6 bB*	17±0.1 bB

[a] All concentrations are expressed as mg · kg^{-1} of dry soil and data are the means of three independent experiments. Statistical multiple pair-wise comparisons were carried out on column means by the LSD Fisher test ($P \leq 0.05$). Same lowercase letters indicate that differences between microcosms (BS, TV, LT) within the same amendment were not significant. Same uppercase letters indicate lack of statistically significant differences between different supplements added within each biostimulation or bioaugmentation treatment. The occurrence of significant differences between each treatment and its respective incubation control is denoted by the presence of an asterisk.

Figure 1

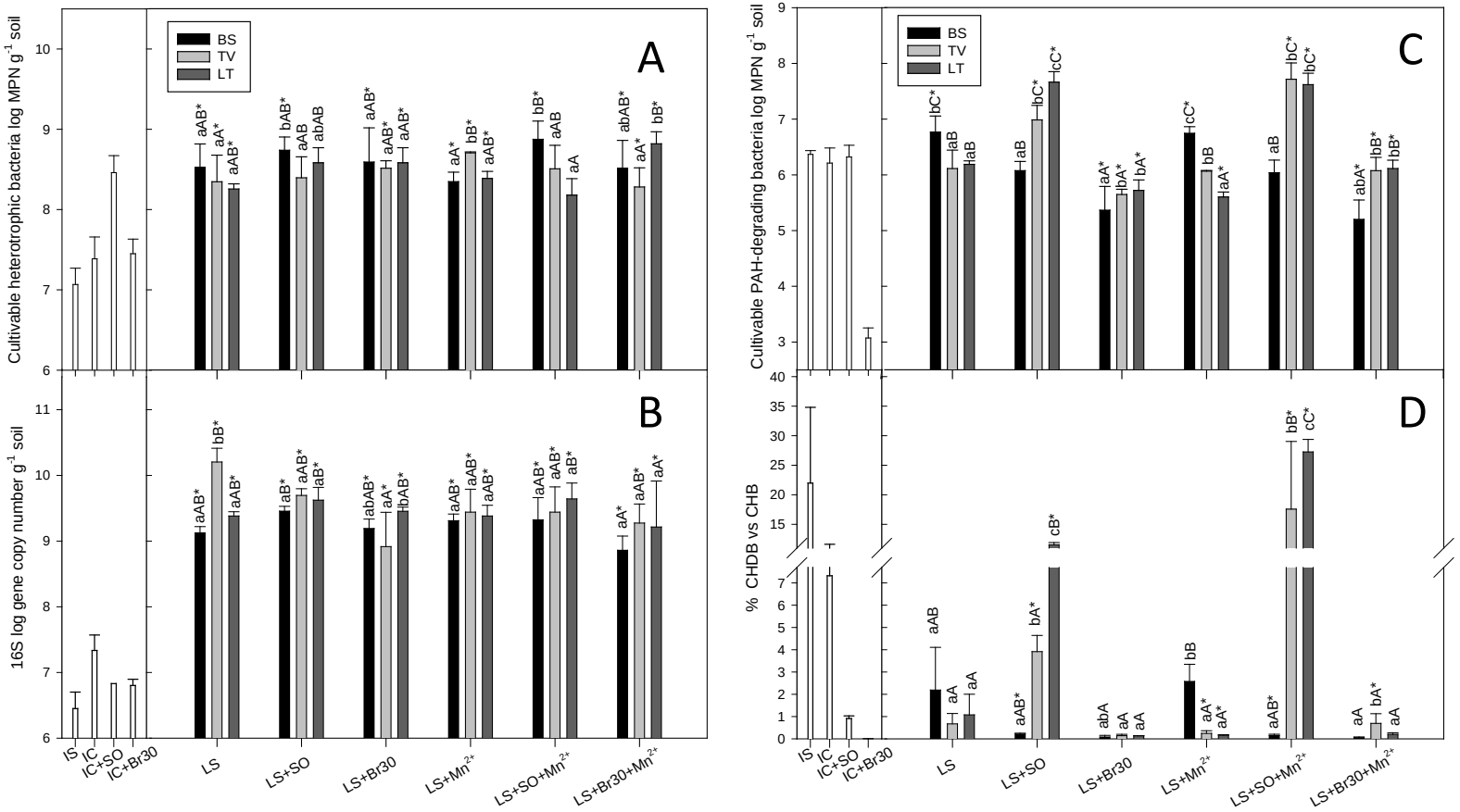

A
B
C
D
BS
TV
LT
Cultivable heterotrophic bacteria log MPN g-1 soil
16S log gene copy number g-1 soil
Cultivable PAH-degrading bacteria log MPN g-1 soil
% CHDB vs CHB
IS
IC
IC+SO
IC+Br30
LS
LS+SO
LS+Br30
LS+Mn2+
LS+SO+Mn2+
LS+Br30+Mn2+

Figure 2

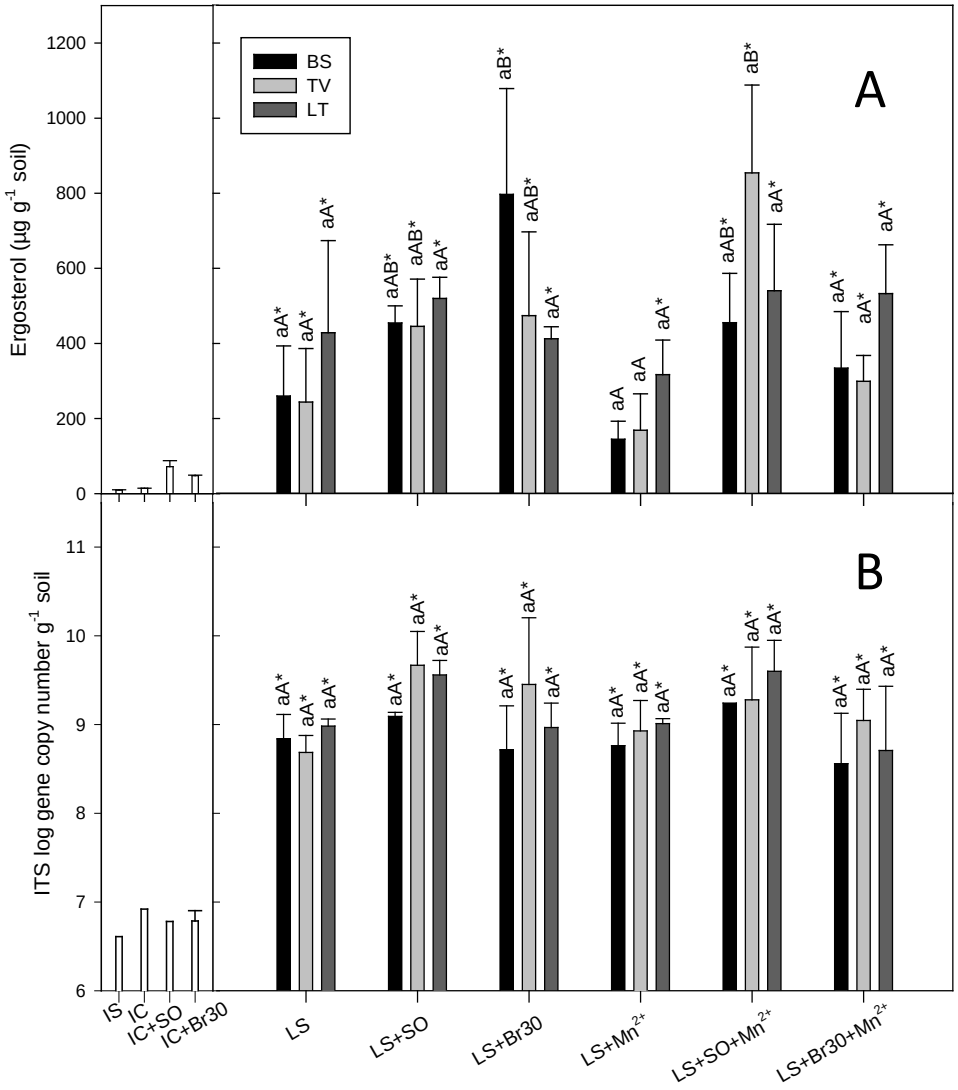
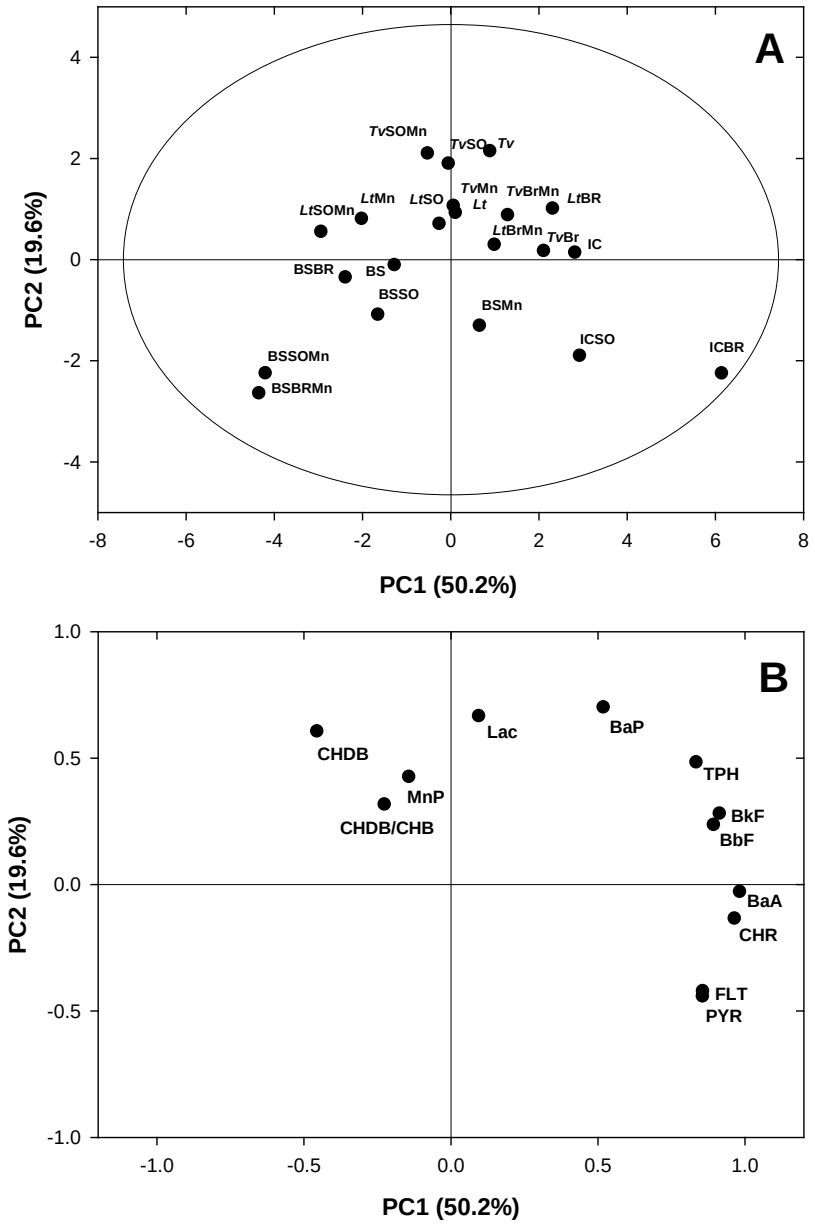

BS
TV
LT
A
B
Ergosterol (µg g-1 soil)
ITS log gene copy number g-1 soil
IS
IC
IC+SO
IC+Br30
LS
LS+SO
LS+Br30
LS+Mn2+
LS+SO+Mn2+
LS+Br30+Mn2+

Figure 3


A
TvSOMn
TvSO
Tv
TvMn
Lt
TvBrMn
LtBR
LtSOMn
LtMn
LtSO
LtBrMn
TvBr
IC
BSBR
BS
BSSO
BSMn
ICSO
ICBR
BSSOMn
BSBRMn
PC2 (19.6%)
PC1 (50.2%)
B
Lac
BaP
CHDB
TPH
MnP
BkF
CHDB/CHB
BbF
BaA
CHR
FLT
PYR
PC2 (19.6%)
PC1 (50.2%)

CAPÍTULO 6 / CHAPTER 6

Combining DGGE and barcoded pyrosequencing for microbial community characterization through different soil bioremediation strategies in an aged creosote-polluted soil

Combining DGGE and Bar-Coded Pyrosequencing for microbial community characterization throughout different soil bioremediation strategies in an aged creosote-polluted soil

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En un estudio realizado previamente (Lladó et al., 2012a submitted), la fracción más recalcitrante de hidrocarburos totales del petróleo (TPH) y de hidrocarburos aromáticos policíclicos de elevado peso molecular (HMW-PAHs), remanentes en un suelo industrial contaminado por creosota después de haberse realizado una biopila dinámica, fue significativamente degradado mediante una estrategia de bioestimulación basada en añadir un sustrato ligninolítico (LS). Además, la degradación de estos compuestos parecía elevarse añadiendo al suelo de forma conjunta agentes movilizadores (MAs) y iones manganeso (Mn^{2+}). Por otro lado, una estrategia paralela de micorremediación no tuvo éxito debido, probablemente, al gran crecimiento de las poblaciones autóctonas, tanto fúngicas como bacterianas, sobre el LS.

Con el objetivo de aumentar nuestro conocimiento sobre los cambios en las dinámicas y estructura de las comunidades microbianas presentes en el suelo, durante las diferentes estrategias de biorremediación (Lladó et al., 2012a submitted), las poblaciones fúngicas y bacterianas fueron estudiadas a través de combinar técnicas dependientes de cultivo como el Número Más Probable (MPN) y técnicas moleculares como la Multiplex bacterial tag-encoded FLX pyrosequencing y el 16SrDNA e ITS DGGE.

En el suelo inicial, procedente de la biopila dinámica, los grupos de bacterias más importantes pertenecían a las familias α y γ-Proteobacteria (Sphingomonadaceae, 18.7%; Caulobacteraceae, 3.4% Xanthomonadaceae, 3.2%). Curiosamente, también fue

hallada una elevada biodiversidad fúngica, donde los géneros *Fusarium* (23.2%) y *Scedosporium* (24.8%) eran predominantes.

Después de 60 días de ensayo de biorremediación a escala de laboratorio, los datos obtenidos de la pirosecuenciación y los DGGE revelaron que i) los cambios poblacionales presentes en las comunidades bacterianas se debían en mayor grado al tipo de MA añadido al suelo y no a la presencia de LS, y ii) el genero bacteriano *Cupriavidus* podía estar llevando a cabo un papel importante en la degradación de HMW-PAHs cuando el LS y Brij 30 eran añadidos al suelo contaminado.

Finalmente, los resultados de pirosecuenciación confirmaron la gran distribución del genero fúngico *Fusarium* en el suelo, así como su gran competitividad para colonizarlo. Sin embargo, no fue posible describir ninguna relación directa entre mayor degradación de HMW-PAHs y la presencia de ningún género fúngico en el suelo contaminado por creosota.

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Abstract

A highly recalcitrant fraction of TPH and HMW-PAH remaining in an industrial creosote-polluted soil after a 180-d pilot-scale biopiling treatment was significantly degraded by a biostimulation approach based on the addition of lignocellulosic substrate (LS) in a previous study (Lladó *et al*., 2012a submitted), while a mycoaugmentation approach with two white-rot fungi strains (WRF) failed due to the LS-promoted growth of indigenous fungal and bacterial populations. In addition, the degradation results may have been further enhanced by the addition of a concomitant mobilizing agent (MA) and Mn^{2+}.

In order to gain insight into the community dynamics and structure throughout the different biostimulation and bioaugmentation treatments performed (Lladó *et al*., 2012a submitted), both the bacterial and fungal biodiversity were analyzed by means of a diversified approach based on combining culture-dependent techniques (MPN), 16SrDNA-DGGE and multiplexed bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP).

In this respect, α- and γ-proteobacteria were the most important bacterial groups (Sphingomonadaceae, 18.7%; Caulobacteraceae, 3.4%; Xanthomonadaceae, 3.2%) following the biopiling treatment and a high fungal biodiversity was also found, with *Fusarium* (23.2%) and *Scedosporium* (24.8%) the two main genera. After a 60-d lab-scale bioremediation assay, the DGGE and pyrosequencing data revealed that: i) the major bacterial community shifts were caused by the type of MA added to the soil and, to a lesser extent, by the addition of LS; and ii) the bacterial genus *Cupriavidus* could play an important role in the biodegradation of HMW-PAHs when LS and Brij 30 are added to the polluted soil.

Finally, the pyrosequencing results confirmed the broad distribution of the *Fusarium* genus in the soil environment, as well as its capacity for competitive colonization. However, it was not possible to describe a significant direct relationship between the higher level of HMW-PAH depletion and the presence of any fungal genus in the polluted soil.

1. Introduction

Microbial biodegradation is the main process in natural decontamination and offers an interesting alternative to the removal of pollutants, such as polycyclic aromatic hydrocarbons (PAHs), from contaminated sites (Chung and Alexander, 1999). Furthermore, PAH-degrading microorganisms have a paramount role in the depletion of these hydrocarbons and are ubiquitous in the environment, for example in soils (bacterial and non-ligninolytic fungal) and woody materials (ligninolytic fungi).

Considering the importance of native microbial communities for bioremediation success (Muckian *et al*., 2009), little is known of the global dynamics of either biostimulated native bacterial or fungal populations in real historically polluted soils (Singleton *et al.*, 2011; Viñas *et al*., 2005). Moreover, because of the growing interest in the use of white-rot fungi (WRF) in soil bioaugmentation strategies (Federici *et al*., 2007), in-depth studies on the cooperative or antagonistic relationships between exogenously added fungi and the native microflora are essential.

In order to study soil microbial community dynamics during bioremediation assays, and bearing in mind that less than 1% of microbial diversity in soils can be cultured (Torsvik and Ovreas, 2002), culture-independent methods, including denaturing gradient gel electrophoresis (DGGE), have been developed and used extensively (Muyzer and Smalla, 1998). The impact of any treatment on the soil

microbial community can be assessed by the interpretation of the DGGE profiles. Moreover, the convenience of comparing a large number of samples corresponding to different treatments means that, these days, the technique constitutes a routine and economic method of obtaining rapid descriptions of soil microbial populations, although it has inherent limitations, such as a low resolving power (threshold of 0.1% of the total), making it impossible to detect changes in the non-abundant members of the community (van Elsas and Boersma, 2011).

However, the current emergence of high-throughput sequencing methods, such as 454-based pyrosequencing, have revolutionized molecular microbial ecology and made it possible to overcome the soil biodiversity resolution limits inherent to DGGE or clone libraries, and new dimensions in the characterization of complex microbial communities have been achieved (Urich *et al*., 2008).

In a recent work, a creosote-polluted soil, previously biotreated in a dynamic biopile and characterized by the presence of a highly recalcitrant PAH fraction, was used to comparatively evaluate the impact of single or combined supplements on both a biostimulation and bioaugmentation approach in terms of biodegradation outcomes and evolution of the resident microbiota (Lladó *et al*., 2012a submitted). Unexpectedly, biostimulating the soil with moisture and a lignocellulosic substrate (LS) (wheat straw/wheat bran mixture (80:20 w/w) achieved significantly better TPH and HMW-PAH degradation values than just biostimulating with moisture or even bioaugmenting the soil with WRF. The colonization rates of the two WRF strains assayed (*i.e.* *Trametes versicolor* and *Lentinus tigrinus*) were lower than expected, probably due to antagonistic relationships with the native populations. The effect on the autochthonous microbial communities of two mobilizing agents (MAs) added to the soil (*i.e.* soybean oil and Brij 30), in order to improve PAH bioavailability, and LS supplementation with

manganese ions, in order to enhance the activity of the fungal extracellular ligninolytic enzymes, was also discussed (Lladó *et al*., 2012a submitted).

In view of the above-mentioned considerations, the main objective of the present study was to achieve a more in-depth understanding of the dynamics and community shifts in both fungal and bacterial populations during the different bioremediation treatments carried out in a historically creosote-contaminated soil (Lladó *et al*., 2012a submitted). Moreover, the bacterial and fungal biodiversity were analyzed through culture-independent molecular ecology methods, such as DGGE and 454-based pyrosequencing. As far as we know, this is the first time both fungal and eubacterial native populations in a real polluted site have been studied by means of pyrosequencing in order to improve our knowledge about native microbial communities and their role in polluted sites. DGGE from MPN cultures grown on PAHs was carried out for all samples and pyrosequencing was performed only for the most representative treatments (Table 1).

2. MATERIALS AND METHODS

2.1. Soil, materials and microorganisms

A composite sample of an aged creosote-contaminated soil previously biostimulated by means of aeration and optimal humidity in a pilot-scale biopile (Realp *et al*., 2008) was obtained. After four months of biostimulation, a composite sample from the soil biopile (20 kg) was sieved (<6 mm) and finally stored at 4ºC until use. Its main properties were as follows: total petroleum hydrocarbon (TPH) content, 2815 mg · kg^{-1}; real acidity (in water), pH 7.5; water-holding capacity, 33.7%; total organic carbon, 3.5%; NO_3^-, 15 mg · kg^{-1}; NH_4^+, 1.6 mg · kg^{-1}; texture: sand (32%), silt (28%) and clay (40%); cultivable heterotrophic bacteria (CHB), $1.3 \cdot 10^7$ MPN · kg^{-1};

cultivable hydrocarbon-degrading bacteria (CHDB), $2.3 \cdot 10^6$ MPN $\cdot$ kg^{-1}; % CHDB vs CHB, 21%.

Brij 30 (dodecyl tetraethylene glycol ether), soybean oil and manganese (II) sulfate ($MnSO_4$) were purchased from Sigma-Aldrich (Saint Louis, MO, USA).

Two different white rot fungi (WRF) strains, *Trametes versicolor* ATCC 42530 and *Lentinus tigrinus* CBS 577.79, were acquired and maintained as described previously (Lladó *et al*., 2012a submitted).

2.2. Microcosm set-up

Previously sterilized soybean oil (SO) and Brij 30 were used for soil pre-treatment in order to reach a concentration of 4.5% (w/w). The pre-treated soil was kept for 6 d at 4ºC prior to use.

Meanwhile, a lignocellulosic substrate (LS), made of a wheat straw/wheat bran mixture (80:20, w/w) and intended for the preparation of the fungal inoculum was transferred to 16 x 3.5 cm test tubes (Covino *et al*., 2010). The LS was previously sterilized (121ºC for 45 min) and then moisture was adjusted at 70% (w/w) with sterile deionized water, while $MnSO_4$ (20 mg $\cdot$ kg^{-1}) was added as a manganese source when the treatment required it. Pre-colonized wheat (*Triticum aestivum*) seeds were used as a fungal inoculum for the LS at 5% (w/w) in those treatments where *T. versicolor* (TV) and *L. tigrinus* (LT) were assayed. Fungal cultures were grown for 7 d at 28ºC under stationary conditions, and non-inoculated LS, to be used in biostimulation experiments (BS), was incubated in parallel under the same conditions. Regardless of the inoculation, the LS mass amounted to 10% of the total dry weight for each microcosm.

Subsequently, a layer of either MA-supplemented or bulk soil (25 g), the moisture content of which had previously been adjusted to 60% of its water-holding

capacity (w/w), was added to the test tubes containing either inoculated or non-inoculated LS. The soil was previously amended by mixing it with an additional 10% (w/w) of non-inoculated sterilized (121ºC for 45 min) LS. All microcosms were incubated at 28ºC for 60 d in the dark and their moisture content kept constant by the periodic addition of sterile deionized water. All experiments were carried out in three parallel replicates under non-axenic conditions. Non-amended controls were prepared and incubated as above and will henceforth be referred to as incubation controls. Subsequent analyses were carried out on the upper soil layer and the results were normalized by taking into account the dilution effect of the LS mixed with the polluted soil.

Procedures for analyzing the organic contaminants, biochemical determinations, CHB and CHDB MPN counts, and quantitative PCR assay of both the bacterial and fungal communities are described in Lladó *et al*., (2012a submitted). A summary of the results is shown in Table 1.

2.3. DGGE molecular profiling

Both total predominant and PAH-degrading eubacteria and fungal microbial communities were studied by means of DGGE and followed different approaches.

Samples for DNA extraction were collected from previously homogenized soil test tubes and the highest positive dilutions from most-probable-number assays on microtiter plates were grown in a PAH mixture (dibenzothiophene, fluorene, anthracene and phenanthrene (Wrenn and Venosa, 1995), at initial time and after 60 days, and placed in sterile Eppendorf tubes stored at –20ºC prior to analysis. Thus, a sample of 50 mg of each microcosm was extracted with a bead-beating protocol using the Power Soil DNA extraction kit (MoBio Laboratories, Solano Beach, CA, USA), in accordance with

the manufacturer's instructions. To obtain DNA from microtiter plates, a composite sample of 1.6 mL containing 200 μL of each replicate (n=8) from the last positive dilution (n=8) was centrifuged to harvest the microbial biomass and then treated as described above.

Two primer sets were used to selectively amplify bacterial and fungal rDNA fragments. Universal eubacterial forward F341-GC and reverse R907 primers were used to amplify the hypervariable V3–V5 region from the 16S rRNA gene, as previously reported (Yu and Morrison, 2004). The fungal first internal transcribed spacer (ITS1) from the ribosomal DNA was amplified with the primer pair ITS5 and ITS2 (White *et al*., 1990). The forward primer ITS5 and F341 contained the GC clamp (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGG-3'). All PCR reactions were performed on a Mastercycler Personal Thermocycler (Eppendorf, Hamburg, Germany). 25 μL of PCR mixture contained 1.25 U Takara Ex Taq DNA Polymerase (Takara Bio, Otsu, Shiga, Japan), 12.5 mM dNTPs, 0.25 μM of each primer and 100 ng of DNA. 16S rRNA gene PCR reactions were run as described previously (Lladó *et al*. 2009) and each ITS gene PCR reaction was run with a hot start of 94ºC for 5 min followed by 25 cycles of 94ºC for 30 s, 50ºC for 30 s (the annealing temperature began at 60ºC and was lowered by 1ºC in each of the first 10 cycles) and 72ºC for 45 s, with a final elongation step of 5 min at 72ºC.

