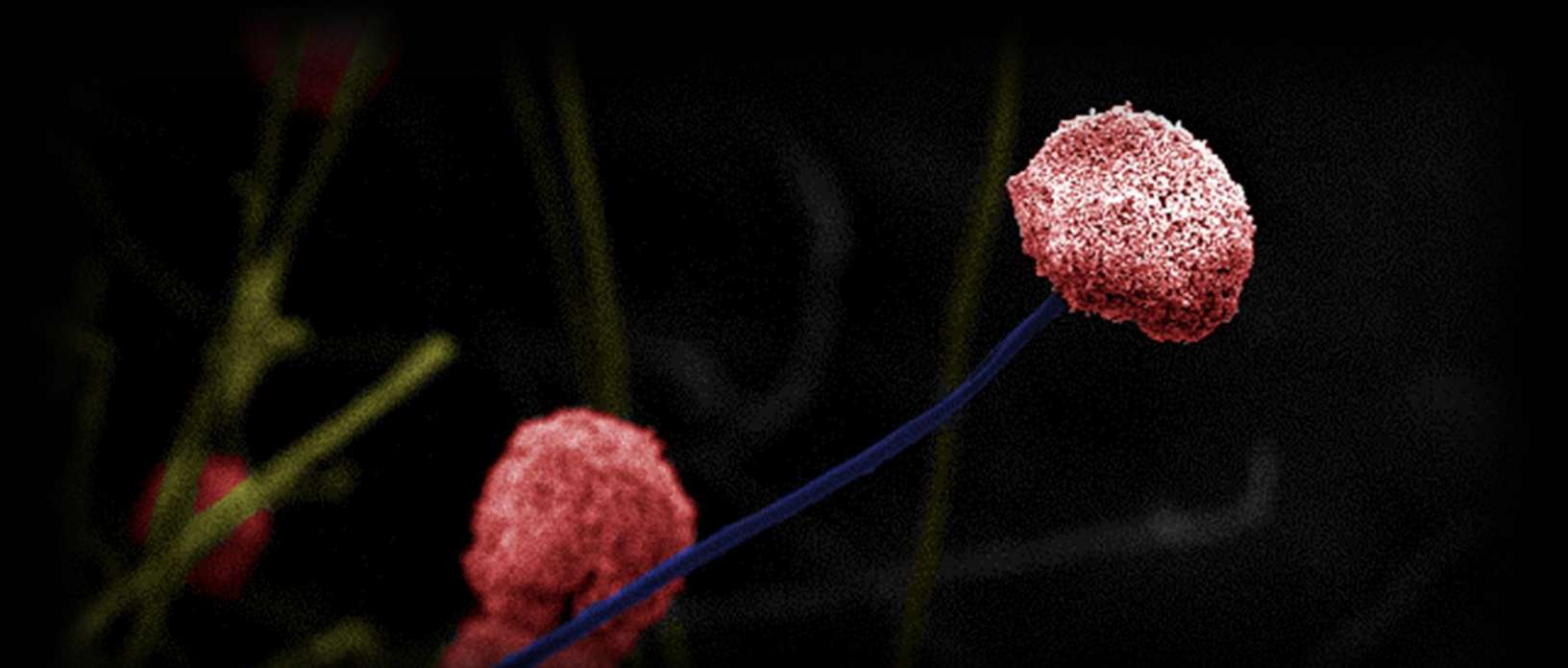
Microcosm Evaluation and Metagenomic Analysis of the Bioremediation of Soils Contaminated with PAHs by Microbial Consortia

German Alexis Zafra Sierra



INSTITUTO POLITÉCNICO NACIONAL
CENTRO DE INVESTIGACIÓN EN BIOTECNOLOGÍA APLICADA

MICROCOSM EVALUATION AND METAGENOMIC ANALYSIS OF THE BIOREMEDIATION OF SOILS CONTAMINATED WITH PAHS BY MICROBIAL CONSORTIA

GERMAN ALEXIS ZAFRA SIERRA

A thesis submitted in fulfillment of the requirements for the degree of
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MICROCOSM EVALUATION AND METAGENOMIC ANALYSIS OF THE BIOREMEDIATION OF SOILS CONTAMINATED WITH PAHs BY MICROBIAL CONSORTIA

Presentada por el alumno:

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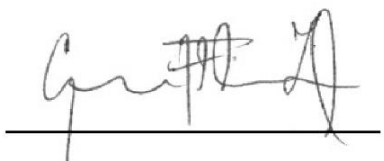

German Alexis Zafra Sierra

Imagination will often carry us to worlds that never were. But without it we go nowhere.

Carl Sagan (1934-1996)

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First and foremost, my greatest thanks to my wonderful family and relatives for their enduring support and encouragement all the way through. Their unconditional support since many years have given me the strength to pursue my goals and dreams, no matter where they take me. To my dear parents, my sister and nephew who have never stopped believing in me, thank you; this thesis is dedicated to you.