The PCR amplicons obtained were loaded in two 8% (w/v) polyacrylamide gels with a chemical denaturing gradient ranging from 40% to 60% (100% denaturant contained 7 M urea and 40% formamide (w/v)) and electrophoretically resolved in a DGGE-4001 system (CBS Scientific Company, Del Mar, CA, USA). Electrophoresis was carried out at 60ºC and at 100 V for 16 h in a 1×TAE buffer solution (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, pH 7.4). The DGGE gels were stained for 45 min in 1×TAE buffer solution containing SybrGold™ (Molecular Probes, Inc., Eugene, OR,

USA) and then scanned under blue light using a blue converter plate (UV Products Ltd, Cambridge, UK). Images of DGGE gels were digitalized and the DGGE bands were processed using the Gene Tools software v. 4.0 (SynGene Synoptics, Frederick, MD, USA) and manually corrected. Furthermore, a covariance principal component analysis (PCA) of band types and relative peak area was carried out. Most predominant DGGE bands were excised with a sterile filter tip, resuspended in 50 µL molecular-grade water and stored overnight at 4ºC. A 1:50 dilution of the supernatants was subsequently reamplified by PCR as described previously and sequenced using R907 and ITS2 primers, for the eubacterial and fungal sequences, respectively. Sequencing was carried out using the ABI Prism Big Dye Terminator Cycle Sequencing Reaction Kit v. 3.1 and an ABI 3700 DNA sequencer (both from Perkin Elmer / Applied Biosystems, Waltham, MA, USA), in accordance with the manufacturer's instructions. Sequences were edited using the BioEdit software package v. 7.0.9 (Ibis Biosciences, Carlsbad, CA, USA), inspected for the presence of ambiguous base assignments and subjected to chimera check with Bellerophon version 3 (Huber *et al*., 2004). The sequences were then aligned with the NCBI genomic database using the BLAST search alignment tool. The nucleotide sequences identified in this study were deposited in the GenBank database under accession numbers JQ894738-JQ894777.

2.4. Multiplexed bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP)

Treatments selected for barcoded 16S rRNA and ITS gene pyrosequencing analysis are shown in Table 1. The same DNA extract as used in the DGGE analyses was used for pyrosequencing purposes. A diluted DNA extract (1:10) in ultra-pure water was used as a template for triplicate PCR reactions. Each sample was amplified separately with both 16S rRNA (eubacteria) and ITS (fungi) gene primers containing

unique multiplex identifier (MID) tags. MID1 through MID6, MID25 and MID26 from the extended MID set recommended by Roche Diagnostics (Diagnostics, 2009) were used for ITS gene amplification (fungi). In addition, MID9 through MID14, MID7 and MID16 were used for 16S rRNA gene amplification (eubacteria). Each forward primer began at the 5' end with the primer adaptor A (5'-CCATCTCATCCCTGCGTGTCTCCGAC-3'), followed by the library key sequence (5'-TCAG-3'), the selected MID sequence, and the template sequence. Reverse primers were designed by replacing adaptor A with adaptor B (5'-CCTATCCCCTGTGTGCCTTGGCAGTC-3'), without key and MID sequences. The template specific to the 16S rRNA gene amplification were the forward primer 341F (5'-CCTACGGGAGGCAGCAG-3') (Kolton *et al*., 2011) and the reverse primer 802R (5'-TACCAGGGTATCTAATCC-3') (dos Santos *et al*., 2011), whilst primer pair ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3') (Buée *et al*., 2009) were used to perform the fungal amplicon libraries. Reactions were carried out following the same PCR program used for the DGGE analyses, as described above. The triplicate reactions for each sample were pooled and purified with a PCR clean-up system (Promega, WI, USA) and eluted in 50 µL of ultra-pure water. The DNA concentration of pooled amplicons was then measured using Quant-iT Picogreen dsDNA Kit (Invitrogen, Carlsbad, CA, USA) prior to combining them all (16S and ITS libraries) into a single sample at a concentration suitable for the pyrosequencing protocol. The sample was finally submitted to the Genomic Department of the Parc Científic de Barcelona (University of Barcelona) for sequencing, using the 454 Life Sciences Titanium Platform (Roche Diagnostics, Branford, CT, USA).

2.5. Pyrosequencing data analysis

The trimming of the 16S barcoded sequences into libraries was carried out using the Ribosomal Data Project-II (RDP-II) pyrosequencing pipeline (http://pyro.cme.msu.edu/). This resulted in eight distinct datasets. Sequence alignment and subsequent clustering were performed using the same pipeline (Cole *et al*., 2009). Datasets were individually classified using the RDP Classifier tool with a bootstrap cutoff of 80%. Chimera check and alpha- and beta-diversity analyses were carried out using MOTHUR software version 1.24.0 (Schloss *et al*., 2009).

Segregation of the ITS barcoded sequences into libraries was carried out using MOTHUR software version 1.24.0. This again resulted in eight distinct datasets. Sequence alignment, clustering, chimera check and alpha and beta-diversity analyses were also performed using MOTHUR software version 1.24.0 (Schloss *et al*., 2009). BLAST+ 2.2.25 was used in order to taxonomically classify the ITS sequences. Analysis of BLAST output files was performed using MEGAN software version 4.0 (Huson *et al*., 2011).

Data from pyrosequencing datasets were submitted to the Sequence Read Archive of the National Center for Biotechnology Information (NCBI) under the study accession number SRA051395.

3. Results and discussion

3.1. DGGE analyses of microbial communities in soil

In order to analyze the predominant bacterial and fungal populations in the creosote-polluted soil and their response to different bioremediation and bioaugmentation treatments, two different DGGEs were carried out (Fig. 1). To obtain further insight into the similarity of the profiles, a multivariate approach based on

principal component analysis (PCA) was performed (Fig. 2). Similar values of cultivable heterotrophic bacteria (CHB) were quantified among the treatments by means of MPN and qPCR (Table 1).

The DGGE results revealed that the addition of MAs (Brij 30 and soybean oil) caused dramatic shifts in the autochthonous bacterial populations (Fig. 2), as described previously (Colores *et al*., 2000). Indeed, a more notable variation in the 16SrRNA DGGE profiles was observed when the soil was biostimulated with moisture and soybean oil (IC+SO) than when the biostimulation was carried out with moisture and the non-ionic surfactant Brij 30 (IC+Br30), or even when no MAs were added (IC) (Fig. 2A). This was also observed when the soil was biostimulated with LS after 60 days of incubation (Fig. 2A). Again, when Brij 30 was added to the soil, the bacterial community seemed to shift differently than when soybean oil or no MA was added. This phenomenon was not so obvious when the soil was bioaugmented with *T. versicolor* or *L. tigrinus*, probably due to the antagonistic effect of an active native population in the creosote-polluted soil (Lladó *et al*., 2012a submitted).

However, the addition of MAs did not promote a fungal population shift when the diversity was studied by means of ITS-DGGE. Figure 1B shows how the most important ITS bands coincide in the soil DGGE profiles in the IC, IC+SO and IC+Br30 treatments. In order to confirm this, pyrosequencing analyses were carried out.

Indeed, the addition of LS and WRF did not seem to produce a remarkable shift in the autochthonous fungal population in microcosms. Furthermore, the differences seen in Figure 2 with respect to the LT-LS+Mn, LT-LS+SO+Mn and LT-LS+Br30+Mn treatments could be caused by a low signal profile (Fig. 1, lane 20) or the different positions of similar bands (Fig. 1, lane 21 and 22). However, as reported elsewhere

(Lladó *et al*., 2012b submitted), the occurrence of multiple ITS banding in single fungal populations could be a problem when working with environmental samples.

Although DGGE is an economic molecular technique that allows the simultaneous observation of the bacterial and fungal community dynamics of multiple real polluted soil samples, further analysis is needed in order to gain more in-depth knowledge about the real composition of complex microbial communities.

3.2. Potential PAH degraders studied by MPN-DGGE

In order to analyze the soil's cultivable hydrocarbon-degrading bacteria that can use PAHs as a sole source of carbon and energy, as well as their response to the different bioremediation treatments, a DGGE analysis (Fig. 3) using DNA obtained from the more diluted wells used to enumerate the CHDB was carried out. Low CHDB percentages with respect to CHB (Table 1) hamper the visualization and successful sequencing of the DNA corresponding to CHDB microorganisms when general DNA from the soil is used for DGGE. Indeed, the great simplicity of the DGGE profiles allowed 42 bands to be excised and successfully sequenced (Table 2). Band 4 and band 39 were considered as chimera.

Firstly, the polluted soil in the initial conditions (post biopile treatment) still presented microorganisms that could be related to PAH-degraders such as different α-Proteobacteria (Sphingomonadaceae and Caulobacteraceae) and Actinobacteria like the *Mycobacterium* genre (Guo *et al*., 2010). It is well known that acclimatization of microbial communities to the soil pollutants is a key factor in increasing degradation (Haritash and Kaushik, 2009). In the present study, this was expected because of the age of the pollutants in the soil and because the levels of CHDB were high after the dynamic biopile reached values of $3.89 \pm 0.85 \cdot 10^7$ MPN $\cdot$ kg^{-1} (Realp *et al*., 2008).

Indeed, because of this high period of acclimatization, it was surprising that new bands belonging to β- and γ-Proteobacteria families (i.e. Comamonadaceae and Xanthomonadaceae, respectively) appeared in the IC treatment, where only the optimum water content was added to the soil. A phylotype (position band B8 in Fig. 3) closely related to the *Pseudoxanthomonas* genus belonging to γ-Proteobacteria is recurrent in this soil (Llado *et al*., 2009) and it has been described as an HMW-PAH degrader (Nayak *et al*., 2010). The *Pseudoxanthomonas* genus was found in practically all of the bioremediation treatments in the present work, even those in which the soil was biostimulated by the addition of LS.

In addition, the toxic effect of the Brij 30 on the CHDB populations observed, in terms of MPN counts, in the IC+Br30 treatment (Lladó *et al*., 2012a submitted), was concomitant with a shift observed in the biodiversity studied by means of MPN-DGGE, compared to the IC and IC+SO treatments. Such a decrease in the size of the PAH-degrading population, as well as the shift in the diversity structure of the CHDB population (MPN-DGGE profiles), could explain the lower degradation rate of 4-ring PAHs detected when Brij 30 was added to the soil, with the exception of the BS-LS+Br30+Mn^{2+} treatment (Lladó *et al*., 2012a submitted).

On the other hand, the addition of soybean oil to the soil in the IC+SO treatment, which was associated with a higher rate of benzo(a)pyrene depletion (Lladó *et al*., 2012a submitted), seemed to promote the occurrence of band 9, a phylotype closely related to α-proteobacteria belonging to the genus *Agrobacterium*. Bacteria in the *Rhizobiaceae* family are commonly found in polluted environments (Keum *et al*., 2006), but no evidence of benzo(a)pyrene degradation enhancement has been found in the literature. Furthermore, band 9 in the IC+SO treatment coincides with band 29 in the BS-LS+SO+Mn^{2+} treatment, in which soybean oil amendment was concomitant with LS

and manganese ions. Indeed, in the BS-LS+SO+Mn^{2+} treatment the degradation rates of 5-ring PAHs were significantly higher than in the other treatments (in the same range as observed with the BS-LS+Br30+Mn^{2+} treatment). It is well known that benzo(a)pyrene may be biodegraded by bacteria by means of co-metabolic pathways (Kanaly and Bartha, 2009); therefore, although it is not demonstrated in the present study, it may also be possible for soil bacteria to use the MA (Brij 30) as a carbon source (Boopathy, 2002), while degrading 5-ring PAHs by co-metabolic pathways.

In addition, band 34 is closely related to β-proteobacteria belonging to the genus *Cupriavidus*. This genus, which has been associated with benzo(a)pyrene degradation in stable-isotope probing (SIP) assays (Jones, 2010), only appeared in the MPN plates when Brij 30 was combined with LS and manganese ions (the BS-LS+Br30+Mn^{2+} treatment), which would suggest a potential key role in HMW-PAH degradation. Further research is needed to better ascertain the PAH-degrading capacity of *Cupriavidus* strains isolated from PAH-polluted soils.

Moreover, wheat straw may also be used as a carbon source by bacteria (Andersson and Henrysson, 1996), since it is another putative activator of co-metabolic PAH-degrading pathways. Therefore, the addition of LS promoted the appearance of phylotypes belonging to Alcaligenaceae (β-proteobacteria) closely related to *Pigmentiphaga*, which was detected in practically all of the LS-biostimulated treatments, except those where *T. versicolor* or *L. tigrinus* were inoculated. Therefore, the present study proposes *Pigmentiphaga*, a genus recently associated with naphthalene, phenanthrene and anthracene degradation (Jones, 2010), as another potential key player in the HMW-and PAH-biodegradation processes.

3.3. 16S barcoded pyrosequencing analyses

In order to reach a more in-depth understanding of the most predominant microbial taxa in the soil, as well as their shifts during the biostimulation and bioaugmentation treatments (Fig. 4), pyrosequencing analysis was carried out. In addition, statistical analyses were performed in order to obtain the richness, diversity and sample coverage indices (Table 3). Indeed, the biodiversity detected was high in the initial soil, where Proteobacteria and Actinobacteria were the dominant phyla (Fig. 4A). There is substantial variability in the abundance of members of different phyla in different soils, but Proteobacteria and Actinobacteria are commonly present at very high levels (Janssen, 2006). Moreover, α- and γ-Proteobacteria were identified as the most important families in the initial soil (Fig. 4B). This fact coincided with a previous study (Viñas *et al*., 2005) carried out with the same creosote-polluted soil, but prior to the field-scale biopile. Indeed, the experimental design was a feasibility assay to ensure the success of a set of different biostimulation strategies applied to the soil. In that previous study, when no nutrients were added, α- and γ-Proteobacteria were the predominant groups in the second and final incubation periods.

In addition, among all the families detected in the initial soil through pyrosequencing, Sphingomonadaceae (α-Proteobacteria) was the most important, making up 18.7% of the total sequence diversity (Supplementary Information Table S1). Due to their broad presence in polluted sites and their wide range of metabolic pathways, members of the Sphingomonadaceae family are considered to be powerful PAH degraders in soils (Edel-Hermann *et al*., 2009; Leys *et al*., 2005). Although *Sphingomonas* genera made up 51.2% of the total family (Supplementary Information Table S2), no members of this family were detected in the MPN-DGGE analyses of the initial soil. Such important differences between the methods (DGGE from MPN plates and pyrosequencing from total soil diversity) could be because it is not possible to

compare culture-based dependent methods with molecular methods. In addition, the pre-growing step in the PAH mixture (MPN) changed the populations to those more closely related to PAH biodegradation; however, no pyrosequencing was performed with MPN samples and therefore no comparisons can be made.

On the other hand, although the Mycobacteriaceae (Actinobacteria) family represented only 2.4% of all the bacterial diversity present in the initial soil, *Mycobacterium* was found in the MPN-DGGE profile, which would suggest that growth of this bacterial genus on the MPN plates is better than growth of other genera with the capacity to metabolize PAHs and with more presence in the soil, or a greater affinity with the V3-V5 DGGE primers used.

In Figure 6A, the bacterial community shifts caused by the different biotreatments are represented by means of a dendrogram. After 60 days, there was not as much change in the bacterial populations in the soil biostimulated with moisture alone (IC) as with the other treatments, and the Shannon diversity index remained almost the same, trivializing the detection of some new bands in the corresponding DGGE-MPN profile. This phenomenon correlated with a study by Viñas *et al*. (2005), in which from days 135 to 200 of soil incubation at optimum water content, the DGGE profiles of the soil showed practically no change, while in the first days of incubation, the same profiles shifted hugely. This fact confirms that, when carbon and nutrients become scarce in this soil, bacterial diversity remains stable.

Nevertheless, in line with the DGGE results of the soil, the addition of both MAs, soybean oil and Brij 30, led to a remarkable shift in the total bacterial population, with the treatment involving addition of the non-ionic surfactant (IC+Br30) causing the greatest change with respect to the initial soil (Fig. 6A). Again, pyrosequencing data suggest that the bacterial population shifts and the decrease observed in the CHDB

MPN counts (Lladó *et al*., 2012a submitted) were caused by the toxic effect of the non-ionic surfactant Brij 30 and could be the main reason for the decrease observed in the degradation rate of the 4-ring PAHs in the treatments where this MA was added to the soil (Lladó *et al*., 2012a submitted).

However, the community shifts observed by means of both DGGE and pyrosequencing in the IC+SO treatment were concomitant with higher CHB-MPN counts but lower HMW-PAH depletion levels (Lladó *et al*., 2012a submitted), suggesting that soybean oil was being used as a carbon source by the bacteria.

On the other hand, in both MA treatments (IC+SO and IC+Br30), the relative importance of Proteobacteria in the community increased, especially when Brij 30 was added. Soybean oil (SO) promoted the presence of Sphingomonadaceae-based phylotypes, which reached 33.6% of all 16SrDNA sequences, while Brij 30 produced an increase in Proteobacteria diversity, particularly the γ-Proteobacteria family (Supplementary Information Table S2). This fact suggests that Sphingomonadaceae may take advantage of other bacteria when soybean oil is utilized as an additive.

Moreover, when the MPN DGGE was analyzed, the class of *Rhizobiaceae*, which supposedly plays a role in co-metabolic benzo(a)pyrene depletion, was present at low percentages (< 1%) in the IC+SO pyrosequencing results. The causes for the presence of the *Mycobacterium* genus in the initial soil, mentioned above in section *3.2.*, could explain these different results when the two molecular techniques are compared with each other. Therefore, further research is needed in order to relate the benzo(a)pyrene degradation detected in the IC+SO treatment to a key bacterial or fungal player.

On the other hand, the addition of LS produced an increase in the Shannon diversity index (Table 3) with respect to the IC+SO and IC+Br30 treatments, suggesting

that a wider range of bacteria can grow from the white straw or synergically with the native fungal population detected (Lladó *et al*., 2012a submitted), which may use the LS as the sole source of carbon and energy, rather than the soybean oil and Brij 30 when they were concomitantly added. However, although the Shannon index was higher, meaning that there was a higher number of bacterial species in the soil, the type of MA added to the soil had a greater effect than the addition of LS on the bacterial community changes (Fig. 6A), which confirms the changes already detected in the general soil DGGE profiles.

Furthermore, the addition of LS led to an important decrease in the relative importance of the Sphingomonadaceae family in the soil (Table S1), but no important or common increase in any bacterial family, including the genus *Pigmentiphaga* detected in the MPN DGGE, was detected in those treatments where the PAH-biodegradation rates were higher (BS-LS+SO+Mn^{2+} and BS-LS+Br30+Mn^{2+}). Instead, a higher relative importance of the Xanthomonadaceae (15.9%) and Burkholderiaceae (10.8%) families was detected in the BS-LS+SO+Mn^{2+} and BS-LS+Br30+Mn^{2+} treatments, respectively (Table S1). The huge ratios of phylotypes closely related to Burkholderiaceae in the BS-LS+Br30+Mn^{2+} treatment confirmed the possible importance of the *Cupriavidus* genre in HMW-PAH depletion when LS and Brij 30 were added to the polluted soil, as mentioned above in Section *3.2*. However, there was no evidence of the importance of the *Agrobacterium* genus in PAH degradation in the soil-pyrosequencing results when soybean oil was mixed with the creosote-polluted soil.

Finally, the 16SrDNA pyrosequencing libraries showed that the bacterial diversity detected in TV-LS+SO+Mn^{2+} and LT-LS+SO+Mn^{2+} was very similar to that found in the BS-LS+SO+Mn^{2+} treatment, which probably confirms the low colonization

rates achieved by both WRF (Lladó *et al*., 2012a submitted), which did not produce any significant pressure on the native bacterial community.

3.4. ITS barcoded pyrosequencing analyses

As far as we know, this is the first study that employs barcoded pyrosequencing in order to study the fungal community shifts in a PAH-polluted soil through different bioremediation treatments. It is also essential to study the roles and dynamics of fungi in historically aged contaminated sites and their potential interactions with the native bacterial communities. Indeed, fungi are highly plastic and tolerant and therefore have advantages over bacteria; they regenerate not only via spores but also via hyphal fragments, and have a higher capacity to reach pollutants due to hyphal elongation and extracellular enzyme utilization (Singh, 2006; Hidayat *et al*., 2012). In the present study, soil moisture was adjusted to 60% of its water-holding capacity in order to stimulate fungal growth conditions, while previous studies with the same creosote soil had always been carried out at 40% (Viñas *et al*., 2005), and temperature was maintained at 28ºC (manuscript in preparation).

Table 4 shows the surprisingly high fungal biodiversity present in a soil polluted with such a highly toxic antifungal as creosote. These results do not coincide with those of the DGGE, probably due to either the low signal present in the initial soil profile or the high signal sharing in numerous DGGE bands, which would confirm that DGGE underestimates the fungal diversity in the microcosms.

Fusarium (23.2%) and *Scedosporium* (24.8%) were the two predominant fungal genera in the soil at the end of the biopile process and before commencement of the biotreatments defined in the present study. Both genera have previously been identified as PAH degraders (Al-Turki, 2009; Lladó *et al*., 2012; Thion *et al*., 2012).

On the other hand, when both soybean oil and Brij 30 were added to the soil, *Fusarium* achieved more than 90% relative importance in the soil, which demonstrates its capacity to adapt to the new soil conditions (Fig. 5). This fact suggests a high rate of adaptation to the soil environment and a huge degree of competitiveness when both moistures achieved optimum values and new carbon sources were added. In fact, *Fusarium* genera are ubiquitous in soils and contain pathogen and saprophyte species that can produce different types of mycotoxins with the capacity to outcompete other fungal species (Summerell *et al*., 2003).

However, the addition of LS involved a less constrictive effect on the fungal biodiversity, although *Fusarium* was still the predominant genus. In these cases, *Scedosporium* also took advantage of the sterile and non-colonized substrate, while other genera maintained a much more marginal growth. The pyrosequencing results proved that both genera were the fastest at colonizing the LS, while the ITS qPCR results (Table 1) confirmed the remarkable growth of the native fungal communities observed in the microcosms when the white straw was added to the system (Lladó *et al*., 2012a submitted). Interestingly, as mentioned above, previous literature associated *Fusarium* and *Scedosporium* with PAH biodegradation in soils based on their laccase production (Saparrat *et al*., 2000; Canero & Roncero, 2008). However, in the present study, where both genera colonized the soil at high rates in all of the samples processed, it is not possible to describe a direct relationship between the higher HMW-PAH depletion of the BS-LS+SO+Mn^{2+} and BS-LS+Br30+Mn^{2+} treatments and the presence of those native fungi in the soil. However, it is certainly the first step to gaining knowledge on the interaction of native fungi with other microbial populations, thus conditioning bioremediation success in aged polluted industrial soils.

Moreover, the growth of *Fusarium* and *Scedosporium* did not produce any appreciable shifts in the bacterial community, which was in line with another previous study (Edel-Hermann *et al.*, 2009), in which a native *Fusarium*, isolated from the same soil treated, was inoculated into the soil. However, as was the case for bacteria, the population-structure shifts in the fungal community also seemed to be caused by the addition of surfactant much more so than by the addition of LS (Fig. 6B).

Finally, by means of pyrosequencing *T. versicolor*, ITS phylotypes were detected at low percentages in the TV-LS+SO+Mn^{2+} treatment, while no sequence of *L. tigrinus* was found in the LT-LS+SO+Mn^{2+} microcosms. This fact confirmed the antagonistic effect experienced by bioaugmented WRF strains (Lladó *et al.*, 2012a submitted), an effect that would obviously be hampered by the outstanding autochthonous fungal and bacterial soil populations.

4. Conclusions

Here we report that barcoded pyrosequencing is a powerful molecular tool for gaining insight into microbial diversity in contaminated soils and its dynamics throughout bioremediation processes. As a complement to DGGE analyses, the genus *Cupriavidus* could play an important role in HMW-PAH degradation in aged creosote-polluted soil. The high capacity for adaptation of the fungal genera *Fusarium* and *Scedosporium* to soil conditions was also demonstrated.

The pyrosequencing results confirmed the failure of exogenous WRF to colonize the creosote-polluted soil, probably due to antagonistic interactions with the highly represented indigenous microbiota, which confirms the importance of increasing knowledge of the role of certain native fungi and bacteria in real industrial soil bioremediation processes.

We also conclude that adding MAs to a contaminated soil in order to enhance the bioavailability of pollutants could lead to important community shifts involving changes in the biodegradability of the compounds.

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References

Al-Turki, A.I., 2009.Microbial polycyclic aromatic hydrocarbons degradation in soil. Res. J. Environ. Toxicol.3,1-8.

Andersson, B.E., Henrysson, T., 1996. Accumulation and degradation of dead-end metabolites during treatment of soil contaminated with polycyclic aromatic hydrocarbons with five strains of white-rot fungi. Appl. Microbiol. Biotechnol.46,647-652.

Boopathy, R., 2002. Effect of food-grade surfactant on bioremediation of explosives-contaminated soil. J. Hazard. Mater.92,103-114.

Buée, M., Reich, M., Murat, C., Morin, E., Nilsson, R.H., Uroz, S., 2009. 454 pyrosequencing analyses of forest soils reveal an unexpectedly high fungal diversity. New Phytol.184,449-456.

Cerniglia, C.E., 1997. Fungal metabolism of polycyclic aromatic hydrocarbons: past, present and future applications in bioremediation. J. Ind. Microbiol. Biotechnol. 19,324-33.

Chung, N., Alexander, M., 1999. Effect of Concentration on Sequestration and Bioavailability of Two Polycyclic Aromatic Hydrocarbons. Environ. Sci. Technol.33: 3605–3608.

Cole, J.R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R.J., 2009. The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucl. Acid. Res.37,141-145.

Colores, G.M., Macur, R.E., Ward, D.M., Inskeep, W.P., 2000. Molecular analysis of surfactant-driven microbial population shifts in hydrocarbon-contaminated soil. Appl. Environ. Microbiol.66,2959-2964.

Covino, S., Cvancarova, M., Muzikár, M., Svobodová, K., D'Annibale ,A., Petruccioli M., Federici, F., Kresinová, Z., Cajthaml, T., 2010. An efficient PAH-degrading Lentinus (Lentinus) tigrinus strain: Effect of inoculum formulation and pollutant bioavailability in solid matrices. J. Hazard. Mater.183,669-676.

Diagnostics Roche. 2009. Using multiple identifier (MID) adaptors for the GS FLX titanium chemistry-extended MID set. Technical Bulletin: Genome Sequencer FLX System.

dos Santos, H.F., Cury, J.C., do Carmo, F.L., dos Santos, A.L., Tiedje, J., 2011.Mangrove Bacterial Diversity and the Impact of Oil Contamination Revealed by Pyrosequencing: Bacterial Proxies for Oil Pollution. PLoS ONE.

Edel-Hermann, V., Brenot, S., Gautheron, N., Aimé, S., Alabouvette, C., Steinberg, C., 2009. Ecological fitness of the biocontrol agent Fusarium oxysporum Fo47 in soil and its impact on the soil microbial communities. FEMS Microbiol. Ecol. 68,37-45.

Federici, E., Leonardi, V., Giubilei, M.A., Quaratino, D., Spaccapelo, R., D'Annibale, A., Petruccioli, M., 2007. Addition of allochthonous fungi to a historically contaminated soil affects both remediation efficiency and bacterial diversity. Appl. Microbiol. Biotechnol.77,203-211.

Guo, C., Dang, Z., Wong, Y., Tam, N.F., 2010. Biodegradation ability and dioxgenase genes of PAH-degrading Sphingomonas and Mycobacterium strains isolated from mangrove sediments. Int. Biodet. Biodeg.64,419-426.

Haritash, A.K., Kaushik, C.P., 2009. Biodegradation aspects of Polycyclic Aromatic Hydrocarbons (PAHs): A review. J. Hazard. Mater.169,1-15.

Huber, T., Faulkner, G., Hugenholtz, P., 2004. Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. Bioinformatics.20, 2317-2319.

Huson, D.H., Mitra, S., Ruscheweyh, H.J., Weber, N., Schuster, S.C., 2011. Integrative analysis of environmental sequences using MEGAN 4. Gen. Res.21,1552-1560.

Janssen, P.H., 2006. Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. Appl. Environ. Microbiol.72,1719-1728.

Jones, M.D., 2010. Stable-isotope probing-based investigations of polycyclic aromatic hydrocarbon-degrading bacteria in contaminated soil. University of North Carolina, Chapel Hill.

Kanaly, R.A., Bartha, R., 2009. Cometabolic mineralization of benzo[a]pyrene caused by hydrocarbon additions to soil. Environ. Toxicol. Chem.18,2186-2190.

Keum, Y.S., Seo, J.S., Hu, Y., Li, Q.X., 2006.Degradation pathways of phenanthrene by Sinorhizobium sp. C4. Appl. Microbiol. Biotechnol.71,935-941.

Kolton, M., Meller, H.Y., Pasternak, Z., Graber, E.R., Elad, Y., Cytryn, E., 2011. Impact of biochar application to soil on the root-associated bacterial community structure of fully developed greenhouse pepper plants. Appl. Environ. Microbiol.77,4924-4930.

Leys, N.M., Bastiaens, L., Verstraete, W., Springael, D., 2005. Influence of the carbon/nitrogen/phosphorus ratio on polycyclic aromatic hydrocarbon degradation by Mycobacterium and Sphingomonas in soil. Appl. Microbiol. Biotechnol.66,726-736.

Lladó, S., Jimenez, N., Vinas, M., Solanas, A.M., 2009.Microbial populations related to PAH biodegradation in an aged biostimulated creosote-contaminated soil. Biodegradation.20,593-601.

Lladó, S., Solanas, A.M., de Lapuente, J., Borràs, M., Viñas, M. 2012. A diversified approach to evaluate biostimulation and bioaugmentation strategies for heavy-oil-contaminated soil. Sci. Total. Environ.435-436,262-269.

Muyzer, G., Smalla, K.,1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie Van Leeuwenhoek.73,127-141.

Nayak, A.S., Sanganal, S.K., Mudde, S.K., Oblesha, A., Karegoudar, T.B., 2010.Acatabolic pathway for the degradation of chrysene by Pseudoxanthomonas sp. PNK-04. FEMS Microbiol. Lett.320,128-134.

Realp, E., Doménech, J.A., Martinez-García, R., Restrepo, C., Lladó, S., Viñas, M., Solanas, A.M., 2008.Ensayo piloto de biorremediación por la tecnología de la biopila dinámica para la descontaminación de suelos contaminados por creosotas provenientes de las actividades dedicadas a la preparación de la madera. Residuos: Revista técnica.102: 38-49.

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., Sahl, J.W., Stres, B., Thallinger, G.G., Van Horn, D.J., Weber, C.F., 2009.Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl. Environ. Microbiol.75,7537-7541.

Singleton, D.R., Richardson, S.D., Aitken, M.D., 2011. Pyrosequence analysis of bacterial communities in aerobic bioreactors treating polycyclic aromatic hydrocarbon-contaminated soil. Biodegradation.22,1061-1073.

Thion, C., Cébron, A., Beguiristain, T., Leyval, C., 2012. PAH biotransformation and sorption by Fusarium solani and Arthrobacter oxydans isolated from a polluted soil in axenic cultures and mixed co-cultures. Int.Biodet. Biodeg.68,28-35.

Torsvik, V., Ovreas, L., 2002. Microbial diversity and function in soil: from genes to ecosystems. Curr. Opin. Microbiol.5,240-245.

Urich, T., Lanzen, A., Qi, J., Huson, DH., Schleper, C., Schuster, S.C., 2008. Simultaneous Assessment of Soil Microbial Community Structure and Function through Analysis of the Meta-Transcriptome. PLoS ONE.

van Elsas, J.D., Boersma, F.G.H., 2011.A review of molecular methods to study the microbiota of soil and the mycosphere. Eur. J. Soil Biol.47,77-87.

Viñas, M., Sabate, J., Espuny, M.J., Solanas, A.M., 2005.Bacterial community dynamics and polycyclic aromatic hydrocarbon degradation during bioremediation of heavily creosote-contaminated soil. Appl. Environ. Microbiol. 71,7008-7018.

Wang, Q., Garrity, G.M., Tiedje, J.M., Cole, J.R., 2007. Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl. Environ. Microbiol.73(16),5261-5267.

White, T.J., Bruns, T.D., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR Protocols: A Guide to Methods and Applications.315-322.

Yu, Z., Morrison, M., 2004. Comparisons of different hypervariable regions of rrs genes for use in fingerprinting of microbial communities by PCR-denaturing gradient gel electrophoresis. Appl. Environ. Microbiol.70,4800-4806.

Figure Captions

Fig. 1. Denaturing Gradient Gel Electrophoresis profiles of PCR-amplified 16S rRNA (V3-V5 regions) (A) and ITS1 gene fragments (B) of soil microbial communities. In both gels the lanes are arranged in the same order. From right to left: Lane 1, Initial Soil; Lane 2, IC; Lane 3, IC+SO; Lane 4, IC+Br30; Lane 5, BS-LS; Lane 6, BS-LS+SO; Lane 7, BS-LS+Br30; Lane 8, BS-LS+ Mn^{2+}; Lane 9, BS-LS+ SO+ Mn^{2+}; Lane 10, BS-LS+Br30+Mn^{2+}; Lane 11, TV-LS ; Lane 12, TV-LS+SO; Lane 13, TV-LS+Br30; Lane 14, TV-LS+Mn^{2+}; Lane 15, TV-LS+SO+Mn^{2+}; Lane 16, TV-LS+Br30+Mn^{2+}; Lane 17, LT-LS ; Lane 18, LT-LS+SO; Lane 19, LT-LS+Br30; Lane 20, LT-LS+Mn^{2+}; Lane 21, LT-LS+SO+Mn^{2+}; Lane 22, LT-LS+Br30+Mn^{2+}. Both gels are carried out at a denaturing concentration from 40% to 60%.

Fig. 2. Principal component analysis (PCA) of the 16SrRNA and ITS DGGE.

Fig. 3. Denaturing Gradient Gel Electrophoresis profiles of PCR-amplified 16S rRNA gene fragments (V3-V5 regions) of last positive dilution of MPN plates. Numbers are disposed at the left side of the corresponding band.Gel was carried out at a denaturing concentration from 40% to 60%.

Fig. 4. Eubacterial biodiversity composition, in relative abundance (%), of different phyla based on the classification of partial 16S rRNA sequences of bacteria from soil microcosms using RDP-classifier. Phyla **(A)**; Proteobacteria classes **(B)**.

Fig. 5. Fungal biodiversity composition, in relative abundance (%), of different genera based on the classification of partial ITS1 sequences of fungi from soil using the BLAST nt database.

Fig. 6. Thetayc cluster tree showing the relationship of bacterial **(A)** and fungal **(B)** communities in the different microcosms to one another based on pyrosequence libraries. The scale bar is the distance between clusters in Thetayc units.

Table 1: Treatment description, molecular analyses carried out and summary of chemical and microbiological results (Lladó et al., 2012a submitted)

Treatment[a]	DGGE 16/ITS	DGGE 16S MPN	Pyrosequencing 16S/ITS	TPH[b]	4-ring PAHs	5-ring PAHs	CHDB[c]	qPCR[d] 16S	qPCR ITS
Initial Soil	+	+	+	2815±233	272±10	117±4	6.37±0.07 (21%)	6.45±0.25	6.56
IC	+	+	+	1439±51	96±10.2	80±3	6.21±0.01 (6.9%)	7.34±0.23	6.92
IC + SO	+	+	+	1395±30	157±8.1	71±2	6.32±0.02 (0.9%)	6.83	6.78
IC + Br30	+	+	+	1515±179	214±8.4	77±3	3.07±0,06 (0.004%)	6.80±0.1	6.74±0.12
BS-LS	+	+	-	1077±242	76±3.3	62±5	6.76±0.16 (2.2%)	9.12±0.1	8.79±0.27
BS-LS + SO	+	+	-	1098±207	77±12	55±6	6.07±0.05 (0.2%)	9.46±0.07	9.04±0.05
BS-LS + Br30	+	+	-	1255±68	115±12	62±3	5.36±0.07 (0.08%)	9.19±0.14	8.67±0.5
BS-LS + Mn^{2+}	+	+	+	1106±26	63±3.9	54±1	6.74±0.26 (2.6%)	9.31±0.1	8.71±0.25
BS-LS + SO + Mn^{2+}	+	+	+	810±27	56±7.8	37±2	6.03±0.01 (0.15%)	9.32±0.34	9.24
BS-LS + Br30 + Mn^{2+}	+	+	-	766±27	58±3.3	33±2	5.20±0.22 (0.05%)	8.86±0.22	8.51±0.57
TV-LS	+	-	-	1545±153	77±5.6	78±6	6.11±0.02 (0.62%)	10.2±0.21	8.64±0.19
TV-LS + SO	+	-	-	1338±204	81±9	68±0.1	6.98±0.18 (3.8%)	9.70±0.1	9.62±0.64
TV-LS + Br30	+	-	-	1552±29	127±10.7	70±3	5.65±0.09 (0.14%)	8.92±0.52	9.41±0.21
TV-LS + Mn^{2+}	+	-	-	1417±155	78±12	70±8	6.06±0.21 (0.24%)	9.44±0.35	8.88±0.34
TV-LS + SO + Mn^{2+}	+	+	+	1449±65	94±11	62±3	7.71±0.06 (16.8%)	9.44±0.38	9.23±0.59
TV-LS + Br30 + Mn^{2+}	+	-	-	1436±60	114±10	67±3	6.07±0.05 (0.65%)	9.27±0.29	9.00±0.35
LT-LS	+	-	-	1396±15	79±6.5	70±2	6.19±0.37 (1.05%)	9.38±0.06	8.94±0.08
LT-LS + SO	+	-	-	1467±170	91±14	64±9	7.66±0.17 (12%)	9.62±0.19	9.51±0.16
LT-LS + Br30	+	-	-	1578±42	131±14	70±3	5.72±0.20 (0.14%)	9.45±0.06	8.92±0.28

LT-LS + Mn^{2+}	+	-	-	1147±53	61±6.7	58±2	5.60±0.03 (0.16%)	9.38±0.16	8.96±0.06
LT-LS + SO + Mn^{2+}	+	+	+	1093±71	72±13	51±2	7.62±0.24 (27.6%)	9.64±0.24	9.55±0.35
LT-LS + Br30 + Mn^{2+}	+	-	-	1260±2	112±3.7	69±0.2	6.11±0.01 (0.19%)	9.21±0.7	8.66±0.72

[a] IC:Incubation Control; BS:Biostimulation; TV:*Trametes versicolor*; LT:*Lentinus tigrinus*; LS:Lignocellulosic Substrate; SO:Soybean Oil; Br30:Brij 30; Mn^{2+}: Manganese ions.

[b] All concentrations are expressed as mg · kg^{-1} of dry soil and data are the means of three independent experiments.

[c] Cultivable PAHs-degrading specialized bacteria (CHDB), expressed as Log MPN g^{-1} soil and CHDB/CHB percent ratios; data are the means of three independent experiments.

[d] 16SrRNA and ITS region gene copies quantified by qPCR, expressed as Log gene copies g^{-1}; data are the means of three independent experiments.

Table 2:Properties of DGGE bands: designations and accession numbers for the band sequences and levels of similarity to related organisms

Band	Band detection[a]									Length (bp)	Closest organism in GenBank database (accession no.)	% similarity[b]	Phylogeneticgroup[c]
	InitialSoil	IC	IC SO	IC Br30	BS	BS SO	BS Br30	TV SO	LT SO				
16S B1	X	-	X	-	-	X	-	-	-	480	*Agrobacterium tumefaciens*(NR_041396.1)	99%	*Rhizobiaceae*(α)
16S B2	X	-	-	-	-	-	-	-	-	455	*Bradyrhizobiumliaoningense*(NR_041785.1)	99%	*Bradyrhizobiaceae*(α)
16S B3	X	-	X	-	-	X	-	X	-	459	*Rhizobium oryzae* (NR_044393.1)	98%	*Rhizobiaceae*(α)
16S B5	X	-	-	-	-	-	-	X	X	495	*Mycobacterium monacense*(NR_041723.1)	100%	*Mycobacteriaceae*(Actinobacteria)
16S B6	-	X	-	-	-	-	-	-	-	352	*Hydrogenophagaintermedia*(NR_024856.1)	99%	*Comamonadaceae*(β)
16S B7	-	X	-	X	-	-	-	-	-	350	*Achromobacterdenitrificans*(NR_042021.1)	99%	*Alcaligenaceae*(β)
16S B8	-	X	X	X[e]	X	X	X	X	-	496	*Pseudoxanthomonasmexicana*(NR_025105.1)	98%	*Xanthomonadaceae* (γ)
16S B12	-	-	-	X	X	X	X	-	-	481	*Pigmentiphagakullae*(NR_025112.1)	98%	*Alcaligenaceae*(β)
16S B16	-	X[e]	X	-	X	-	-	-	-	468	*Azoarcusindigens*(NR_024851.1)	97%	*Rhodocyclaceae*(β)
16S B33	-	-	-	-	-	X	-	-	-	486	*Mycobacterium rutilim*(NR_043761.1)	100%	*Mycobacteriaceae*(Actinobacteria)
16S B34	-	-	-	-	-	-	X	-	-	501	*Cupriaviduscampinensis*(NR_025137.1)	100%	*Burkholderiaceae*(β)
16S B36	-	-	-	-	-	-	X	-	-	507	Uncultured soil bacterium clone from a PAHs polluted soil (DQ907006)	100%	-
16S B41	-	-	-	-	-	-	-	-	X	503	*Bordetellahimzii*(NR_027537.1)	98%	*Alcaligenaceae*(β)

[a]Band detection (+) above 1% of relative intensity.
[b]SequenceswerealignedagainsttheGenBankdatabasewiththe BLAST searchalignmenttool.
[c]Phylogenetic groups weredefinedbyusing the Ribosomal Data Project (RDP) Naive BayesianClassifier (Wang et al., 2007). Family is represented.α, β, γ represent α-proteobacteria, β-proteobacteriaand γ-proteobacteria, respectively.
[d]B1=B9=B29; B3=B10=B19=B20=B22=B31;B5=B38=B42;B7=B14; B8=B11=B17=B21=B25=B28=B32=B37=B40; B12=B13=B15=B18=B23=B24=B26=B30=B35; B16=B27
[e]Band detected by means of gel migration. Band not sequenced.

Table 3: Estimated richness, diversity and sample coverage for 16S rRNA and ITS1 libraries of creosote polluted soil microcosms.

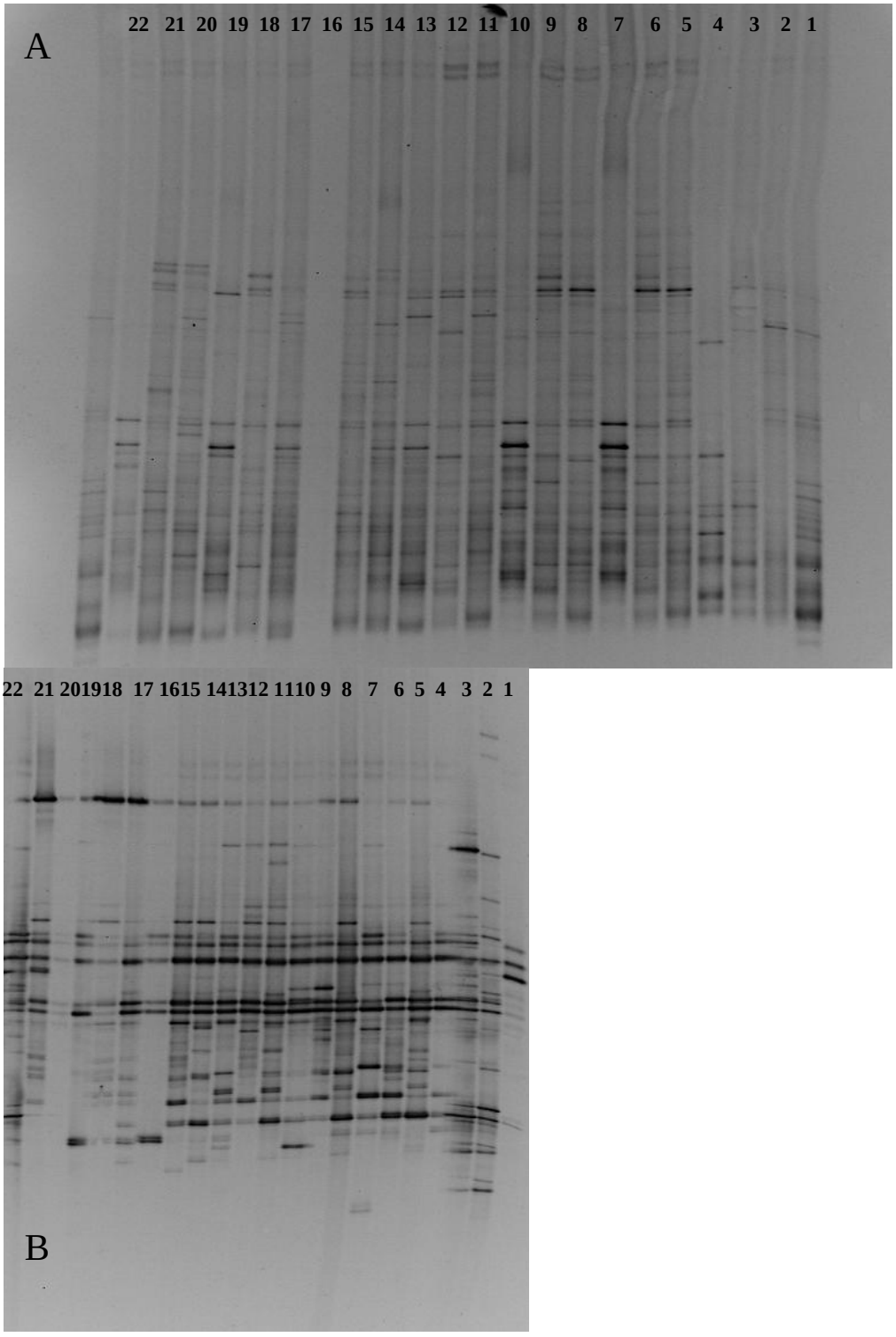
Treatment	NS[a]	OTUs[b]	Chao1[c]	Shannon[d]	ESC[e]
16S					
InitialSoil	18137	3339	8156 (7580; 8810)	5,52 (5,47; 5,55)	0,88
IC	11390	2630	6857 (6297; 7503)	5,64 (5,59; 5,69)	0,85
IC+SO	5304	1017	2547 (2230; 2946)	4,82 (4,75; 4,88)	0,88
IC+Br30	2557	554	1515 (1249; 1881)	4,62 (4,54; 4,71)	0,86
BS-LS+SO+Mn	1984	728	2122 (1795; 2550)	5,24 (5,14; 5,34)	0,74
BS-LS+Br30+Mn	1591	546	1885 (1515; 2395)	4,89 (4,78; 5,00)	0,75
TV-LS+SO+Mn	2030	746	2034 (1743; 2412)	5,10 (5,00; 5,20)	0,73
LT-LS+SO+Mn	27657	4613	11312 (10611; 12093)	5,67 (5,63; 5,70)	0,90
ITS					
InitialSoil	11712	2167	7729 (6891;8715)	4,76 (4,71; 4,81)	0,86
IC	-	-	-	-	-
IC+SO	2067	507	1443 (1189; 1792)	4,23 (4,14; 4,34)	0,83
IC+Br30	1055	314	1223 (906; 1711)	4,16 (4,03; 4,29)	0,77
BS-LS+SO+Mn	225	117	563 (339; 1013)	4,09 (3,90; 4,28)	0,58
BS-LS+Br30+Mn	173	93	458 (263; 879)	3,94 (3,73; 4,14)	0,55
TV-LS+SO+Mn	2195	608	2044 (1665; 2560)	4,83 (4,74; 4,92)	0,80
LT-LS+SO+Mn	1213	367	1388 (1044; 1905)	4,55 (4,44; 4,66)	0,78

[a]Number of sequences for each library.
[b]Calculated with MOTHUR at the 3% distance level.
[c]Chao1 richness index calculated using MOTHUR at the 3% distance level (values in brackets are 95% confidence intervals).
[d]Shannon diversity index calculated using MOTHUR at the 3% distance level (values in brackets are 95% confidence intervals).
[e]Estimated sample coverage: Cx=1-(Nx/n), where Nx is the number of unique sequences and n is the total number of sequences.

Table 4: Percent relative abundance (%) of genera in ITS gene pyrosequencing libraries in the initial creosote polluted soil.