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LIST OF PUBLICATIONS

This is a thesis by publication, in which each article form the individual chapters of the thesis. Articles are either published, accepted for publication or to be submitted for publication in peer-reviewed journals and are listed as follows:

CHAPTER 2

Zafra G., Absalón A.E., Cuevas M.C., Cortés-Espinosa D.V. Isolation and selection of a highly tolerant microbial consortium with potential for PAH biodegradation from heavy crude oil-contaminated soils. *Water Air and Soil Pollution* 2014; 225(2):1826. DOI: 10.1007/s11270-013-1826-4
JCR impact factor: 1.685

CHAPTER 3

Zafra G., Moreno-Montaño A., Absalón A.E., Cortés-Espinosa D.V. Degradation of Polycyclic Aromatic Hydrocarbons in soil by a tolerant strain of *Trichoderma asperellum*. *Environmental Science and Pollution Research,* 2014. *In press* (Epub ahead of print). DOI: 10.1007/s11356-014-3357-y
JCR impact factor: 2.757

CHAPTER 4

Zafra G., Absalón A.E., Cortés-Espinosa D.V. Morphological changes and growth of filamentous fungi in presence of high concentrations of PAHs. Accepted for publication in *Brazilian Journal of Microbiology*.
JCR impact factor: 0.762

CHAPTER 5

Zafra G., Absalón A.E., Anducho-Reyes M.A., Cortés-Espinosa D.V. Construction of PAH-degrading Mixed Microbial Consortia by induced Selection in Soil. To be submitted to *Microbial Ecology*
JCR impact factor: 3.118

CHAPTER 6

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JCR impact factor: 2.757

CHAPTER 7

Zafra G., Absalón A.E., Taylor T.D., Cortés-Espinosa D.V. Comparative metagenomic analysis of PAH bioremediation in soil by mixed microbial consortia. To be submitted to *Environmental Science & Technology*
JCR impact factor: 5.481

CHAPTER 8

Zafra G. & Cortés-Espinosa D.V. Degradation of aromatic hydrocarbons by *Trichoderma* species: an overview. (Review). To be submitted to *Fungal Biology Reviews*
JCR impact factor: 1.362

STATEMENT OF CONTRIBUTIONS BY OTHERS

I declare that the work presented in this thesis was primarily designed, experimentally executed, interpreted and written by the first author of the individual manuscripts (German Zafra). Contributions by other authors are described in the following:

CHAPTER 2

Diana V. Cortés-Espinosa provided significant contributions to the conception and design of this study. Ma. del Carmen Cuevas provided the soil samples used for microbial isolation. Diana V. Cortés-Espinosa and Angel Absalón provided critical and through revision of the manuscript.

CHAPTER 3

Diana V. Cortés-Espinosa provided significant contributions to the conception and design of this study. Angélica Moreno provided technical support in the realization of enzymatic assays. Diana V. Cortés-Espinosa and Angel Absalón provided critical and through revision of the manuscript.

CHAPTER 4

Diana V. Cortés-Espinosa provided contributions to the conception and design of this study. Diana V. Cortés-Espinosa and Angel Absalón provided critical revision of the manuscript.

CHAPTER 5

Diana V. Cortés-Espinosa and Angel Absalón provided contributions to the conception and design of this study. Miguel A. Anducho provided logistical support for the realization of DGGE assays. Diana V. Cortés-Espinosa and Angel Absalón provided critical and through revision of the manuscript.

CHAPTER 6

Diana V. Cortés-Espinosa provided significant contributions to the conception and design of this study. Miguel A. Anducho provided logistical support for the realization of DGGE assays. Diana V. Cortés-Espinosa and Angel Absalón provided critical revision of the manuscript.

CHAPTER 7

Diana V. Cortés-Espinosa and Angel Absalón provided important contributions to the conception and design of this study. Todd Taylor provided facilities for computational analysis and contributed in the analysis and interpretation of metagenomic data. All coauthors provided critical revision of the manuscript.

CHAPTER 7

All coauthors contributed equally to the preparation of the manuscript.

CONFERENCE PRESENTATIONS

Zafra G., Absalón A.E., Cortés-Espinosa D. Identification of Highly Tolerant Fungal Isolates to Polycyclic Aromatic Hydrocarbons. XV National Congress of Biotechnology and Bioengineering. Cancún, México. June 2013 (Poster presentation).

Zafra G., Absalón A.E., Cortés-Espinosa D. Determination of Bacterial Tolerance to High Concentrations of PAHs. Second International Symposium on Bioremediation and Sustainable Environmental Technologies. Jacksonville, USA. June 2013. (Poster presentation).
In extenso paper published in: *Proceedings of the Second International Symposium on Bioremediation and Sustainable Environmental Technologies*. 2013. A-13, pp. 1-6. ISBN 978-0-9819730-7-4

Zafra G., Absalón A.E., Taylor T.D., Cortés-Espinosa D. Functional metagenomic analysis of the bioremediation of a hydrocarbon-polluted soil with a mixed microbial consortium. 4th International Symposium on Environmental Biotechnology and Engineering. Mexico City, Mexico. September 2014. (Oral presentation).
In: *Environmental Biotechnology and Engineering - 2014*. ISBN 978-607-9023-24-9.