Main Genera	Class/Family	Initial Soil
Alternaria	Pleosporaceae	1,5
Aspergillus	Trichocomaceae	3,6
Chaetomium	Chaetomiaceae	4,6
---	Coriolaceae	4,6
Fusarium	Nectriaceae	23,2
Hebeloma	Hymenogastraceae	8,6
Lasiosphaeris	Sordariomycetes	3,2
Leptographium	Ophiostomataceae	1,0
Malassezia	Malasseziaceae	6,7
Mortierella	Mortierellaceae	4,8
Nectriaceae	Nectriaceae	<1
Peziza	Pezizaceae	5,1
Phaeoisaria	Calosphaeriaceae	1,1
Rhizophlycitis	Rhizidiaceae	<1
Scedosporium	Microascaceae	24,8
Scytalidium	Leotiomycetes	1,2
Sebacina	Sebacinaceae	3,2
Stachybotrys	Sordariomycetes	<1
Trichoderma	Hypocreaceae	<1

Figure 1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22 21 20 19 18 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1
22 21 201918 17 1615 141312 1110 9 8 7 6 5 4 3 2 1
B

Figure 2

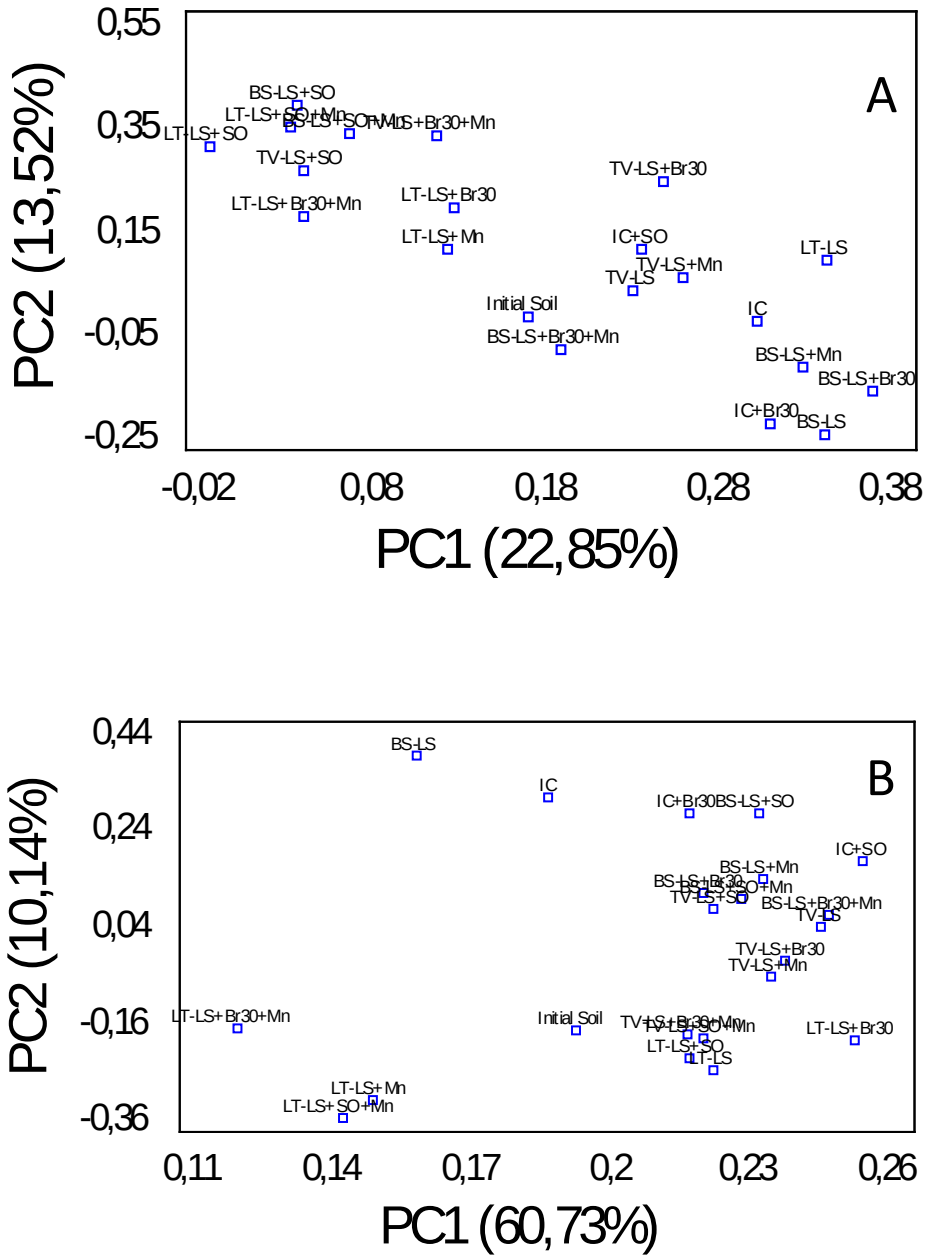
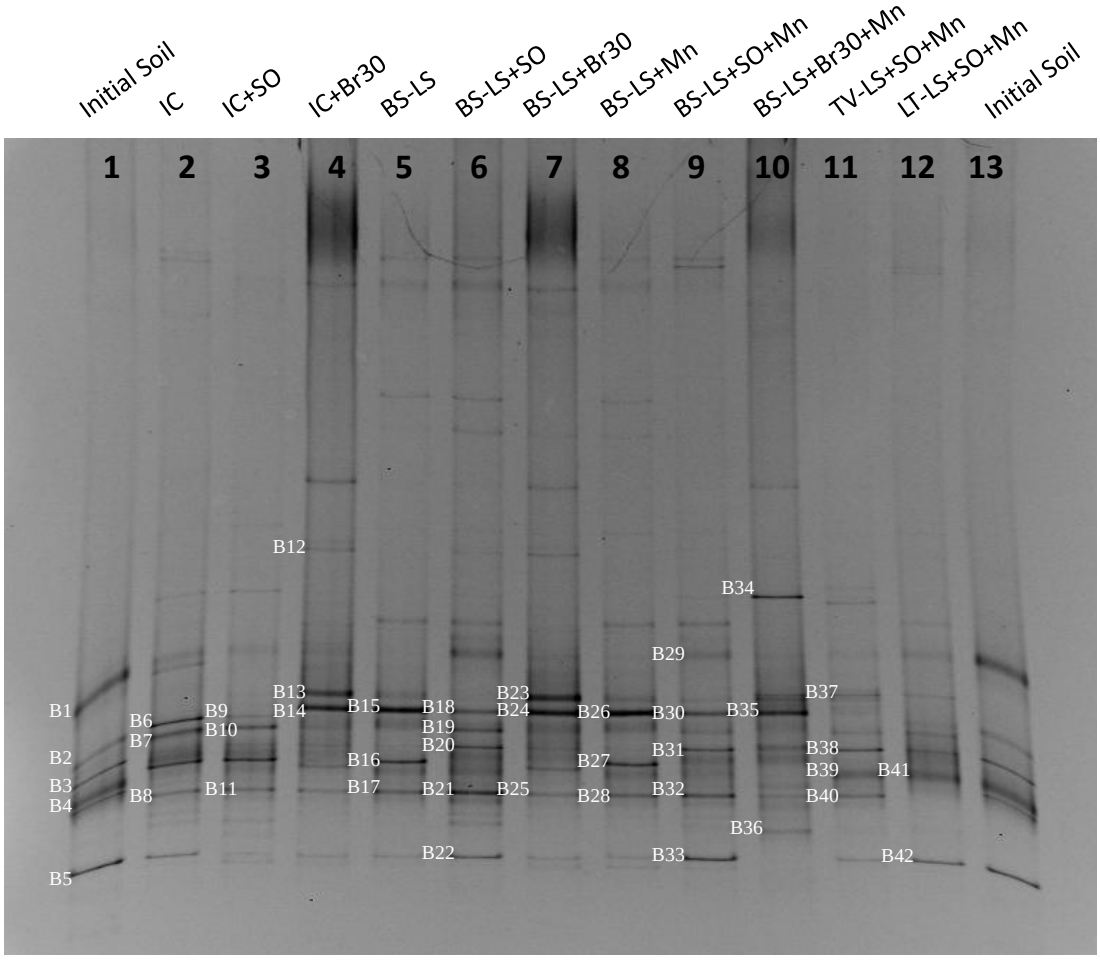

A
PC2 (13,52%)
PC1 (22,85%)
0,55
0,35
0,15
-0,05
-0,25
-0,02
0,08
0,18
0,28
0,38
BS-LS+SO
LT-LS+SO
TV-LS+SO
TV-LS+Br30
LT-LS+Br30
LT-LS+Br30+Mn
LT-LS+Mn
IC+SO
TV-LS+Mn
TV-LS
LT-LS
Initial Soil
IC
BS-LS+Br30+Mn
BS-LS+Mn
BS-LS+Br30
IC+Br30
BS-LS
B
PC2 (10,14%)
PC1 (60,73%)
0,44
0,24
0,04
-0,16
-0,36
0,11
0,14
0,17
0,2
0,23
0,26
BS-LS
IC
IC+Br30
BS-LS+SO
IC+SO
BS-LS+Mn
BS-LS+Br30+Mn
TV-LS
TV-LS+Br30
TV-LS+Mn
LT-LS+Br30+Mn
Initial Soil
LT-LS+Br30
LT-LS+SO
LT-LS
LT-LS+Mn
LT-LS+SO+Mn

Figure 3


Initial Soil
IC
IC+SO
IC+Br30
BS-LS
BS-LS+SO
BS-LS+Br30
BS-LS+Mn
BS-LS+SO+Mn
BS-LS+Br30+Mn
TV-LS+SO+Mn
LT-LS+SO+Mn
Initial Soil
1 2 3 4 5 6 7 8 9 10 11 12 13
B1
B2
B3
B4
B5
B6
B7
B8
B9
B10
B11
B12
B13
B14
B15
B16
B17
B18
B19
B20
B21
B22
B23
B24
B25
B26
B27
B28
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B37
B38
B39
B40
B41
B42

Figure 4

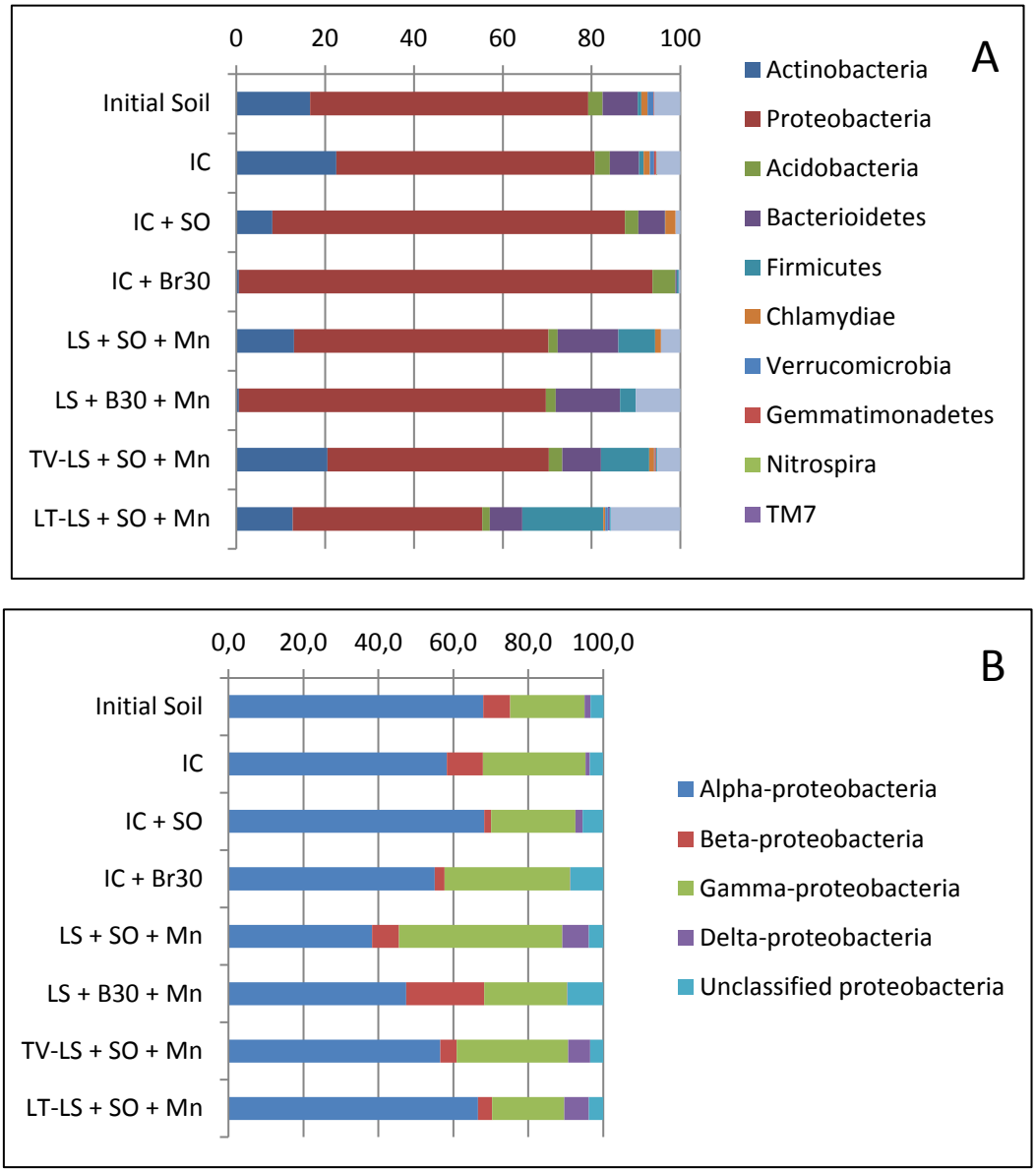

A
0
20
40
60
80
100
Initial Soil
IC
IC + SO
IC + Br30
LS + SO + Mn
LS + B30 + Mn
TV-LS + SO + Mn
LT-LS + SO + Mn
Actinobacteria
Proteobacteria
Acidobacteria
Bacterioidetes
Firmicutes
Chlamydiae
Verrucomicrobia
Gemmatimonadetes
Nitrospira
TM7
B
0,0
20,0
40,0
60,0
80,0
100,0
Initial Soil
IC
IC + SO
IC + Br30
LS + SO + Mn
LS + B30 + Mn
TV-LS + SO + Mn
LT-LS + SO + Mn
Alpha-proteobacteria
Beta-proteobacteria
Gamma-proteobacteria
Delta-proteobacteria
Unclassified proteobacteria

Figure 5

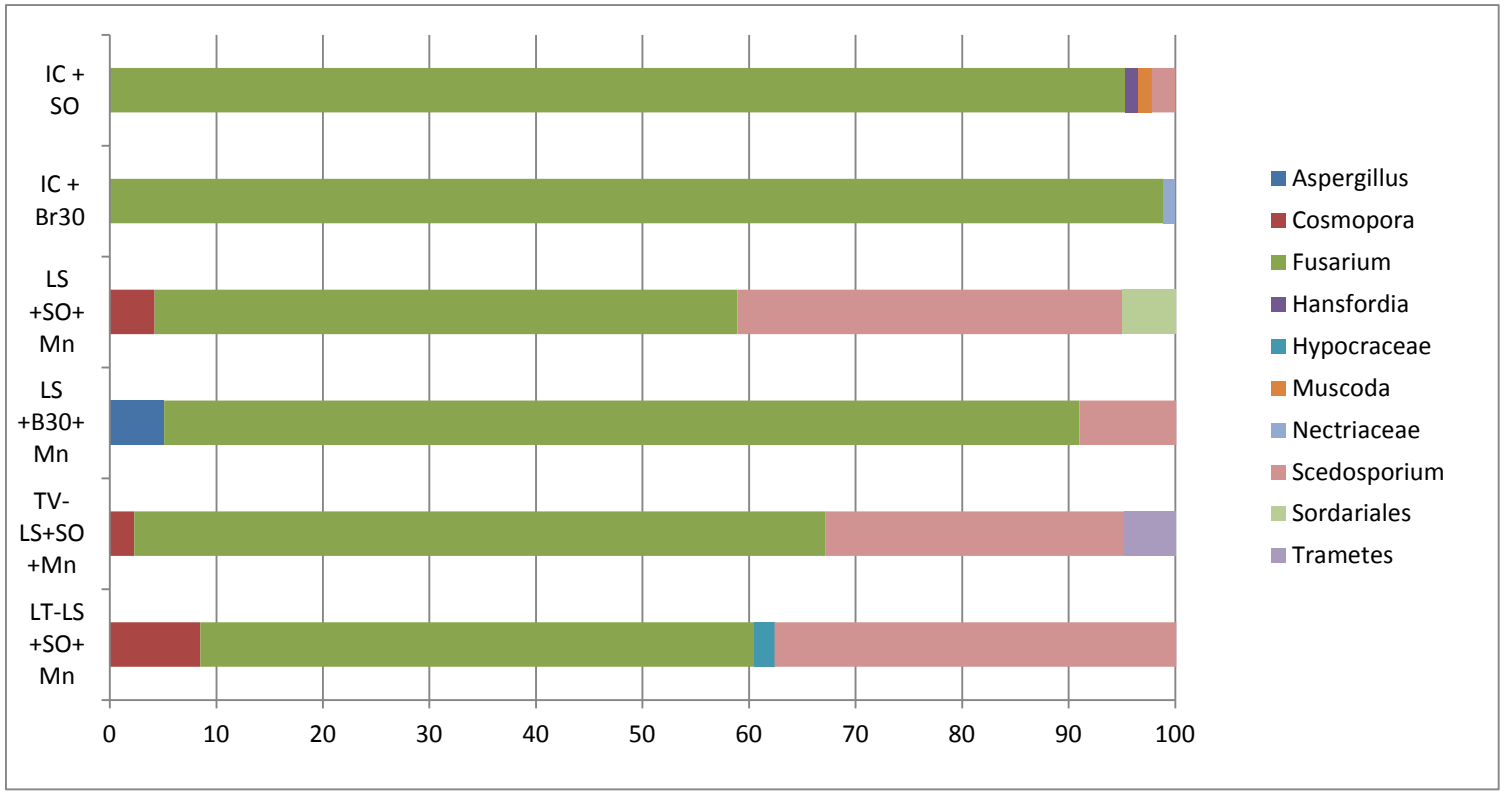
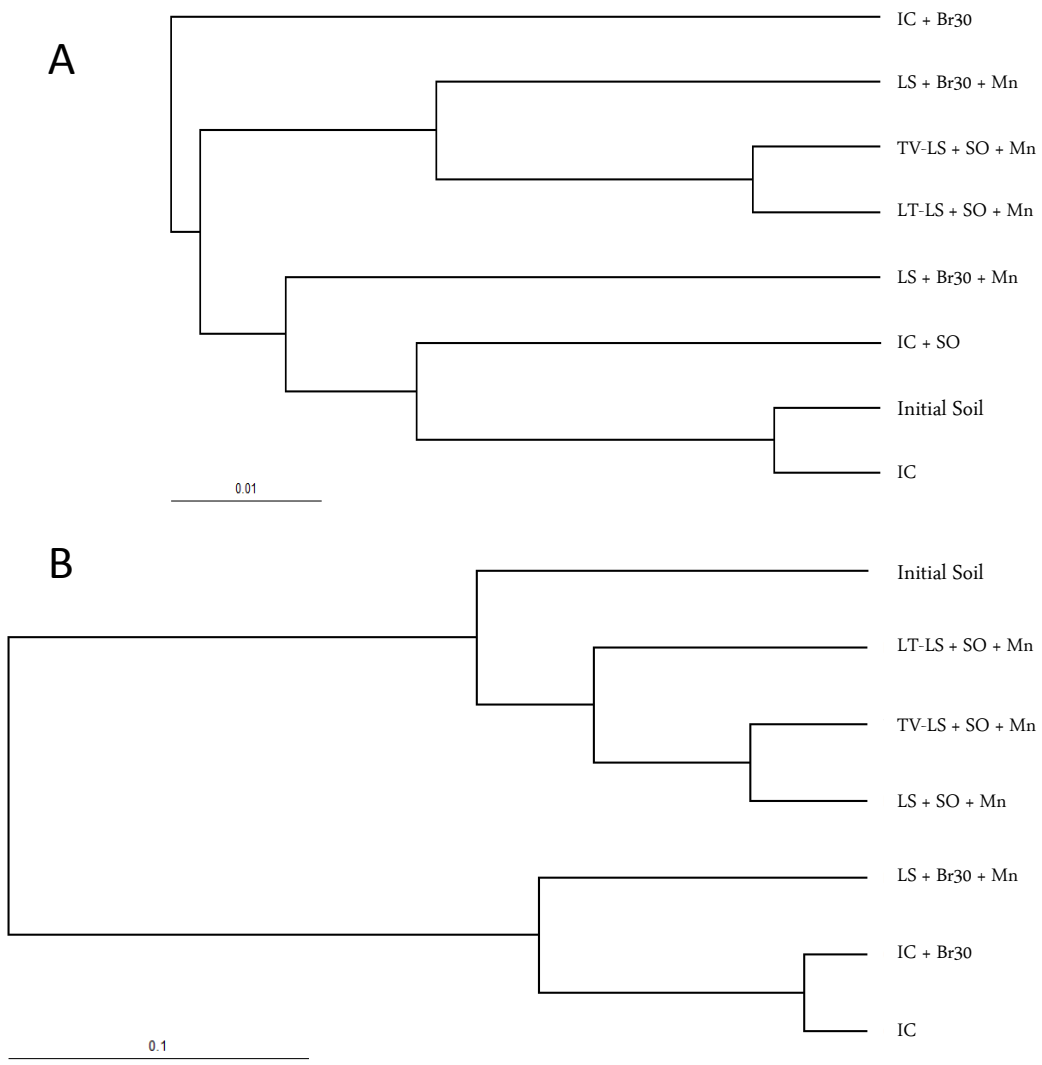

IC + SO
IC + Br30
LS +SO+ Mn
LS +B30+ Mn
TV-LS+SO +Mn
LT-LS +SO+ Mn
0
10
20
30
40
50
60
70
80
90
100
Aspergillus
Cosmopora
Fusarium
Hansfordia
Hypocraceae
Muscoda
Nectriaceae
Scedosporium
Sordariales
Trametes

Figure 6


A
IC + Br30
LS + Br30 + Mn
TV-LS + SO + Mn
LT-LS + SO + Mn
LS + Br30 + Mn
IC + SO
Initial Soil
IC
0.01
B
Initial Soil
LT-LS + SO + Mn
TV-LS + SO + Mn
LS + SO + Mn
LS + Br30 + Mn
IC + Br30
IC
0.1

Overview

OVERVIEW

The hazards associated with the high molecular weight hydrocarbons can be overcome by the use of conventional methods which involve removal, alteration, or isolation of the pollutant. Such techniques involve excavation of contaminated soil and its incineration or containment. These technologies are expensive, and in many cases transfer the pollutant from one phase to another (Haritash & Kaushik, 2009). On the other hand, bioremediation offers an environmentally friendly and cost-competitive approach for the clean-up of contaminated sites, although natural biodegradation may be slower than other technologies (Alexander, 1999).

However, the application of bioremediation technologies to soils contaminated with the heaviest hydrocarbon fractions of mineral oils or HMW-PAHs are still a challenge because of the low bioavailability and complex chemical composition of these products (Lee et al., 2008; Farmer et al., 2003). Furthermore, as the time of contact between contaminant and soil increases there is a decrease in chemical and biological availability, a process termed ‘ageing’ (Hatzinger & Alexander,1995) that involve sequestration of the contaminants by the soil mineral and organic fractions. As a consequence of ‘ageing’ processes, bioremediation may be strongly constrained on historically polluted soils like those described in the present thesis. Taking into account that with this class of products it is particularly difficult to decrease their concentration below the limits established by legislation, efforts should be made to minimize the presence of these compounds, as well as to better understand their effect on soil ecotoxicity.

In order to increase hydrocarbon bioavailability in soils, compounds like surfactants and vegetable oils may be used in bioremediation treatments (Wick et al., 2011). Unfortunately, organic matter content and clay mineralogy also affect surfactant performance, but the effect differs depending on the type of surfactant used (Rodriquez-Cruz et al., 2005). Anionic and nonionic surfactants are more commonly used in remediation because they are less likely to sorb onto soil surfaces (Mulligan et al., 2001).

Lack of catabolic abilities of the indigenous soil communities is another of the most important bioremediation limiting factors in aged soils where the pollutant profile is dominated by high molecular weight hydrocarbons (Mueller et al., 1989). Bioaugmentation of a specific competent microorganism or group of microorganisms to improve the metabolic capacity of the indigenous population of microbes could be a good approach for enhancing hydrocarbon biodegradation in historically polluted sites (Gentry et al., 2004).

In this respect, WRF are endowed with extracellular, non-specific and radical-based ligninolytic machinery that confers them the ability to degrade a wide range of contaminants, including high molecular mass PAHs which are seldom prone to bacterial attack (Bhatt et al., 2002).

Chapter 1 of this thesis was designed to aim the study of different biostimulation and bioagumentation bioremediation strategies for a heavily-oil polluted soil. The soil used (sandy-loam texture) was from the site of a former screw industry, which had been operating several decades before this work. Previous studies developed in our research group had reported a remarkable slowdown of degradation kinetics, after the first 6 months of microscoms incubation in all bioremediation treatments, and therefore heavy mineral oil enrichment (Sabaté et al., 2004). The additional supplementation of nutrients and a reinoculation of a bacterial consortium had no effect on this kinetics. This fact and failure of the surfactant tween 80 in enhancing TPH degradation led us to attempt a similar microcosms strategy but assessing white rot fungi bioaugmentation and addition of biosurfactants instead of tween 80.

Interestingly, data from chapter 1 show that that after 280 days, the highest TPH degradation in the heavily-oil polluted soil was reached after the WFR *Trametes versicol*or inoculation, with a reduction of 50% of TPH (Figure 1.1). Although the majority of previous *Trametes versicolor* bioaugmentation studies in polluted soils have been mainly focused on PAHs biodegradation, its effect on a non-sterile industrial mineral oil-polluted soil had also been described (Yateem et al., 1997).

In contrast, among the biostimulation agents, the addition of the rhamnolipids produced by the strain AT10 from *P. aeruginosa* did not improve the biodegradation

achieved by treatments. The preferential utilization of surfactants as carbon source by hydrocarbon degraders could explain the inhibited biodegradation of the pollutants (Deschenes et al., 1996).

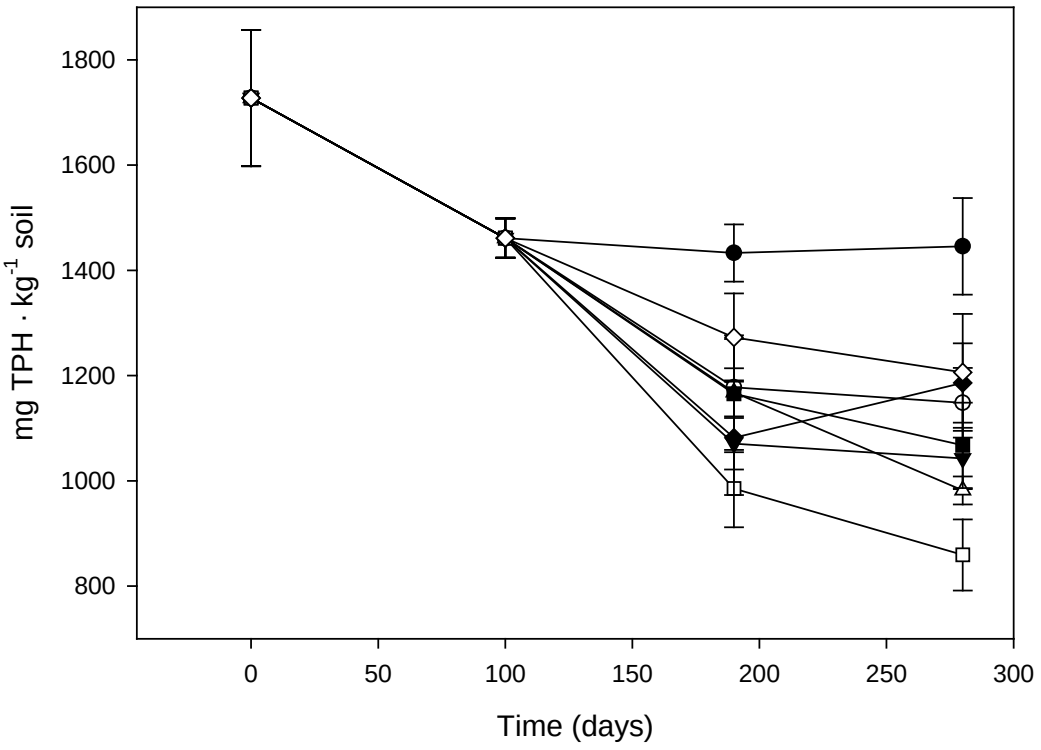


Fig. 1.1. Concentration of TPH in bioremediation treatments.●, control; ○, basic; ▼,nutrients; △, nutrients and glucose; ■, nutrients and TD consortium; □, nutrients and *Trametes versicolor*; ◆, nutrients and surfactant 10 fold its critical micelle concentration CMC; ◇, nutrients and surfactant at 100 folds higher than its CMC (Chapter 1).

It is noteworthy that in the acute toxicity test performed using *Eisenia fetida*, no lethaly was observed in any of the monitoring times. This finding could be explained by the low bioavailability of the pollutant product. However, bioremediation treatments altered worm weight during the incubation period and with respect to their controls (Figure 1.2). This finding suggests a positive correlation between length of treatment and health status of organisms.

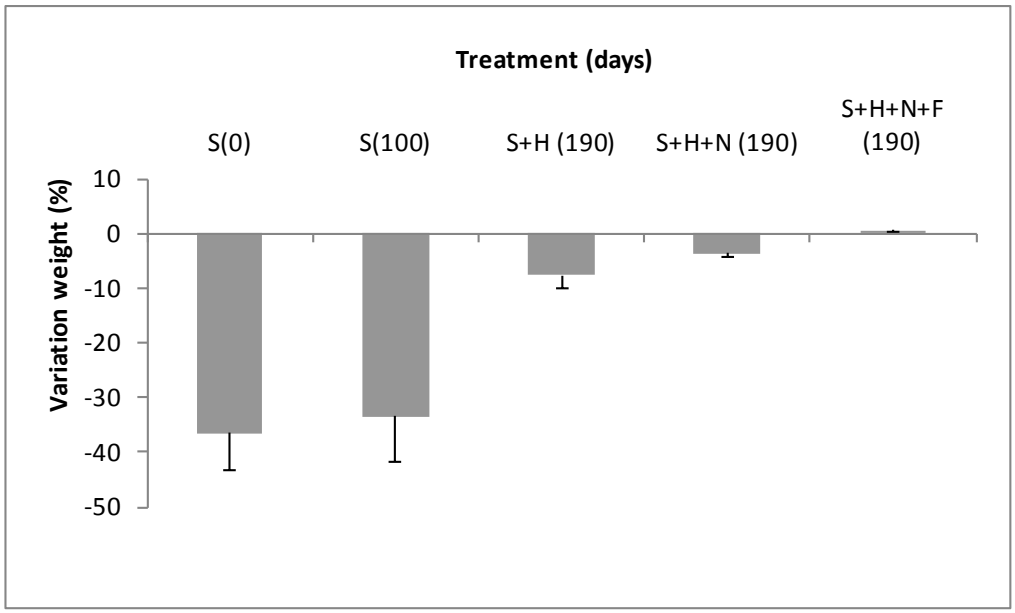


Fig. 1.2. Evolution of *Eisenia fetida* weight during the soil experiment. From left to right: S(0); Soil at 0 days, S(100); Soil at 100 days, S+H(190); Soil + humidity at 190 days, S+H+N(190); Soil + humidity + nutrients at 190 days, S+H+N+F(190); Soil + humidity + nutrients + fungus at 190 days (Chapter 1).

These promising results observed when a WRF was inoculated into a heavy hydrocarbon polluted site and extensive literature for PAHs contaminated soils, suggested the optimization and implementation of the technology in a creosote polluted soil, obtained from a wood treatment plant near Barcelona, in which our research group had previously been working with and that has been already introduced in chapter 2.

In a previous feasibility assay performed in our laboratory with the same creosote polluted soil (Viñas et al., 2005) it was concluded that the kinetics of TPH degradation displayed a biphasic pattern for all bioremediation treatments. This behavior may have been due to a number of factors, such as a decrease in PAH bioavailability (Alexander, 2000), or enrichment of more recalcitrant compounds. In a parallel study, a decrease in bioavailability was demonstrated and theoretical bioavailability threshold for each PAH was defined (Sabaté et al., 2005). In addition, a reduction in soil toxicity was reported by Microtox assays. It was also demonstrated that, in this particular soil, water content and aeration became the key factors for bioremediation. Nutrient addition had a negative effect on late-stage biodegradation of 4-ring PAHs and TPHs. The presence of an excess of nutrients could have inhibited the

degradation of HMW-PAHs by enhancing the growth of a non-specific PAHs degrading population, fact that will be considered later in this overview, when talking about microbial communities.

As a consequence of the positive results obtained in the feasibility assay, it was decided to carry out a pilot-scale biopile in order to assess if the same processes described in laboratory conditions, which triggered satisfactory bioremediation levels, could be extrapolated to the real field conditions, with the same industrial polluted soil. The biodegradation results of the pilot-scale biopile were reported in chapter 2.

After 180 days of soil biopiling, the PAHs degradation kinetics reported different behaviours depending on the number or rings of the hydrocarbons (Figure 1.3). 3-ring PAHs were completely depleted whereas the 4-ring PAHs were still being degraded, suggesting that six months were not time enough in order to reach their theoretical bioavailability threshold. Finally, a very slight 5-ring PAHs biodegradation was reported at the end of the study.

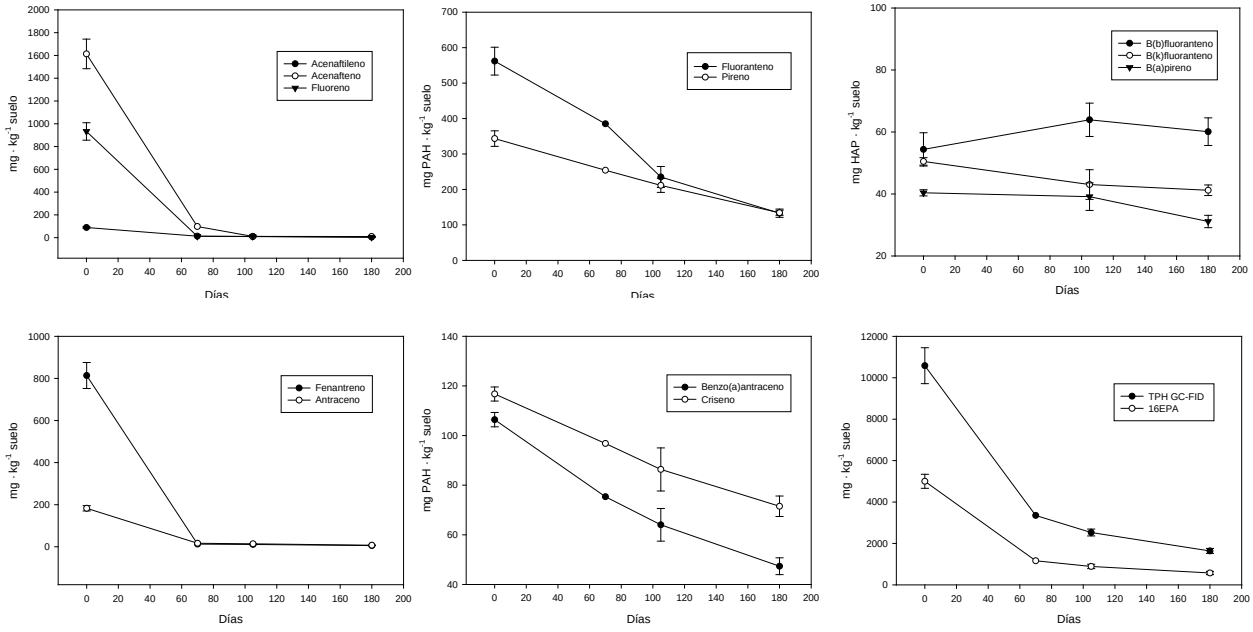


Fig. 1.3. TPH and PAHs degradation kinetics (Chapter 2).

However, although HMW-PAHs were still above their legal reference values for industrial land use, the soil was considered non-toxic by means of the Microtox toxicity assay. This fact suggested again very low levels of bioavailability of this recalcitrant compounds, due to low solubility properties and high adsorption rates to the clay particles of the creosote polluted soil.

This study reported the paramount importance of taking into account bioavailability and toxicity and not only pollutant concentration levels in order to perform proper risk assessment studies in contaminated soils. Nevertheless, this previously biotreated soil represented a paradigmatic opportunity to study other bioremediation technologies oriented to increase the degradation of high recalcitrant hydrocarbons. With this intention, the final biopile soil was sieved and stored at 4ºC and used in the different studies reported from chapter 4 onwards in the present thesis work, whereas in chapter 3 soil from the lab-scale feasibility assay carried out was used (Viñas et al., 2005).

In chapter 3, in order to enhance the bioavailability of the target HMW-PAHs (benzo(a)anthracene and chrysene) that remain in the bioremediated soil, an experimental strategy based on the incubation in slurry was carried out. Mineral medium conditions were selected because, as described in Viñas et al., 2005, addition of nutrients did not promote HMW-PAHs degradation in this particular case. The soil, which can be considered as an exhausted soil in terms of the more easily biodegradable PAHs, was incubated with and without additional B(a)A and Chry.

The results showed that the microbial population present in the bioremediated soil was able to degrade 89% and 53% of the added benzo(a)anthracene and chrysene, but was unable to reduce the residual concentration of these two PAHs in the slurry of the bioremediated soil (Figure 1.4), concluding that bioavailability was a key factor in the lack of degradation of 4-ring PAHs, both of which could be tightly sorbed to clay particles.

However, the same strategy conducted with 5-ring PAHs obtained very different results. Even increasing bioavailability, a lack of degradation by the indigenous microbial populations was observed.

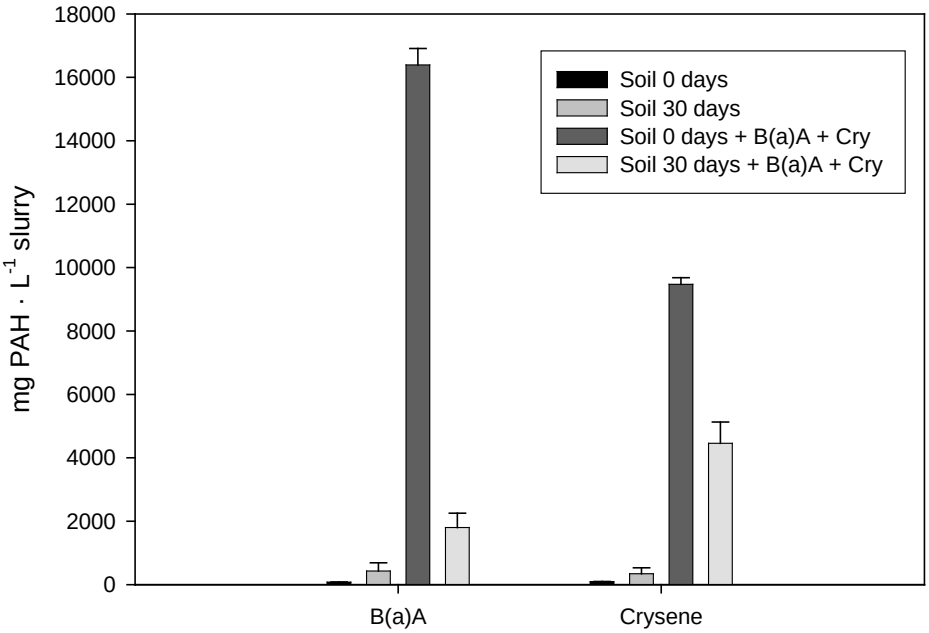


Fig. 1.4. Benzo(a)anthracene and chrysene concentration at 0 and 30 days, in creosote-contaminated soil with and without supplementation of these two PAHs (Chapter 3).

This unsuccessful attempt led us to assess a similar slurry method but optimizing the fungal bioaugmentation strategy with a view to enhance the biodegradation of the most recalcitrant PAHs, described in chapter 4. The white rot fungus *Trametes versicolor* was chosen because its laccase has been widely described as optimal for degrading HMW-PAHs (Collins et al., 1996).

The results obtained in chapter 4 reported the highest 4-ring PAHs degradation was achieved by the native microbiota while growing in carbon limiting conditions (values between 51% and 54% for fluoranthene and pyrene and between 26% and 28% for benzo(a)anthrance and chrysene) (Table 1). It is important to note that these degradation values were achieved during the first 15 days of incubation. Further degradation was not obtained between the day 15 and 30, confirming that at certain threshold concentrations the HMW-PAHs were no longer available, even in a liquid medium where active degrading bacteria were present, and none nanoporousity exists hampering microbial accessibility to those PAHs that could be strongly sorbed onto soil mineral surfaces (Bosma et al., 1997). On the other hand, the carbon rich MEG medium promoted a lower biodegradation of the 4-ringed PAHs, probably because of the

utilization of glucose, and other carbon sources such as malt extract, as preferred source of carbon and energy by the autochthonous degrading populations.

However, the 5-ring PAHs depletion was only enhanced by the *Trametes versicolor* bioaugmentation in MEG medium, confirming its biodegradation capabilities. In addition, when autoclaved soil was utilized, the 5-ringed PAHs degradation was significantly better than in the other treatments, but with low percentages (15% of degradation). These facts suggest that more incubation time would probably lead to a higher biodegradation of 5-ring PAHs by the WRF.

Results obtained in chapter 3 and 4 encouraged us in order to attempt fungal bioaugmentation of the biotreated creosote polluted soil under unsaturated solid-phase conditions. Furthermore, spite of the negative results previously obtained with tween 80 and rhamnolipids, other non-ionic surfactant as brij 30 (Br30) and a vegetable oil like soybean oil (SO) were used in order to enhance mass transfer rate and bioavailability of HMW-PAHs, as a consequence of the collaboration with a research group from the University of Tuscia in Viterbo (Italy), with great previous experience in using mobilizing agents while performing fungal bioaugmentation (Leonardi et al., 2007; Leonardi et al., 2008).

The use of WRF in soil, however, requires the addition of lignocellulosic wastes (LS), so one of the main objectives of the study reported in chapter 5 was to comparatively evaluate the impact of single or combined supplements (Mobilizing agents (MAs) and Mn^{2+}) on both a biostimulation with a lignocellulosic substrate and a bioaugmentation approach to the aged creosote soil in terms of biodegradation outcomes.

Surprisingly, best TPH removals, ranging from 71% to 73%, were achieved in biostimulated microcosms (BS) supplemented with MAs and Mn^{2+} ions. Anyhow, regardless of the supplementation, the residual TPH contents in biostimulated microcosms were significantly lower than those found in respective incubation controls (Table 1).

Table 1: Initial concentrations of TPH and 4- and 5-ring PAHs in soil and residual concentrations observed after 60 days of incubation at 28ºC in all different bioremediation treatments (Chapter 3).

Treatment	TPH[a]	Fluor	Pyr	B(a)A	Chry	B(b)F	B(k)F	B(a)P
Initial Soil	2815±233	83±4	84±4	37±1	68±1	57±2	38±2	22±1
IC	1439±51	30±0,4	35±3	21±0,8	40±6	39±1	24±1	17±1
IC + SO	1395±30	43±2	48±3	19±0,1	47±3	38±1	24±0,3	9±0,3
IC + Br30	1515±179	61±3	70±2	28±0,4	55±3	38±2	25±0,7	14±0,3
BS-LS	1077±242 aB*	17±1 aA*	18±0,7 aB*	15±0,6 aB*	26±1 aBC*	29±3 aD*	18±1 aC*	15±0,7 aC
BS-LS + SO	1098±207 aB*	16±2 aA*	18±2 aB*	14±3 aB*	29±5 aC*	26±2 aCD*	16±2 aB*	12±1 aB
BS-LS + Br30	1255±68 aB*	28±4 aB*	34±4 aC*	18±2 aC*	35±2 aD*	28±1 aD*	19±1 aC*	15±1 aC
BS-LS + Mn^{2+}	1106±26 aB*	13±1 aA*	15±2abAB*	12±0,5 aA*	23±0,4 aAB*	25±0,5 aC*	16±0,4 aB*	13±0,5 aB*
BS-LS + SO + Mn^{2+}	810±27 aA*	13±3 aA*	14±2 aA*	10±0,8 aA*	20±2 aA*	18±1 aB*	11±0,7 aA*	8±0,2 aA
BS-LS + Br30 + Mn^{2+}	766±27 aA*	13±0.2 aA*	16±0.4aAB*	10±0,7 aA*	19±2 aA*	15±1 aA*	10±0,9 aA*	8,5±0,4 aA*
TV-LS	1545±153 bA	14±2 aA*	16±2 aA*	17±0,6 bAB*	30±1 bAB*	35±2 bC*	23±1 bC*	19±2 aA
TV-LS + SO	1338±204 aA	14±2 aA*	17±3 aA*	17±2 aAB*	33±2 aB*	32±1 aBC*	21±1 aB*	15±0,4 bA
TV-LS + Br30	1552±29 bA	31±4 aC*	36±5 aC*	21±0,9 aC*	39±0,8 abC*	32±2 bBC*	21±0,2 abB*	17±0,1 bA
TV-LS + Mn^{2+}	1417±155 bA	16±3 aA*	17±3 bA*	16±2 bA*	29±4 aA*	32±4 bBC*	21±2 bB*	17±2 bA
TV-LS + SO + Mn^{2+}	1449±65 cA	23±4 bB*	24±4 bB*	16±1 bA*	31±2 cAB*	27±2 bA*	18±0,5 bA*	17±4 bA
TV-LS + Br30 + Mn^{2+}	1436±60 cA	28±3 bBC*	30±3 bBC*	19±2 bBC*	37±2 bC*	31±0,8 bB*	20±1 bB*	16±1 bA
LT-LS	1396±15 abB	15±3 aAB*	17±2 aAB*	16±0,5 abB*	31±1 bAB*	32±1abC*	21±1 bB*	17±3 aC
LT-LS + SO	1467±170 aBC	20±7 aB*	22±7 aB*	16±3 aB*	33±8 aB*	30±4 aBC*	20±3 aB*	14±1 abB
LT-LS + Br30	1578±42 abC	32±5 aC*	35±5 aC*	21±1 aC*	43±3 bC*	31±2 bC*	21±1 bB*	17±0,5 bC
LT-LS + Mn^{2+}	1147±53 aAB*	11±2 aA*	13±1 aA*	13±0,7 aA*	24±3 aA*	26±2 aAB*	18±0,1 aAB*	13±0,6aAB*
LT-LS + SO + Mn^{2+}	1093±71 bA*	16±4 abAB*	18±3 aAB*	13±2 bA*	25±4 bA*	24±2 bA*	15±3 bA*	11±0,9aA
LT-LS + Br30 + Mn^{2+}	1260±2 bB*	25±0,1 bBC *	31±3 bC*	19±0,4 bC*	37±0,2 bBC*	30±0,2 bBC*	21±0,6 bB*	17±0,1 bC

[a] All concentrations are expressed as µg g^{-1} of dry soil and data are the means of three independent experiments. Statistical multiple pair-wise comparison was carried out on row means by the LSD Fisher test (P≤0.05). Same lowercase letters indicate that differences between microcosms (BS, TV, LT) within the same amendment. Same uppercase letters indicate lack of statistically significant difference within each biostimulation or bioaugmentation treatment at different supplements. Significant differences between each soil treatment and its respective incubation control are represented by an *.

Conversely, bioaugmented microcosms failed to significantly lead to lower TPH contents than respective incubation controls with the only exceptions of Mn^{2+}-supplemented *L. tigrinus* microcosms.

With regard to the PAH fraction, it is noteworthy that, unlike that observed for TPH; all treatment typologies were able to yield significantly lower residual PAH contents than those in incubation controls. However, Brij30 appeared to exert a negative impact on biodegradation of 4-ring PAHs while best removal efficiencies 5-ring compounds were observed in biostimulated microcosms that underwent concomitant supplementation with Mn^{2+} and MAs (Table 2).

In order to better understand these results it is important to note that at the beginning of the mycoremediation test, the colonization of the upper (soil) layer by the WRF inoculants underneath was clearly hindered by the outstanding growth capabilities of indigenous soil fungi. In fact, it has been described that the colonization of the LS by native soil populations restrains the growth and activity of white-rot fungi and inhibits fungal lignocellulose decomposition, reducing enzyme release (Magan et al. 2010). This antagonistic effect between autochthonous communities and exogenous fungi could be the reason for lower TPH and PAH biodegradation rates in those microcosms where *Trametes versicolor* or *Lentinus tigrinus* were inoculated, compared to those where the native communities were biostimulated with pre-sterilized LS addition.

Unfortunately, little information is so far available on the relationships between exogenously added fungi and the indigenous microflora and it is mostly limited to artificially spiked soils (Mougin et al.1997; Andersson et al. 2003). In this respect, both cooperation (Kotterman et al. 1998) and antagonism (Radtke et al. 1994) between bacterial microflora and fungi in degradation and mineralization of contaminants have been reported. Results of chapter 5 encourage us in order to carry out a depth biodiversity study of the most important soil samples through molecular ecology culture independent methods, as DGGE and 454-based/pyrosequencing, described in chapter 6.

In addition, throughout the thesis work, all studies had focused and emphasized the paramount importance of the correlation between native microbial biodiversity and biodegradation of pollutants in soils. On the one hand, it is useful to describe which

microrganisms could be related to degradation of high recalcitrant hydrocarbons (chapters 1, 3, 4 and 5) and in the other hand, it is necessary to study biodiversity dynamics of both fungal and bacterial autochthonous populations, when exogenous strains are inoculated in soil as bioremediation treatments, in order to better understand success or failures of these technologies (chapter 1, 4 and 5).

In chapter 1, in order analyze the initial bacterial population in the heavily-oil polluted soil and its response to different bioremediation treatments, a DGGE analysis of PCR-amplified 16S rRNA gene fragments was performed (Figure 1.5).

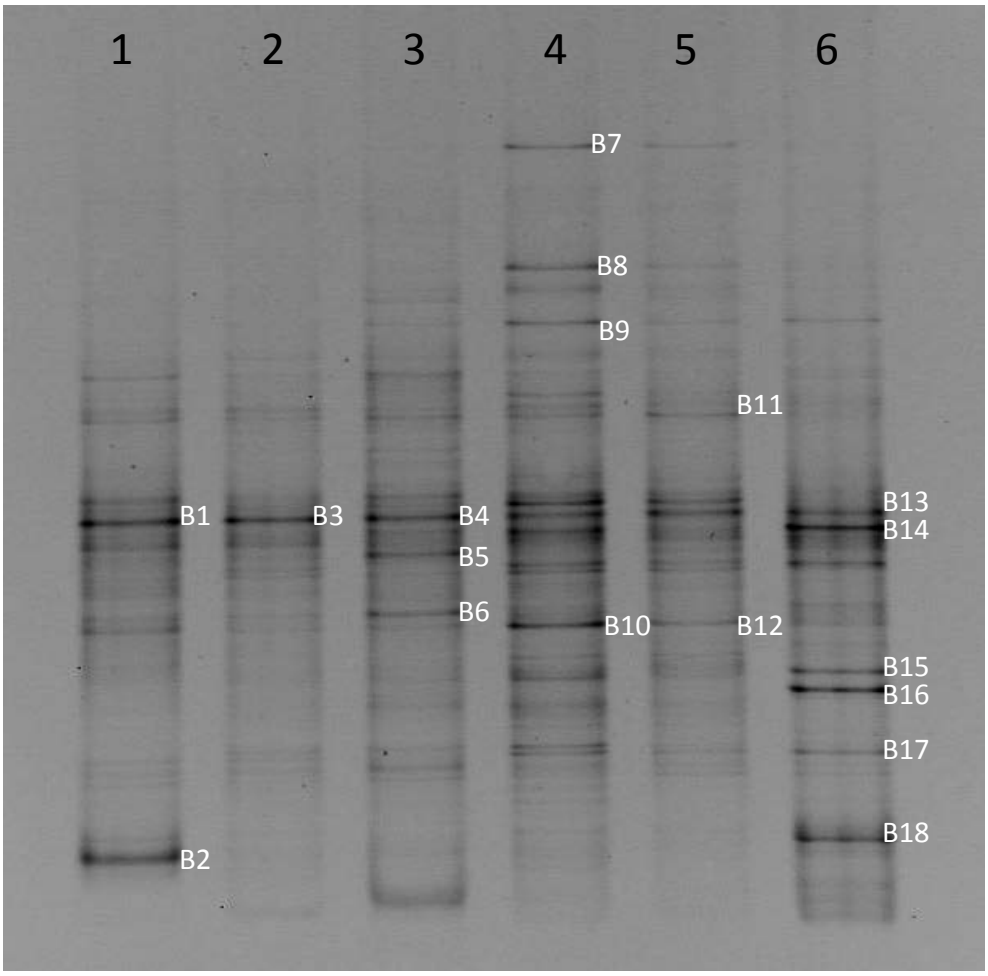


Fig. 1.5. A) Denaturing gradient gel electrophoresis (40% to 60% denaturant) profiles of eubacterial biodiversity from the original and five treated soils. From left to right: Lane 1, 0 days; Lane 2, 100 days; Lane 3, 100 days plus rice straw addition; Lane 4, basic treatment at 280 days; Lane 5, nutrient treatment at 280 days; Lane 6, nutrient and *Trametes versicolor*

treatment at 280 days. Numbered DGGE bands were successfully excised and sequenced and are shown in Table 2.

The DGGE profile of the initial contaminated soil showed low diversity, which is common in polluted environments.

Soil biostimulation with water or water plus nutrients for 280 days resulted in similar DGGE profiles and TPH degradation rates (Lanes 4 and 5 in Figure 1.5). However, other studies report that the DGGE profiles for a hydrocarbon-polluted soil biostimulated with water or water plus nutrients differ greatly (Wu et al., 2008). These distinct diversity patterns suggest that similar biostimulation treatments produce population changes that differ, depending on the polluted soil matrix and the microbial community involved.

The results described in chapter 1 confirmed that the presence of *T. versicolor* and its ligninolytic substrate in the soil changed substantially bacterial biodiversity during the 280 days of incubation, promoting the enrichment of Gram-positive bacteria belonging to the *Actinobacteria* and *Bacillus* groups. It is important to point out that microbial diversity changes promoted after *T. versicolor* inoculation were concomitant with both the high proportion of hydrocarbon degraders encountered in the MPN assays and the higher TPH biodegradation observed in the white-rot fungus bioaugmentation treatment.

Following with microbial characterization of microorganisms involved in the complex process of biodegradation of high recalcitrant hydrocarbons in real historically polluted sites, it was decided in chapter 3 to analyze the microbial population initially present in the soil and its response to the presence of high amounts of B(a)A and Chry in the spiked experiments. Two DGGE analyses were carried out: one on the total DNA in the slurries with and without the spiked PAHs, and the other from the more diluted wells of the microtiters used to enumerate the PAH degraders in both types of slurry.

At the end of the experiment, four additional bands (B2, B5, B6, B7) appeared in the total DNA profile of the spiked slurry in comparison to the unspiked one (lane 2 and 3, Figure 1.6). In relation to the microrganisms identified, the branch of

Sphingobacteriales, of the CFB group, was the main bacterial family detected in the soil slurries. This is in accordance with previous results obtained with the same creosote-contaminated soil (Viñas et al., 2005).

Moreover, the DGGE profiles of the more diluted positive PAH-degrading populations obtained from microtiter plates from the slurries with and without additional B(a)A and Chry corresponded to lane 5 and lane 6 respectively (Figure 1.6). In both profiles *Mycobacterium* and *Pseudoxanthomonas* genres were detected, suggesting an important role in HMW-PAHs degradation.

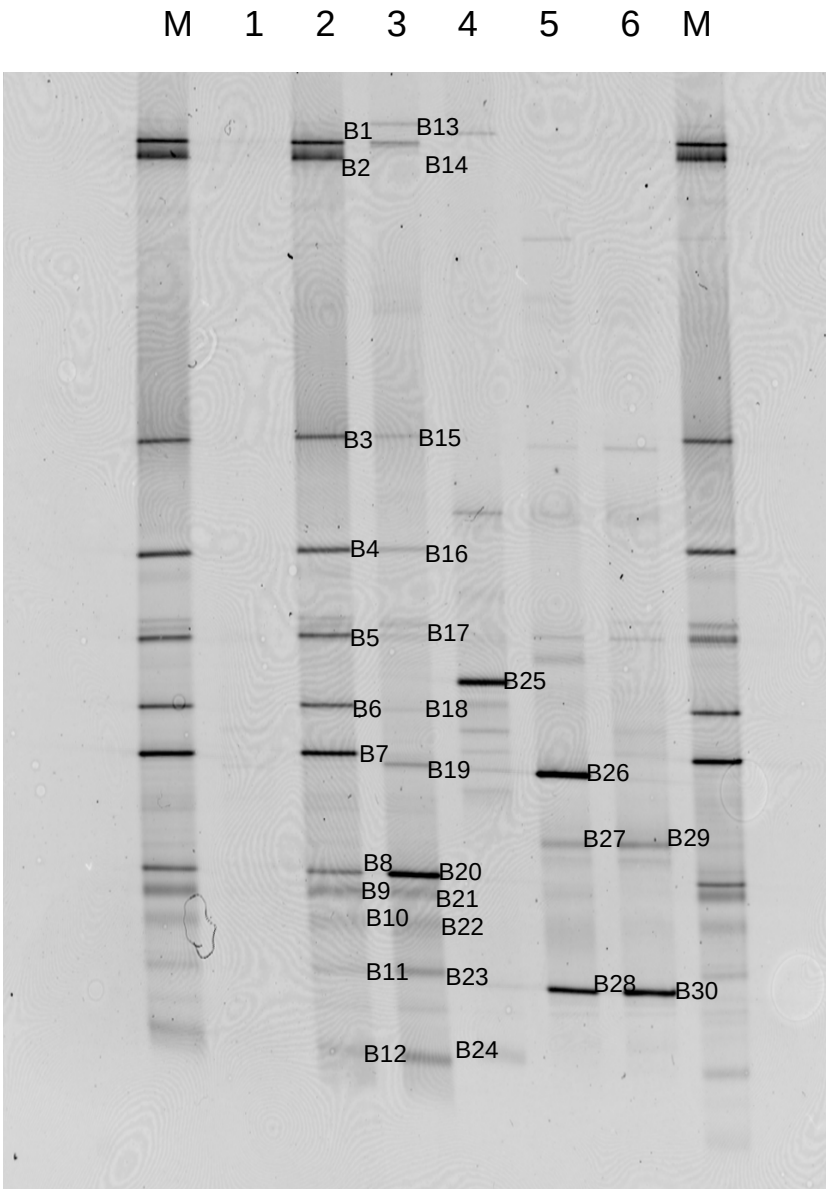


Fig. 1.6. Denaturing gradient gel electrophoresis (50% to 70% denaturant) profiles of PCR-amplified 16S rRNA gene fragments of total DNA from the original exhausted soil (Lane 1) and from slurries with and without additional B(a)A and Chry (Lanes 2 and 3, respectively) at day 30, and from PAH-degrader MPN plates from the original exhausted soil (Lane 4) and from spiked and unspiked slurries (Lane 5 and 6). Lane M contains the same DNA sample as Lane 2 and was used as a marker.

As a consequence of the results obtained in chapter 1 and 3 and the fungal bioaugmentation strategy assessed in chapter 4, it was decided to obtain a depth view of microbial communities by studying also the fungal native biodiversity of the creosote polluted soil and its dynamics during different biostimulation and bioaugmentation treatments through DGGE analysis of PCR-amplified ITS1 region. To date, no similar studies have been found in the literature which has provided an exploration of autochthonous fungal diversity by means of a DNA-based approach in historically polluted sites throughout a bioremediation process.

Data of chapter 4 showed that regardless to the carbon content of the liquid medium used, *Fusarium solani* is the main fungus detected after 30 days of incubation, in spite of the presence of *T. versicolor*, although was not one of the most important fungi in the initial soil (Table 3). This fact would suggest that a rich carbon medium (treatments 1S and 3S) could be promoting the growth of *F. solani* versus microorganisms that are more acclimated to the initial polluted soil conditions, probably because of faster growth kinetics. (Wu et al., 2010). Moreover, *Fusarium* spp. were also detected after 30 days of biostimulation in carbon limitant conditions (treatment 6S), which could indicate that this genus is playing a role throughout the biodegradation process.

Table 3. Properties of ITS DGGE bands: designations and accession numbers for the band sequences and levels of similarity to related organisms

Band	1S 30d	3S 30d	5S 30d	6S 30d	Length (bp)	Closest organism in GenBank database (accession no.)	% similarity[a]	Phylogenetic group[b]
ITS B1[b]	X		X		208	***Trametes versicolor* FP1022316sp (JN164984)**	100%	*Polyporaceae* (Basidiomycota)
ITS B2		X			201	*Peziza pseudoviolacea* 16504 (JF908564)	97%	*Pezizaceae* (Ascomycota)
ITS B3		X			164	*Chromelosporium* sp. CID601 (EF89890)	96%	*Pezizaceae* (Ascomycota)
ITS B6[c]	X	X		X	171	***Fusarium solani* isolate 177 (JN232143)**	100%	*Nectriaceae* (Ascomycota)
ITS B39				X	152	*Scedosporium prolificans* strain 776497 (GU594770)	90%	*Microascaceae* (Ascomycota)
ITS B41				X	161	*Fusarium oxysporum* isolate 1 (JN558555)	93%	*Nectriaceae* (Ascomycota)
ITS B42				X	161	*Cosmopora* sp. strain GJS96186 (JN995635)	100%	*Nectriaceae* (Ascomycota)

[a]Sequences were matched with the closest relative from the Genbank database.
[b]Band ITS: B1=B4=B9=B16=B17=B18=B19=B20=B26=B37
[c]BandITS:B5=B6=B7=B8=B10=B11=B12=B13=B14=B15=B21=B22=B23=B24=B25=B27=B28=B29=B30=B31=B32=B33=B34=B35=B36=B40

The ability of *Fusarium* spp. to degrade HMW-PAHs has been demonstrated elsewhere (Chulalaksananukul et al., 2006), although the presence of *F. solani* in treatments 1S and 3S cannot be related to an improvement in PAH degradation. It is important to point out that fungal diversity seemed to be higher on treatment 6S after 30 days of incubation, probably due to the lack of glucose and *T. versicolor* in the mineral medium.

Like in chapter 3, to analyze the bacterial population, the total DNA present in the slurries was compared to the DNA obtained from the more diluted wells used to enumerate the PAH degraders (Figure 1.7).

After soil addition, the bacterial diversity profile shifted dramatically although divergently with regard to the carbon content of the liquid medium. The shift produced by the presence of either glucose (treatment 3S) or the presence of the white-rot fungus (treatment 1S) was different when compared to the shift produced by only activating the soil with water and mineral nutrients (treatment 6S). Surprisingly, the *T. versicolor* inoculation and growth in the rich carbon medium did not noticeably change the detectable bacterial biodiversity compared to when no WRF was bioaugmented. The same behaviour was also observed for DGGE profiles of PAH-degrading populations.

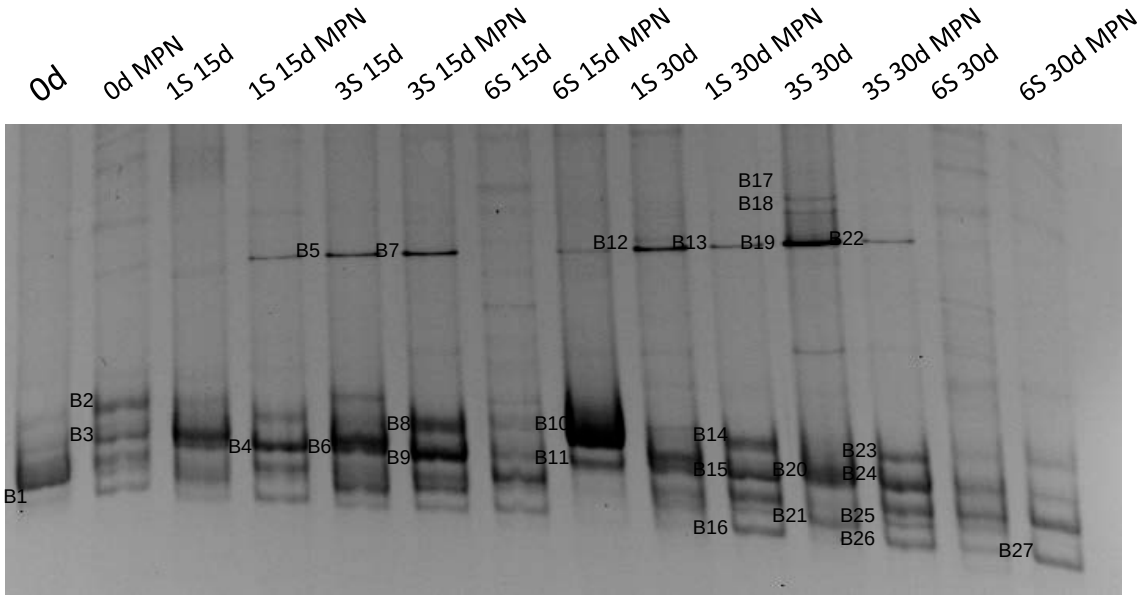


Fig. 1.7. Denaturing Gradient Gel Electrophoresis profiles of PCR-amplified 16S rRNA gene fragments (V3–V5 regions) of slurry communities and MPN plates. Numbers are disposed at the left side of the corresponding band.

In chapter 4, also fungal/bacterial ratio was quantified by quantitative PCR (Figure 1.8). As expected, the fungal/bacterial ratio was three-fold higher in those flasks where *T. versicolor* was inoculated (1S), compared to those treatments where only the autochthonous population was present (3S and 6S) (Figure 1.8B).

Moreover, where native populations grew with an easily assimilable carbon source but without the *T. versicolor* bioaugmentation (3S), the number of ITS copies suffered a three-fold increase during the first 15 days of incubation due to the large amount of glucose present in the medium, producing a two-fold increase of the fungal/bacterial ratio; in the presence of the white-rot fungus (1S) however, the growth of heterotrophic bacteria, combined with a slightly but statistically significant loss of ITS gene copies, produced a reduction by two orders of magnitude of the ratio. This fact could be a consequence of an antagonistic effect of an active bacterial autochthonous population against *T. versicolor*. This process was accentuated at 30 days of incubation.

The antagonistic effect between autochthonous microbial populations and exogenous WRF described a historically creosote polluted soil in chapter 4 and also in chapter 5 lead us into give a deeper approximation on both fungal and bacterial

populations dynamics and community shifts during the different bioestimulation and bioaugmentation treatments carried out under unsaturated solid-phase in chapter 5.

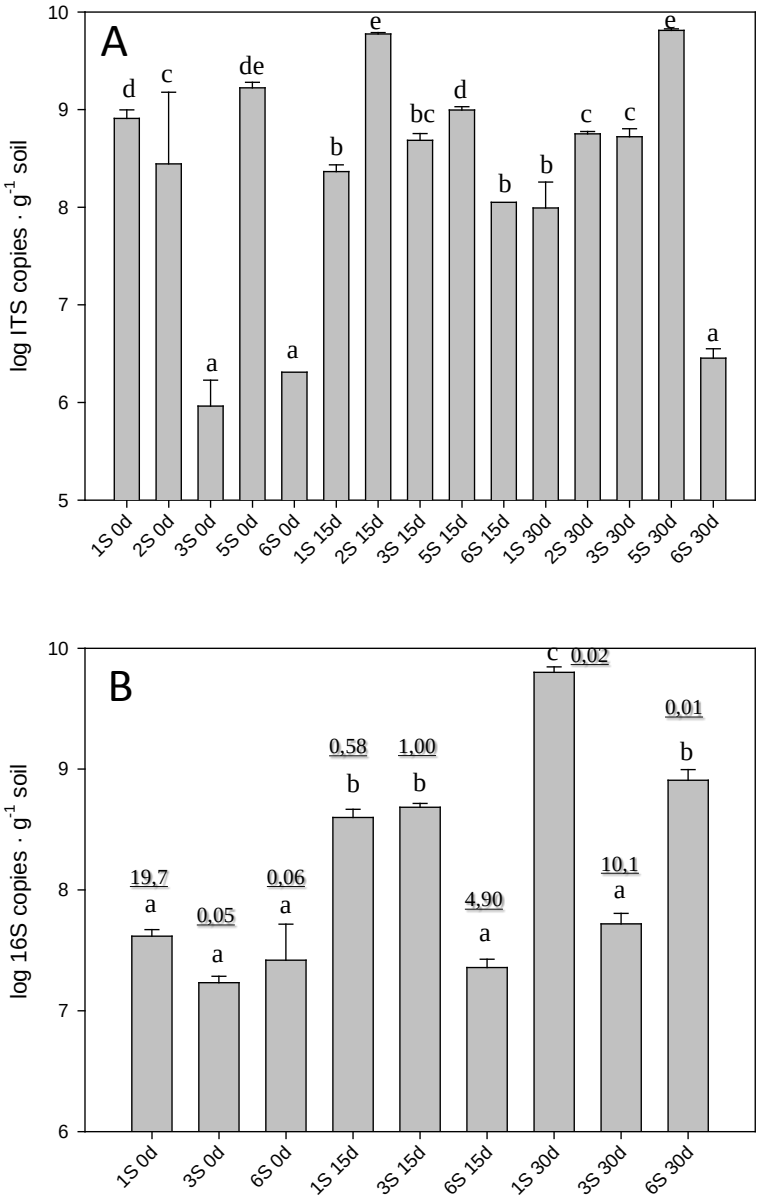


Fig. 1.8. ITS region (A) and 16SrRNA (B) gene copies quantified by qPCR in soil slurry treatments over the course of 30 days of incubation. Different letters indicate significant differences between treatments (*P<0.05*). Fungal/bacterial ratio is also indicated (B).

In chapter 6, in order to take a deeper view, than the DGGE can offer, on the bacterial and fungal communities in the soil and its shifts during the bioremediation treatments and their respective incubation controls (Figure 1.9), pyrosequencing analysis was carried out.

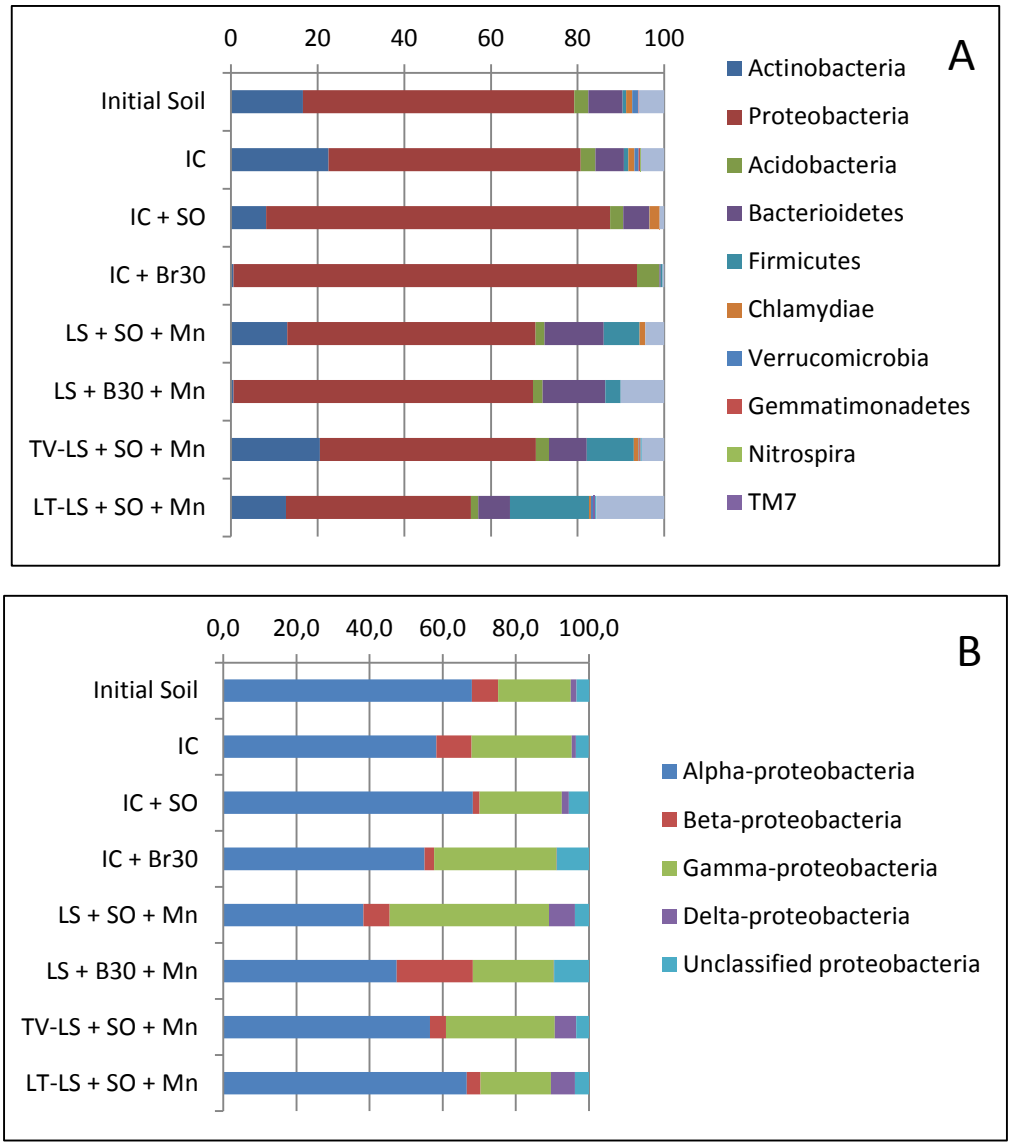


Fig 1.9. Eubacterial biodiversity composition, in relative abundance (%), of different phyla based on the classification of partial 16S rRNA sequences of bacteria from soil microcosms using RDP-classifier. Phyla (A); Proteobacteria classes (B).

Indeed, diversity was high in the initial soil, where proteobacteria and actinobacteria were the dominant phyla. Moreover, α and γ-proteobacteria are the most important families in the pristine soil (Figure 1.9B). This fact was coincident with the DGGE performed by Viñas et al., 2005, with the same creosote polluted soil, but prior to the field scale biopile.

Furthermore, bacterial community shifts caused by the different biotreatments, described in chapter 5, are represented by a dendogram (Figure 1.10A).

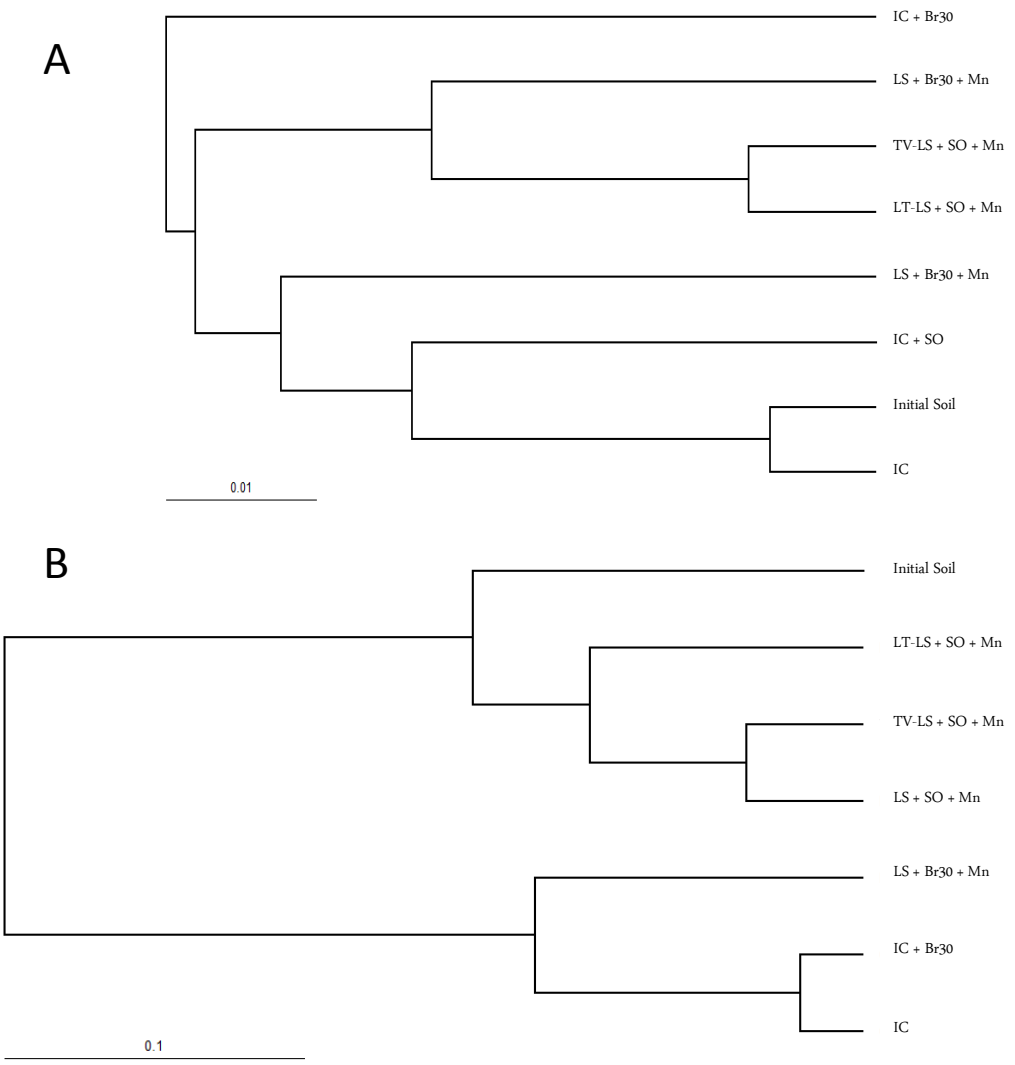


Fig. 1.10. Thetayc cluster tree showing the relationship of bacterial (A) and fungal (B) communities in the different microcosms to one another based on pyrosequence libraries. The scale bar is the distance between clusters in Thetayc units.

It is noteworthy that after 60 days of biostimulating the soil with moisture (IC), the bacterial populations did not change as much as in other treatments. This fact suggests that in this soil, when carbon and nutrients start to be scarce, bacterial diversity remain stable. The stagnation of diversity was well related also to the lack of bacterial growth observed in chapter 5.

However, the addition of surfactants, soybean oil or brij 30, produced a remarkable shift in the bacterial population, being the addition of the non-ionic surfactant the cause of the major change compared to the initial soil. In both treatments, Proteobacteria raised its relative importance in the community, especially where brij 30

was added. This community shifts correlated with lower PAHs depletion levels in both treatments, suggesting a use of surfactants as carbon source by soil bacteria or a toxic effect, as described in chapter 5.

On the other hand, LS addition produced an increase on soil biodiversity with respect to the respective incubation controls, suggesting that a wider range of bacteria could grow from the white straw or in a synergic way with the native fungal population which uses the LS as source of carbon and energy, although soybean oil and brij 30 were added to the soil. However, although biodiversity is higher, it is really noteworthy that the community shifts were caused in a higher degree by the type of surfactant added to the soil than to the LS addition (Figure 1.10A).

In addition, 16S rRNA pyrosequencing libraries showed that the bacterial diversity detected, when *Trametes versicolor* or *Lentinus tigrinus* were inoculated, was very similar to that found when no WRF was present, probably confirming that the low colonization rates achieved by WRF did not produce any remarkable bacterial community shift.

As it was aforementioned, it is of paramount importance to study also the roles and dynamics of fungi in historically aged contaminated sites, not to focusing only in the bacterial communities, because it is well-known that also fungi have multiple metabolic capabilities for PAHs depletion (Cerniglia, 1997) and their dynamics could be affecting the bacterial populations.

Surprisingly, high fungal biodiversity was detected in the initial soil, which had been polluted for years with a highly toxic antifungal as is creosote. *Fusarium* (23,2%) and *Scedosporium* (24,8) were the two main genera in the soil before the biotreatments were started. Both genera have been identified as PAHs degraders (Al-Turki, 2009; Thion et al., 2012).

On the other hand, when soybean oil or brij 30 were added to the soil, *Fusarium* achieved more than a 90% of relative importance in the soil, demonstrating its capability for adapting the new soil conditions (Figure 1.11). This fact suggests high

adaptation to the soil environment and such a huge competitiveness degree when moisture achieved optimum values and new carbon sources were added.

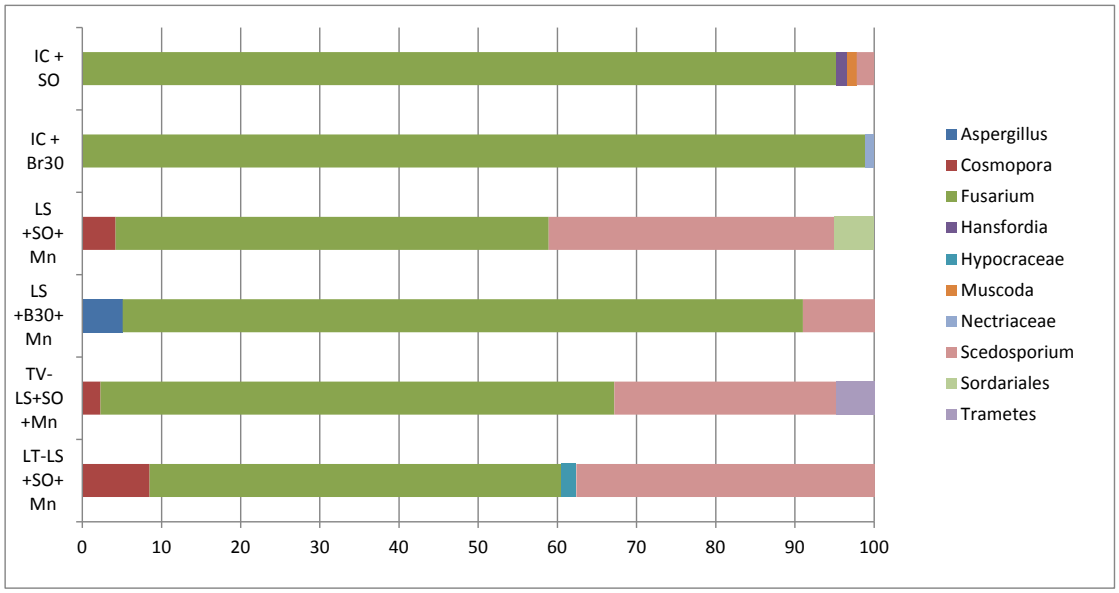


Figure 1.11. Fungal biodiversity composition, in relative abundance (%), of different genera based on the classification of partial ITS1 sequences of fungi from soil using the BLAST nt database.

Conclusions

CONCLUSIONS

Chapter 1:

- Mycoremediaton by means of allochthnous bioaugmentation with a white-rot fungus like *T. versicolor* is as a valuable remediation and detoxifying strategy, for soils contaminated with heavy mineral oil.
- The use of an external fungal inoculum produces a significant shift in the detectable biodiversity of the autochthonous bacterial community.
- A polyphasic approach in bioremediation tests in order to ensure reliable risk assessment of industrially polluted soils is strongly recommended.

Chapter 2:

- The lab-scale feasibility assay was very useful in order to determine the best incubation conditions for the creosote polluted soil in real field-scale.
- After the 180 days pilot-scale biopiling, 3-ring PAHs were almost completely depleted while an active bacterial population with the capacity to continue to degrade the 4-ring fraction was also detected. However, no remarkable degradation of 5-ring PAHs was reported.
- The quantification of pollutants in contaminated sites should not be the sole criterion for establishing the level of risk, since factors such as bioavailability and toxicity may be important as additional parameters.

Chapter 3:

- PAH-spiked slurry approach coupled with molecular ecology may help us to understand biodegradation and microbial aspects encountered in aged hydrocarbon-polluted environments.
- Benzo(a)anthracene and chrysene further biodegradation in the aged creosote-polluted soil is hampered by lack of bioavailability.
- *Mycobacterium sp.* and *Pseudomonas sp.* may contribute to the degradation of both 3- and 4-ringed PAHs, in which Sphingobacteriales of the CFB group could also have a role.

Chapter 4:

- A slurry incubation strategy was a feasible assay in order to enhance our knowledge of fungal-bacterial interactions in real historically polluted soils.
- The native microbial populations in soil were able to degrade 4-ring PAHs at high rates, but only under carbon limiting conditions.
- The microbial genera *Chryseobacterium, Pusillimonas, Sphingobium* and *Fusarium* could be playing an important role in HMW-PAH depletion.
- *T. versicolor* bioaugmentation was not able to improve autochthonous HMW-HAP degrading capabilities in non-sterile slurries, due to an antagonistic effect of the autochthonous populations.

Chapter 5:

- A highly recalcitrant TPH and HMW-PAHs fraction, remaining in an actual creosote-polluted soil after a 180-d pilot-scale biopiling treatment might be significantly degraded by a, biostimulation approach, based on LS addition.
- Degradation results might be further boosted by the presence of a concomitant mobilizing agent and Mn^{2+}.
- Mycoaugmentation approaches, which strictly require the concomitant LS addition with fungal inoculants might fail due to the LS-promoted growth of indigenous fungal and bacterial populations.
- The implementation of bioremediation technologies, based on exogenous inoculants, strictly require a lab-scale assessment of interactions between indigenous microbiota and the selected allochthonous species.

Chapter 6:

- Barcoded pyrosequencing is a powerful molecular tool to gain insight on microbial diversity present in contaminated soils and its dynamics through bioremediation processes.

- The eubacterial genus *Cupriavidus* could be playing an important role in HMW-PAHs degradation and, therefore, the high adaptation of the fungal genera *Fusarium* and *Scedosporium* to soil conditions was also evidenced.
- The amendment of a polluted soil with MAs could lead into important community shifts coinvolving changes in biodegradability of the compounds.
- Pyrosequencing results confirmed the failure of exogenous WRF to colonize the creosote-polluted soil, probably due to antagonistic interactions with the highly represented indigenous microbiota, confirming the importance of increasing knowledge in determining the role of certain native fungi and bacteria in real industrial soil bioremediation processes.
- It was not possible to describe a direct significant relationship between higher HMW-PAHs depletion and the presence of any fungal genus in the polluted soil.

References

REFERENCES

Aitken, M.D., Stringfellow, W.T., Nagel, R.D., Kazunga, C., Chen, S. 1998. Characteristics of phenanthrene-degrading bacteria isolated from soils contaminated with polycyclic aromatic hydrocarbons. *Can. J. Microbiol.,* **44**(8), 743-752.

Al-Turki, A.I. 2009. Microbial polycyclic aromatic hydrocarbons degradation in soil. *Res. J. Environ. Toxicol*. **3**(1): 1-8.

Alexander, M. 2000. Aging, bioavailability, and overestimation of risk from environmental pollutants. *Env. Sci. Technol.,* **34**, 4259-4265.

Alexander, M. 1999. Biodegradation and bioremediation, Academic Press. London.

Alexander, M. 1995. How toxic are toxic chemicals in soil? *Env. Sci. Technol.,* **29**, 2713-2717.

Amann, R.I., Ludwig, W., Schleifer, K.H. 1995. Phylogenetic Identification and In Situ Detection of Individual Microbial Cells without Cultivation. *Microb. Rev.*, **59**, 143-169.

Amend, A., Seifert, K.A., Bruns, T.D. 2010. Quantifying microbial communities with 454 pyrosequencing: does read abundance count? *Molec. Ecol.*, **19**(24), 5555-5565.

Andersson, B.E., Lundstedt, S., Tornberg, K., Schnürer, Y., Öberg, L.G., Mattiasson, B. 2003. Incomplete degradation of polycyclic aromatic hydrocarbons in soil inoculated with wood-rotting fungi and their effect on the indigenous soil bacteria. *Environ. Chem.* **22**(6):1238-1243.

Atlas, R.M. 1993. Bioaugmentation to enhance microbial bioremediation. in: *Biotreatment of industrial and Hazardous Waste*, M.A. Gealt (Ed.). McGraw-Hill Inc. New York.

Atlas, R.M. 1981. Microbial degradation of petroleum hydrocarbons: an environmental perspective. *Microbiol. Rev.,* **45**(1), 180-209.

Avramova, T., Sotirova, A., Galabova, D., Karpenko, E. 2008. Effect of Triton X-100 and rhamnolipid PS-17 on the mineralization of phenanthrene by Pseudomonas sp. cells. *Int. Biodet. Biodegr.,* **62**(4), 415-420.

Bamforth, S.M., Singleton, I. 2005. Bioremediation of polycyclic aromatic hydrocarbons: current knowledge and future directions. *J. Chem. Technol. Biotechnol.,* **80**, 723-736.

Banitz, T., Wick, L.Y., Fetzer, I., Frank, K., Harms, H., Johst, K. 2011. Dispersal networks for enhancing bacterial degradation in heterogeneous environments. *Environ. Pollut.*, **159**(10), 2781-8.

Barajas-Aceves, M., Hassan, M., Tinoco, R., Vazquez-Duhalt, R. 2002. Effect of pollutants on the ergosterol content as indicator of fungal biomass. *J. Microbiol. Meth.*, **50**, 227-236.

Bernhardt, R. 2006. Cytochromes P450 as versatile biocatalysts. *J. Biotechnol.*, **124**(1), 128-45.

Bhatt, M., Cajthaml, T., Sasek, V. 2002. Mycoremediation of PAH-contaminated soil. *Fol. Microbiol.* **47**(3), 255-258.

Blumer, M. 1976. Polycyclic aromatic compounds in nature. *Sci. Am.* **234**(3), 35-45.

Boominathan, K., Reddy, C.A. 1992. cAMP-mediated differential regulation of lignin peroxidase and manganese-dependent peroxidase production in the white-rot basidiomycete Phanerochaete chrysosporium. *Proc. Natl. Acad. Sci. U.S.A.*, **89**(12), 5586-90.

Boopathy, R. 2002. Effect of food-grade surfactant on bioremediation of explosives-contaminated soil. *J. Hazard. Mater.*, **92**(1), 103-114.

Boschker, H.T.S., Nold, S.C., Wellsbury, P., Bos, D., de Graaf, W., Pel, R., Parkers, R.J., Cappenberg, T.E. 1998. Direct linking of microbial populations to specific biogeochemical processes by 13C-labelling of biomarkers. *Nature*, **392**, 801-804.

Bosma, T.N.P., Middeldorp, P.J.M., Schraa, G., Zehnder, A.J.B. 1997. Mass transfer limitations of biotransformation: quantifying bioavailability. *Environ. Sci.Technol.*, **31**, 248–252.

Bossert, I.D., Bartha, R. 1984. The fate of petroleum in soil ecosystems. in: *Petroleum Microbiology*, R.M. Atlas (Ed.). MacMillan Publishing Co. New York, pp. 453-473.

Breedveld, G.D., Sparrevik, M. 2001. Nutrient-limited biodegradation of PAH in various soil strata at a creosote contaminated site. *Biodegradation*, **11**, 391-399.

Burns, R.G. 1982. Enzyme activity in soil: Location and a possible role in microbial ecology. *Soil. Biol. Biochem.*, **14**(5), 423-427.

Casellas, M., Fernandez, P., Bayona, J.M., Solanas, A.M. 1995. Bioassay-directed chemical analysis of genotoxic components in urban airborne particulate matter from Barcelona (Spain). *Chemosphere*, **30**(4), 725-40.

Casellas, M., Grifoll, M., Bayona, J.M., Solanas, A.M. 1997. New metabolites in the degradation of fluorene by *Arthrobacter* sp. strain F101. *Appl. Environ. Microbiol.*, **63**, 819-826.

Casillas, R.P., Crow, S.A., Jr., Heinze, T.M., Deck, J., Cerniglia, C.E. 1996. Initial oxidative and subsequent conjugative metabolites produced during the metabolism of phenanthrene by fungi. *J. Ind. Microbiol.*, **16**(4), 205-15.

Castillo, M.P. 1997. Degradation of pesticides by Phanerochaete chrysosporium in solid substrate fermentation. in: *Department of Microbiology*, Swedish University of Agricultural Sciences. Uppsala.

Cerniglia, C.E. 1992. Biodegradation of polycyclic aromatic hydrocarbons. *Biodegradation*, **3**, 351-368.

Cerniglia, C.E. 1997. Fungal metabolism of polycyclic aromatic hydrocarbons: past, present and future applications in bioremediation. *J. Ind. Microbiol. Biotechnol.*, **19**(5-6), 324-33.

Clements, W.H., Oris, J.T., Wissing, T.E. 1994. Accumulation and food chain transfer of fluoranthene and benzo(a)pyrene in Chironomus riparus and Lepomis macrochirus. *Arch. Environ. Contam. Toxicol.*, **26**, 261-266.

Collins, P.J., Dobson, A.D.W. 1995. Extracellular lignin and manganese peroxidase production by the white rot fungus Coriolus versicolor 290. *Biotechnol. Lett.*, **17**, 989-992.

Collins, P.J., Kotterman, M., Field, J.A., Dobson, A. 1996. Oxidation of anthracene and benzo[a]pyrene by laccases from *Trametes versicolor*. *Appl. Environ. Microbiol.*, **62**(12), 4563-4567.

Chang, B.V., Chang, I.T., Yuan, S.Y. 2008. Anaerobic degradation of phenanthrene and pyrene in mangrove sediment. *Bull. Environ. Contam. Toxicol.*, **80**(2), 145-9.

Chulalaksananukul, S., Gadd, G.M., Sangvanich, P., Sihanonth, P., Piapukiew, J., Vangnai, A.S. 2006. Biodegradation of benzo(a)pyrene by a newly isolated *Fusarium* sp. *FEMS Microbiol. Lett.*, **262**(1), 99-106.

Dagley, S. 1981. New perspectives in aromatic catabolism. in: *Microbial degradation of xenobiotics and recalcitrant compounds*, A.M.C. T. Leisinger, R. Hütter and J. Nüesch editors (Ed.). Academic Press. New York, pp. 181-186.

Das, N., Chandran, P. 2011. Microbial degradation of petroleum hydrocarbon contaminants: an overview. *Biotechnol. Res. Int.*, 2011, 941810.

Dejonghe, W., Boon, N., Seghers, D., Top, E.M., Verstraete, W. 2001. Bioaugmentation of soils by increasing microbial richness: missing links. *Environ. Microbiol.*, **3**(10), 649-57.

Deschenes, L., Lafrance, P., Villeneuve, J.P., Samson, R. 1996. Adding sodium dodecyl sulfate and Pseudomonas aeruginosa UG2 biosurfactants inhibits polycyclic aromatic hydrocarbon biodegradation in a weathered creosote-contaminated soil. *Appl. Microbiol. Biotechnol*., **46**, 638-46

Dittmer, J.K., Patel, N.J., Dhawale, S.W., Dhawale, S.S. 1997. Production of multiple laccase isoforms by Phanerochaete chrysosporium grown under nutrient sufficiency. *FEMS Microbiol. Lett.*, **149**, 65-70.

Dorn, P.B., Salanitro, J.P. 2000. Temporal ecological assessment of oil contaminated soils before and after bioremediation. *Chemosphere*, **40**(4), 419-26.

Dorn, P.B., Vipond, T.E., Salanitro, J.P., Wisniewski, H.L. 1998. Assessment of the acute toxicity of crude oils in soils using earthworms, microtox®, and plants. *Chemosphere*, **37**(5), 845-860.

Dumont, J., Schultz, T.W., Buchanan, M., Kao, G. 1983. Frog embryo teratogenesis assay-Xenopus (FETAX)- a short-term assay applicable to complex environmental mixtures. in: *Short-Term Bioassays in the Analysis of Complex Environmental Mixtures*, S. Waters, C. Claxton, N. Chernoff (Ed.). Plenum Press. New York, pp. 393-405.

Edwards, D.A., Luthy, R.G., Liu, Z. 1991. Solubilization of polycyclic aromatic hydrocarbons in micellar nonionic surfactant solutions. *Env. Sci. Technol.*, **25**(1), 127-133.

Efroymson, R.A., Alexander, M. 1995. Reduced mineralization of low concentrations of phenanthrene because of sequestering in nonaqueous-phase liquids. *Environ. Sci. Technol.*, **29**(2), 515-21.

Eisler, R. 1987. Polycyclic aromatic hydrocarbon hazards to fish, wildlife and invertebrates: a synoptic review. *U.S. Fish and Wildlife Service Biological Report*, 81-85.

El Fantroussi, S., Verschuere, L., Verstraete, W., Top, E.M. 1999. Effect of phenylurea herbicides on soil microbial communities estimated by analysis of 16S rRNA gene fingerprintings and community-level physiological profiles. *Appl. Environ. Microbiol.*, **65**, 982-988.

Exner, J.H. 1994. *Bioremediation: Field Experience*. Lewis publishers, FL.

Farmer, P.B., Singh, R., Kaur, B., Sram, R.J., Binkova, B., Kalina, I., Popov, T.A., Garte, S., Taioli, E., Gabelova, A., Cebulska-Wasilewska, A. 2003. Molecular epidemiology studies of carcinogenic environmental pollutants: effects of polycyclic aromatic hydrocarbons (PAHs) in environmental pollution on exogenous and oxidative DNA damage. *Mutat. Res.*, **544**, 397–402.

Federici, E., Leonardi, V., Giubilei, M.A., Quaratino, D., Spaccapelo, R., D'Annibale, A., Petruccioli, M. 2007. Addition of allochthonous fungi to a historically contaminated soil affects both remediation efficiency and bacterial diversity. *Appl. Microbiol. Biotechnol.*, **77**(1), 203-11.

Fischer, S.G., Lerman, L.S. 1979. Length-independent separation of DNA restriction fragments in two-dimensional gel electrophoresis. *Cell*., **16**(1), 191-200.

Galhaup, C., Wagner, H., Hinterstoisser, B., Haltrich, D. 2002. Increased production of laccase by the wood-degrading basidiomycete Trametes pubescens. *Enz. Microbiol. Technol*., **30**, 529-536.

Gandolfi, I., Sicolo, M., Franzetti, A., Fontanarosa, E., Santagostino, A., Bestetti, G. 2010. Influence of compost amendment on microbial community and ecotoxicity of hydrocarbon-contaminated soils. *Bioresour. Technol.*, **101**(2), 568-75.

Gans, J., Wolinsky, M., Dunbar, J. 2005. Computational improvements reveal great bacterial diversity and high metal toxicity in soil. *Science*, **309**(5739), 1387-90.

Garcia Frutos, F.J., Escolano, O., Garcia, S., Babin, M., Fernandez, M.D. 2010. Bioventing remediation and ecotoxicity evaluation of phenanthrene-contaminated soil. *J. Hazard. Mater.*, **183**(1-3), 806-13.

Gentry, T., Rensing, C., Pepper, I. 2010. New approaches for bioaugmentation as a remediation technology. *Crit. Rev. Environ. Sci. Technol.* **34**(5):447-494.

Goldman, R. 2001. Smoking increase carcinogenic polycyclic aromatic hydrocarbons in human lung tissue. *Cancer Research*, **61**, 6367-6371.

Gramss, G., Voigt, K.D., Kirsche, B. 1999. Degradation of polycyclic aromatic hydrocarbons with three to seven aromatic rings by higher fungi in sterile and unsterile soils. *Biodegradation*, **10**(1), 51-62.

Gray, N.D., Head, I.M. 2001. Linking genetic identity and function in communities of uncultured bacteria. *Environ. Microbiol.*, **3**(8), 481-92.

Green, S.J., Leigh, M.B. and Neufeld, J.D. 2009. Denaturing gradient gel electrophoresis (DGGE) for microbial community analysis. in: *Microbiology of*

Hydrocarbons, Oils,Lipids, and Derived Compounds, K.N. Timmis (Ed.), Springer. Heidelberg (Germany), pp. 4137-4158.

Greene, J.C., Bartels, C.L., Warren-Hicks, W.J., Parkhurst, B.R., Linder, G.L., Peterson, S.A., Miller, W.E. 1988. Protocols for short term toxicity screening of hazardous waste sites, Vol. EPA/600/3-88/029, US-EPA.

Grifoll, M., Solanas, A.M., Bayona, J.M. 1990. Characterization of genotoxic components in sediments by mass spectometric techniques combined with Salmonella/microsome test. *Arch. Environ. Contam. Toxicol.,* **19**, 175-184.

Grifoll, M., Solanas, A.M., Bayona, J.M. 1992a. Bioassay-directed chemical characterization of genotoxic agents in the dissolved and particulate water phases of the Besos and Llobregat rivers (Barcelona, Spain). *Arch. Environ. Contam. Toxicol.,* **23**, 19-25.

Grimalt, J.O., Grifoll, M., Solanas, A.M., Albaigés, J. 1991. Microbial degradation of marine evaporitic crude oils. *Geochim. Cosmo. Act.,* **55**, 1903-1913.

Grishchenkov, V.G., Townsend, R.T., McDonald, T.J., Autenrieth, R.L., Bonner, J.S., Boronin, A.M. 2000. Degradation of petroleum hydrocarbons by facultative anaerobic bacteria under aerobic and anaerobic conditions. *Process Biochem.*, **35**(9), 889-896.

Guthrie, E.A., Pfaender, F.K. 1998. Reduced pyrene bioavailability in microbially active soils. *Env. Sci. Technol.*, **32**, 501-508.

Ha, H.C., Honda, Y., Watanabe, T., Kuwahara, M. 2001. Production of manganese peroxidase by pellet culture of the lignin-degrading basidiomycete, Pleurotus ostreatus. *Appl. Microbiol. Biotechnol.*, **55**(6), 704-11.

Haider, K., Lim, S., Flaig, W. 1964. Experimente und Theorien über den Ligninabbau bei der Weibfäule des Holzes und bei der Verrottung pflanzlicher Substanz im Boden. *Holzforschung*, **18**, 81-88.

Hammel, K.E. 1995. Mechanisms for polycyclic aromatic hydrocarbon degradation by ligninolytic fungi. *Environ. Health Perspect,* **103** Suppl 5, 41-3.

Harayama, S., Rekik, M. 1989. Bacterial aromatic ring-cleavage enzymes are classified into two different gene families. *J. Biol. Chem.,* **264**(26), 15328-33.

Haritash, A.K., Kaushik, C.P. 2009. Biodegradation aspects of Polycyclic Aromatic Hydrocarbons (PAHs): A review. *J. Hazard. Mater.*, **169**(1-3), 1-15.

Harms, H., Bosma, T.P.M. 1996. Mass transfer limitation of microbial growth and pollutant degradation. *J. Ind. Microbiol.*, **16**, 1-9.

Harms, H., Schlosser, D., Wick, L.Y. 2011. Untapped potential: exploiting fungi in bioremediation of hazardous chemicals. *Nat. Rev. Microbiol.*, **9**(3), 177-92.

Hatakka, A. 1994. Lignin-modifying enzymes from selected white-rot fungi: production and role in lignin degradation. *FEMS Microbiol. Rev.*, **13**, 125-133.

Hatzinger, P.B., Alexander, M. 1995. Effect of ageing of chemicals in their biodegradability and extractability. *Environ. Sci. Technol.*, **29**, 537-545.

He, S., Kunin, V., Haynes, M., Martin, H.G., Ivanova, N., Rohwer, F., Hugenholtz, P., McMahon, K.D. 2010. Metatranscriptomic array analysis of 'Candidatus Accumulibacter phosphatis'-enriched enhanced biological phosphorus removal sludge. *Environ. Microbiol.*, **12**(5), 1205-17.

Hofrichter, M. 2002. Review: lignin conversion by manganese peroxidase. *Enzyme Microbiol. Technol.*, **30**, 454-466.

Holliger, C., Zehnder, A.J. 1996. Anaerobic biodegradation of hydrocarbons. *Curr. Opin. Biotechnol.*, **7**(3), 326-30.

Hund, K., Traunspurger, W. 1994. Ecotox-evaluation strategy for soil bioremediation exemplified for a PAH-contaminated site. *Chemosphere*, **29**(2), 371-90.

Hundt, K., Jonas, U., Hammer, E., Schauer, F. 1999. Transformation of diphenyl ethers by Trametes versicolor and characterization of ring cleavage products. *Biodegradation*, **10**, 279-286.

Ibekwe, A.M., Kennedy, A.C., Frohne, P.S., Papiernik, S.K., Yang, C.H., Crowley, D.E. 2002. Microbial diversity along a transect of agronomic zones. *FEMS Microbiol. Ecol.*, **39**(3), 183-91.

Juhasz, A.L., Naidu, R. 2000. Bioremediation of high molecular weight polycyclic aromatic hydrocarbons: a review of the microbial degradation of benzo(a)pyrene. *Int. Biodet. Biodeg.*, **45**, 57-88.

Juvonen, R., Martikainen, E., Schultz, E., Joutti, A., Ahtiainen, J., Lehtokari, M. 2000. A battery of toxicity tests as indicators of decontamination in composting oily waste. *Ecotoxicol. Environ. Saf.*, **47**(2), 156-66.

Kanaly, R.A., Harayama, S. 2000. Biodegradation of high-molecular-weight polycyclic aromatic hydrocarbons by bacteria. *J. Bacteriol.*, **182**(8), 2059-67.

Kästner, M. 2000. Degradation of aromatic and polyaromatic compounds. in: *Environmental processes II: soil decontamination, biotechnology*, R.G. Rehm H.J., Pühler A., Stadler P. (Ed.), Vol. 11b, Wiley. New York, pp. 212-239.

Keddy, C.J., Greene, J.C., Bonnell, M.A. 1995. Review of whole-organism bioassays: soil, freshwater sediment, and freshwater assessment in Canada. *Ecotoxicol. Environ. Saf.*, **30**(3), 221-51.

Keith, L.H., Telliard, W.A. 1979. Priority pollutants: a perspective view. *Env. Sci. Technol.*, **13**, 416-423.

Kelley, I., Freeman, J.P., Cerniglia, C.E. 1990. Identification of metabolites from degradation of naphthalene by a Mycobacterium sp. *Biodegradation*, **1**(4), 283-290.

Klein, J. 2000. Possibilities, limits and future developments of soil bioremediation. in: *Environmental processes II. Soil Decontamination, Biotechnology*, G. Reed (Ed.), Vol. 11b, Wiley-VCH. Weinheim, pp. 465-476.

Kotterman, M.J., Rietberg, H.J., Hage, A., Field, J.A. 1998. Polycyclic aromatic hydrocarbon oxidation by the white-rot fungus Bjerkandera sp. strain BOS55 in the presence of nonionic surfactants. *Biotechnol. Bioeng.*, **57**(2), 220-7.

Kremer, S., Anke, H. 1997. Fungi in bioremediation. in: *Fungal Biotechnology*, T. Anke (Ed.), Chapman & Hall. Weinheim, pp. 275-295.

Leatham, G.F., Kirk, T.K. 1983. Regulation of ligninolytic activity by nutrient nitrogen in white-rot basidiomycetes. *FEMS Microbiol. Lett.*, **16**, 65-67.

Lee, S.H., Oh, B.I., Kim, J.G. 2008. Effect of various amendments on heavy mineral oil bioremediation and soil microbial activity. *Biores. Technol.*, **99**, 2578-2587.

Leonardi, V., Sasek, V., Petruccioli, M., D'Annibale, A., Erbanová, P., Cajthaml, T. 2007. Bioavailability modification and fungal biodegradation of PAHs in aged industrial soils. *Int. Biodet. Biodeg.*, **60**(3), 165-170.

Leonardi, V., Giubilei, M.A., Federici, E., Spaccapelo, R., Sasek, V., Novotny, C. 2008. Mobilizing agents enhance fungal degradation of polycyclic aromatic hydrocarbons and affect diversity of indigenous bacteria in soil. *Biotechnol Bioeng.*, **101**, 273-85.

Leonowicz, A., Cho, N.S., Luterek, J., Wilkolazka, A., Wojtas-Wasilewska, M., Matuszewska, A., Hofrichter, M., Wesenberg, D., Rogalski, J. 2001. Fungal laccase: properties and activity on lignin. *J. Basic Microbiol.*, **41**(3-4), 185-227.

Leutwein, C., Heider, J. 1999. Anaerobic toluene-catabolic pathway in denitrifying Thauera aromatica: activation and beta-oxidation of the first intermediate, (R)-(+)-benzylsuccinate. *Microbiology*, **145** (Pt 11), 3265-71.

Li, D., Alic, M., Brown, J.A., Gold, M.H. 1995. Regulation of manganese peroxidase gene transcription by hydrogen peroxide, chemical stress, and molecular oxygen. *Appl. Environ. Microbiol.*, **61**(1), 341-5.

Li, J., Chen, B. 2009. Surfactant-mediated Biodegradation of Polycyclic Aromatic Hydrocarbons. *Materials*, **2**, 76-94.

Liang, Y., Britt, D.W., McLean, J.E., Sorensen, D.L., Sims, R.C. 2007. Humic acid effect on pyrene degradation: finding an optimal range for pyrene solubility and mineralization enhancement. *Appl. Microbiol. Biotechnol.*, **74**(6), 1368-75.

Lu, X.Y., Zhang, T., Fang, H.H. 2011. Bacteria-mediated PAH degradation in soil and sediment. *Appl. Microbiol. Biotechnol.*, **89**(5), 1357-71.

Llimona, X. 1991. Fongs i líquens. in: *Enciclopedia Catalana*, Vol. 5.

Mackay, N., Betts, W.B. 1991. The fate of chemicals in soil. in: *Biodegradation: natural and synthetic materials*, W.B. Betts (Ed.), Springer Verlag. London, pp. 89-117.

Magan, N., Fragoeiro, S., Bastos, C. 2010. Environmental Factors and Bioremediation of Xenobiotics Using White Rot Fungi. *Mycobiol*., **38**, 238-248.

Maltseva, O.V., Niku-Paavola, M.L., Leontievsky, A.A., Myasoedova, N.M., Golovleva, L.A. 1991. Ligninolytic enzymes of the white-rot fungus Panus tigrinus. *Biotechnol. Appl. Biochem.*, **13**, 291-302.

Manefield, M., Whiteley, A.S., Griffiths, R.I., Bailey, M.J. 2002. RNA stable isotope probing, a novel means of linking microbial community function to phylogeny. *Appl. Environ. Microbiol.*, **68**(11), 5367-73.

Marquez-Rocha, F.J., Hernandez-Rodriguez, V.Z., Vazquez-Duhalt, R. 2000. Biodegradation of soil-adsorbed polycyclic aromatic hydrocarbons by the white rot fungus Pleurotus ostreatus. *Biotechnol. Lett.*, **22**, 469-472.

Massias, D., Grossi, V., Bertrand, J. 2003. In situ anaerobic degradation of petroleum alkanes in marine sediments: preliminary results. *Comptes Rendus Geoscience*, **335**(5), 435-439.

Meckenstock, R.U., Mouttaki, H. 2011. Anaerobic degradation of non-substituted aromatic hydrocarbons. *Curr. Opin. Biotechnol.*, **22**(3), 406-14.

Mille-Lindblom, C., von Wachenfeldt, E., Tranvik, L.J. 2004. Ergosterol as a measure of living fungal biomass: persistence in environmental samples after fungal death. *J. Microbiol. Methods*, **59**(2), 253-62.

Mougin, C., Pericaud, J., Dubroca, J., Asther, M. 1997. Enhanced mineralization of lindane in soils supplemented with the white rot basidiomycete *Phanerochaete chrysosporium. Soil. Biol. Biochem.* **29**:1321-1324.

Mueller, J.G., Chapman, P.J., Pritchard, P.H. 1989. Action of a fluoranthene-utilizing bacterial community on polycyclic aromatic hydrocarbon components of creosote. *Appl. Environ. Microbiol.*, **55**(12), 3085-90.

Mulligan, C.N., Yong, R.N., Gibbs, B.F. 2001. Surfactant-enhanced remediation of contaminated soil: a review. *Eng. Geol.*, **60**(1-4), 371-380.

Nestler, F.H.M. 1974. The characterization of wood-preserving creosote by physical and chemical methods of analysis. *US Department of Agriculture Forest Service Research Paper, FPL 195 (1974) Madison, Wisc.*

Nisbet, D., La Goy, P. 1992. Toxic equivalence factors (TEFs) for polycyclic aromatic hydrocarbons (PAHs). *Regul. Toxicol. Pharmacol.*, **16**, 290-300.

Novotný, C., Svobodová, K., Kasinath, A., Erbanová, P. 2004. Biodegradation of synthetic dyes by Irpex Lacteus under various growth conditions. *Int. Biodet. Biodeg.*, **54**, 215-223.

Pirnik, M.P., Atlas, R.M., Bartha, R. 1974. Hydrocarbon metabolism by Brevibacterium erythrogenes: normal and branched alkanes. *J. Bacteriol.*, **119**(3), 868-78.

Pointing, S.B. 2001. Feasibility of bioremediation by white-rot fungi. *Appl. Microbiol. Biotechnol.*, **57**(1-2), 20-33.

Poretsky, R.S., Bano, N., Buchan, A., LeCleir, G., Kleikemper, J., Pickering, M., Pate, W.M., Moran, M.A., Hollibaugh, J.T. 2005. Analysis of microbial gene transcripts in environmental samples. *Appl. Environ. Microbiol.*, **71**(7), 4121-6.

Pozdnyakova, N.N., Leontievsky, A.A., Golovleva, L.A. 1999. Extracellular oxidases from solid-state culture of the ligninolytic fungus panus tigrinus 8/18. *Biochemistry (Mosc)*, **64**(4), 442-7.

Prince, R.C., Garrett, R.M., Bare, R.E., Grossman, M.J., Townsend, T., Suflita, J.M., Lee, K., Owens, E.H., Sergy, G.A., Braddock, J.F., Linstrom, J.E., Lessard, R.R. 2003. The roles of photooxidation and biodegradation in long-term weathering of crude and heavy fuel oils spill. *Sci. Technol. Bulletin*, **8**, 145-156.

Quantin, C., Joner, E.J., Portal, J.M., Berthelin, J. 2005. PAH dissipation in a contaminated river sediment under oxic and anoxic conditions. *Environ. Pollut.*, **134**(2), 315-22.

Quaratino, D., Federici, F., Fenice, M., D'Annibale, A. 2006. Mn-peroxidase production by Panus tigrinus CBS 577.79: response surface optimisation and bioreactor comparison. *J. Chem. Technol. Biotechnol.*, **81**, 832-840.

Quaratino, D., Federici, F., Petruccioli, M., Fenice, M., D'Annibale, A. 2007. Production, purification and partial characterisation of a novel laccase from the white-rot fungus Panus tigrinus CBS 577.79. *Antonie Van Leeuwenhoek*, **91**(1), 57-69.

Radajewski, S., Ineson, P., Parekh, N.R., Murrell, J.C. 2000. Stable-isotope probing as a tool in microbial ecology. *Nature*, **403**(6770), 646-9.

Radtke, C., Cook, W.S., Anderson, A. 1994. Factors affecting antagonism of the growth of *Phanerochaete chrysosporium* by bacteria isolated from soils. *Chem. Mat. Sci.*, **41**(2), 274-280.

Reddy, C.A., Dsouza, T.M. 1994. Physiology and molecular biology of the lignin peroxidases of Phanerochaete chrysosporium. *FEMS. Microbiol. Rev.*, **13**, 137-152.

Reddy, C.A., Mathew, Z. 2001. Bioremediation potential of white rot fungi. in: *Fungi in bioremediation*, G.M. Gadd (Ed.), Cambridge University Press. Cambridge, pp. 52-78.

Riesenfeld, C.S., Schloss, P.D., Handelsman, J. 2004. Metagenomics: genomic analysis of microbial communities. *Annu. Rev. Genet.*, **38**, 525-52.

Roche, N., Venague, A., Desgranges, C., Durand, A. 1993. Use of chitin measurement to estimate fungal biomass in solid state fermentation. *Biotechnol. Adv.*, **11**(3), 677-683.

Rodríguez-Cruz, M.S., Sanchez-Martin, M.J., Sanchez-Camazano, M. 2005. A comparative study of adsorption of an anionic and a non-ionic surfactant by soils based on physicochemical and mineralogical properties of soils. *Chemosphere*. **61**(1):56-64.

Rosini, F.D. 1960. Hydrocarbons in petroleum. *J. Chem. Educ.*, **39**, 554-561.

Rothschild, N., Levkowitz, A., Hadar, Y., Dosoretz, C.G. 1999. Manganese deficiency can replace high oxygen levels needed for lignin peroxidase formation by Phanerochaete chrysosporium. *Appl. Environ. Microbiol.*, **65**, 483-488.

Rutherford, P.M., Banerjee, D.K., Luther, S.M., Gray, M.R., Dudas, M.J., McGill, W.B., Pickard, M.A., Salloum, M.J. 1998. Slurry-phase bioremediation of creosote- and petroleum-contaminated soil. *Env. Technol.*, **19**, 683-696.

Sabaté, J., Grifoll, M., Viñas, M., Solanas, A.M. 1999. Isolation and characterization of 2-methylphenanthrene utilizing bacterium: identification of ring cleavage metabolites. *Appl. Microbiol. Biotechnol.,* **52**, 704-712.

Sabaté, J. Viñas, M. Solanas, A.M. 2004. Laboratory-scale bioremediation experiments on hydrocarbon-contaminated soils. *Int. Biodet. Biodeg.,* **54**, 19-25

Sabaté, J., Viñas, M., Solanas, A.M. 2006. Bioavailability assessment and environmental fate of polycyclic aromatic hydrocarbons in bioestimulated creosote-contaminated soil. *Chemosphere.,* **63**(10), 1648-1659.

Salanitro, J.P., Dorn, P.B., Huesemann, M.H., Moore, K.O., Rhodes, I.A., Jackson, L.M., Vipond, T.E., Western, W.W., Wisniewski, H.L. 1997. Crude oil hydrocarbon bioremediation and soil ecotoxicity assessment. *Env. Sci. Technol.,* **31**, 1769-1776.

Saparrat, M.C., Guillen, F., Arambarri, A.M., Martinez, A.T., Martinez, M.J. 2002. Induction, isolation, and characterization of two laccases from the white rot basidiomycete Coriolopsis rigida. *Appl. Environ. Microbiol.*, **68**(4), 1534-40.

Sariaslani, F.S. 1989. Microbial enzymes for oxidation of organic molecules. *Crit. Rev. Biotechnol.*, **9**(3), 171-257.

Saterbak, A., Toy, R.J., Wong, D.C.L., McMain, B.J., Williams, M.P., Dorn, P.B., Brzuzy, L.P., Chai, E.Y., Salanitro, J.P. 1999. Ecotoxicological and analytical assessment of hydrocarbon-contaminated soils and application to ecological risk assessment. *Env. Toxicol. Chem.*, **18**, 1591-1607.

Scheel, T., Hofer, M., Ludwig, S., Holker, U. 2000. Differential expression of manganese peroxidase and laccase in white-rot fungi in the presence of manganese or aromatic compounds. *Appl. Microbiol. Biotechnol.*, **54**(5), 686-91.

Scheller, U., Zimmer, T., Kargel, E., Schunck, W.H. 1996. Characterization of the n-alkane and fatty acid hydroxylating cytochrome P450 forms 52A3 and 52A4. *Arch. Biochem. Biophys.*, **328**(2), 245-54.

Schloss, P.D., Handelsman, J. 2006. Toward a Census of Bacteria in Soil. *PLoS Comp. Biol.*, **2**(7), e92. doi:10.1371/journal.pcbi.0020092.

Schneider, J., Grosser, R., Jayasimhulu, K., Xue, W., Warshawsky, D. 1996. Degradation of pyrene, benz[a]anthracene, and benzo[a]pyrene by Mycobacterium sp. strain RJGII-135, isolated from a former coal gasification site. *Appl. Environ. Microbiol.*, **62**(1), 13-9.

Seghezzi, W., Sanglard, D., Fiechter, A. 1991. Characterization of a second alkane-inducible cytochrome P450-encoding gene, Cyp52A2, form Candida tropicalis. *Gene*, **106**, 51-60.

Semple, K.T., Morris, A.W.J., Paton, G.I. 2003. Bioavailability of hydrophobic organic contaminants in soils: fundamental concepts and techniques for analysis. *Europ. J. Soil Sci.*, **54**, 809-819.

Simon, C., Daniel, R. 2011. Metagenomic Analyses: Past and Future Trends. *Appl. Environ. Microbiol.*, **77**(7), 1153-1161.

Singleton, D.R., Richardson, S.D., Aitken, M.D. 2011. Pyrosequence analysis of bacterial communities in aerobic bioreactors treating polycyclic aromatic hydrocarbon-contaminated soil. *Biodegradation*, **22**(6), 1061-73.

Sogin, M.L., Morrison, H.G., Huber, J.A., Mark Welch, D., Huse, S.M., Neal, P.R., Arrieta, J.M., Herndl, G.J. 2006. Microbial diversity in the deep sea and the underexplored "rare biosphere". *Proc. Natl. Acad. Sci. U.S.A.*, **103**(32), 12115-20.

Solanas, A.M. 1981. Contaminación y biodegradación de hidrocarburos en las aguas superficiales del Barcelonés. Tesis doctoral. Universitat de Barcelona. Departament de Microbiologia. Barcelona.

Solanas, A. M., Pares, R., Bayona, J.M., Albaigés, J. 1984. "Degradation of aromatic petroleum hydrocarbons by pure microbial cultures. *Chemosphere*., **13**, 593-601.

Sutherland, J.B., Rafii, F., Khan, A.A., Cerniglia, C.E. 1995. Mechanisms of polycyclic aromatic hydrocarbon degradation. in: *Microbial transformation and degradation of toxic organic chemicals*, C.E. Cerniglia (Ed.). Wiley-Liss. New York, pp. 269-306.

Thion, C., Cébron, A., Beguiristain, T., Leyval, C. 2012. PAH biotransformation and sorption by Fusarium solani and Arthrobacter oxydans isolated from a polluted soil in axenic cultures and mixed co-cultures. *Int. Biodet. Biodeg*., **68**, 28-35.

Thurston, C.F. 1994. The structure and function of fungal laccases. *Microbiology*, **140**, 19-26.

Tien, M., Kirk, T.K. 1988. Lignin peroxidase of Phanaerochaete chrysosporium. *Method. Enzymol.*, **161**, 238-249.

Torsvik, V., Ovreas, L., Thingstad, T.F. 2003. Prokaryotic Diversity - Magnitude, Dynamics and Controlling Factors. *Science*, **296**, 1064-1066.

Trower, M.K., Buckland, R.M., Higgins, R., Griffin, M. 1985. Isolation and Characterization of a Cyclohexane-Metabolizing Xanthobacter sp. *Appl. Environ. Microbiol.*, **49**(5), 1282-9.

Tsai, J.C., Kumar, M., Lin, J.G. 2009. Anaerobic biotransformation of fluorene and phenanthrene by sulfate-reducing bacteria and identification of biotransformation pathway. *J. Hazard. Mater.*, **164**(2-3), 847-55.

Twiss, M.R., Granier, L., Lafrance, P., Campbell, P.G.C. 1999. Bioaccumulation of 2,2',5,5'-tetrachlorobiphenyl and pyrene by picoplankton (Synechococcus leopoliensis, Cyanophyceae): influence of variable humic acid concentrations and pH. *Environ. Toxicol. Chem.*, **18**, 2063-2069.

Ulrici, W. 2000. Contaminated soil areas, different countries and contaminants, monitoring of contaminants. in: *Environmental processes II. Soil Decontamination Biotechnology*, G. Reed (Ed.), Vol. 11b. Weinheim, pp. 5-42.

Urich, T., Lanzen, A., Qi, J., Huson, D.H., Schleper, C., Schuster, S.C. 2008. Simultaneous assessment of soil microbial community structure and function through analysis of the meta-transcriptome. *PLoS One*, **3**(6), e2527.

US-EPA. 2000. Engineered Approaches to In Situ Bioremediation of Chlorinated Solvents: Fundamentals and Field Applications, Vol. EPA-542-R-00-008.

US-EPA. 1995. How to evaluate alternative clean-up technologies for underground storage tank sites: a guide for corrective action plan, Vol. EPA-510-B-95-007.

US-EPA. 1998. Seminar Series on Monitored Natural Attenuation for Ground Water, Vol. EPA/625/K-98/001.

van Beilen, J.B., Funhoff, E.G. 2005. Expanding the alkane oxygenase toolbox: new enzymes and applications. *Curr. Opin. Biotechnol.*, **16**(3), 308-314.

van Beilen, J.B., Funhoff, E.G., van Loon, A., Just, A., Kaysser, L., Bouza, M., Holtackers, R., Rothlisberger, M., Li, Z., Witholt, B. 2006. Cytochrome P450 alkane hydroxylases of the CYP153 family are common in alkane-degrading eubacteria lacking integral membrane alkane hydroxylases. *Appl. Environ. Microbiol.*, **72**(1), 59-65.

van Beilen, J.B., Witholt, B. 2003. Diversity, function and biocatalytic applications of alkane oxygenases. in: *Petroleum Microbiology*, American Society for Microbiology Press. Washington DC, pp. 317-336.

van Elsas, J.D., Boersma, F.G.H. 2011. A review of molecular methods to study the microbiota of soil and the mycosphere. *Europ. J. Soil Biol.*, **47**(2), 77-87.

Viñas, M., Sabate, J., Espuny, M.J., Solanas, A.M. 2005. Bacterial community dynamics and polycyclic aromatic hydrocarbon degradation during bioremediation of heavily creosote-contaminated soil. *Appl. Environ. Microbiol.*, **71**(11), 7008-18.

Volkering, F., Breure, A.M., Rulkens, W.H. 1997. Microbiological aspects of surfactant use for biological soil remediation. *Biodegradation*, **8**(6), 401-417.

von Mering, C., Hugenholtz, P., Raes, J., Tringe, S.G., Doerks, T., Jensen, L.J., Ward, N., Bork, P. 2007. Quantitative Phylogenetic Assessment of Microbial Communities in Diverse Environments. *Science*, **315**(5815), 1126-1130.

Vyas, B.R.M., Bakowski, S., Šašek, V., Matucha, M. 1994. Degradation of anthracene by selected white-rot fungi. *FEMS Microbiol. Lett.*, **14**, 65-70.

Waarishi, H., Gold, M.H. 1989. Lignin peroxidase Compound III: formation, inactivation and conversion to the native enzyme. *FEBS Lett.*, **243**(2), 165-168.

Watkinson, R.J., Morgan, P. 1990. Physiology of aliphatic hydrocarbon-degrading microorganisms. *Biodegradation*, **1**(2-3), 79-92.

Weckx, S., Van der Meulen, R., Allemeersch, J., Huys, G., Vandamme, P., Van Hummelen, P., De Vuyst, L. 2010. Community dynamics of bacteria in sourdough fermentations as revealed by their metatranscriptome. *Appl. Environ. Microbiol.*, **76**(16), 5402-8.

Weissenfels, W.D., Klewer, H.J., Langhoff, J. 1992. Adsorption of polycyclic aromatic hydrocarbons (PAHs) by soil particles: influence on biodegradability and biotoxicity. *Appl. Microbiol. Biotechnol.*, **36**(5), 689-96.

Wesenberg, D., Kyriakides, I., Agathos, S.N. 2003. White-rot fungi and their enzymes for the treatment of industrial dye effluents. *Biotechnol. Adv.*, **22**(1-2), 161-87.

Wick, A.F., Haus, N.W., Sukkariyah, B.F., Haering, K.C., Daniels, W.L. 2011. Remediation of PAH-contaminated soils and sediments: A literature review. Virginia Polytechnic Institute and State University.

Wu, Y., Luo, Y., Zou, D., Ni, J., Liu, W., Teng, Y. 2008. Bioremediation of polycyclic aromatic hydrocarbons contaminated soil with Monilinia sp.: degradation and microbial community analysis. *Biodegradation.*, **19**, 247-57.

Wu, Y.R., Luo, Z.H., Vrijmoed, L.L. 2010. Biodegradation of anthracene and benz[a]anthracene by two *Fusarium solani* strains isolated from mangrove sediments. *Bioresour. Technol.*, **101**(24), 9666-9672.

Yateem, A., Balba, M., Al-Awadhi, N., El-Nawawy, A. 1997. White rot fungi and their role in remediating oil-contaminated soil. *Environ. Int.* **24**, 181-187.

Ye, D., Siddiqi, M.A., Maccubin, A.E., Kumar, S., Sikka, H.C. 1995. Degradation of polynuclear aromatic hydrocarbons by Sphingomonas paucimobilis. *Env. Sci. Technol.*, **30**(1), 136-142.

Zhao, X.R., Lin, Q., Brookes, P.C. 2005. Does soil ergosterol concentration provide a reliable estimate of soil fungal biomass? *Soil Biol. Biochem.*, **37**, 311-317.

Zheng, Z., Obbard, J.P. 2001. Effect of non-ionic surfactants on elimination of polycyclic aromatic hydrocarbons (PAHs) in soil-slurry by Phanaerochaete chrysosporium. *J. Chem. Technol. Biotechnol.*, **76**(4), 423-429.

Zhou, H.W., Guo, C.L., Wong, Y.S., Tam, N.F. 2006. Genetic diversity of dioxygenase genes in polycyclic aromatic hydrocarbon-degrading bacteria isolated from mangrove sediments. *FEMS Microbiol. Lett.*, **262**(2), 148-57.

Zimmermann, W. 1990. Degradation of lignin by bacteria. *J. Biotechnol.*, **13**, 119-130.