Zafra G., Absalón A.E., Taylor T.D., Cortés-Espinosa D. Microbial diversity shifts during the bioremediation of a heavy crude oil-contaminated soil at microcosm. 1st Biotechnology World Symposium. Tlaxcala, Mexico. October 2014 (Poster presentation).
In: *Journal of Chemical, Biological and Physical Sciences.* ISSN 2249-1929.

Zafra G., Absalón A.E., Anducho-Reyes M.A., Cortés-Espinosa D. Selection of Optimal Culture Conditions for PAH Removal in Soils by Mixed Microbial Consortia. 2014-Contaminated Site Management: Sustainable Remediation & Management of Soil, Sediment and Water. San Diego, USA. November 2014 (Oral presentation).

TABLE OF CONTENTS

ABSTRACT

This thesis focuses on the use of fungal-bacterial consortia composed of native and genetically engineered microorganisms (GEMs) in the bioremediation of soils contaminated with Polycyclic Aromatic Hydrocarbons (PAHs). To that end, the optimal conditions for the bioremediation of soils by combining biostimulation and bioaugmentation were evaluated, using also a metagenomic approach to analyze the interactions and effects produced between soil microbiota and inoculated consortia.

Chapter 2 describes the isolation, identification and selection of bacterial and fungal isolates with high tolerance to PAHs from heavy crude oil-contaminated soils, able to degrade low (LMW) and high molecular weight (HMW) PAHs. Fifty microbial isolates were identified by morphological and molecular studies and evaluated for their potential to tolerate high concentrations of different molecular weight PAHs, such as phenantrene (Phe), pyrene (Pyr) and benzo[a]pyrene (BaP). *Trichoderma asperellum* H15, *Aspergillus nomius* H7, *Aspergillus flavus* H6, *Pseudomonas aeruginosa* B7, *Klebsiella* sp. B10, and *Stenotrophomonas maltophilia* B14 grew using PAHs as sole carbon source and presented a remarkably high tolerance to PAHs, up to 6,000 mg L^{-1}. A degrading consortium composed of 12 fungal and bacterial PAH-tolerant strains was constructed for the bioremediation of a PAH-contaminated soil, leading to a degradation of 87.76 % Phe, 48.18 % Pyr and 56.55 % BaP after 14 days. This is the first study to evaluate microbial tolerance to extreme concentrations of PAHs, resulting in a degrading and highly tolerant consortium capable of using PAHs as sole carbon source.

Trichoderma asperellum H15, the strain showing the highest tolerance to PAHs, was tested for its ability to degrade PAHs in soil microcosms in **Chapter 3**. *T. asperellum* H15 rapidly adapted to PAH-contaminated soils and degraded up to 74% Phe, 63% Pyr and 81% of BaP. Catechol 1,2-dioxygenase, laccase, and peroxidase enzyme activities were found to be involved in the degradation of PAHs. The results demonstrated the potential of *T. asperellum* H15 to be used by itself in a bioremediation process. This is the first report of the involvement of *T. asperellum* in LMW and HMW-PAH degradation in soils, providing evidence to consider *T. asperellum* as a promising and efficient PAH-degrading microorganism.

The effect of PAHs on the growth, morphology and sporulation ability of fungal degrading strains was evaluated in **Chapter 4**. PAHs produced significant detrimental effects on the radial growth of *A. nomius* H7 as well changes in mycelium pigmentation, abundance and sporulation ability. In contrast, growth of *T. asperellum* H15 was not affected, although sporulation was observed only up to 4000 mg L^{-1} and some visible changes in sporulation patterns and mycelium pigmentation were seen. The results suggest that PAHs could trigger important cell membrane

modifications and defense mechanisms responsible for pigment production, as well affect central sporulation pathways leading to a delay in the conidiation process.

Chapter 5 focuses on the construction of two improved PAH-degrading microbial consortia using an induced microbial selection method using PAH-spiked soils. Native fungal and bacterial strains, along with two GEM degrading fungal strains expressing LiP and MnP-encoding genes from *Phanerochaete chrysosporium*, were used to construct the consortia. After screening for microbial antagonists, the induced selection process was performed in microcosms during 14 days. Resulting consortia (C1 and C2) were able to degrade up to 91.5% Phe, 64.2% Pyr and 64.9% BaP after a two week incubation period. The results indicated that constructed microbial consortia presented high potential for soil bioremediation and may be effective for the treatment of sites polluted with PAHs and other hydrocarbons due to their elevated tolerance to aromatic compounds, their capacity to utilize them as energy source and the self-selection process.

In **Chapter 6**, the optimal conditions for PAH degradation in soil by C1 (containing only native strains) and C2 (containing native and GEM strains) consortia were established in microcosms by bioaugmentation and biostimulation with sugarcane bagasse and corn stover. Assays were carried out using sterile soils spiked with 2000 mg Kg^{-1} of a mixture of Phe, Pyr and BaP. Optimal conditions for PAH degradation consisted of 30% relative humidity and a ratio of 95:5 soil/agroindustrial residue. The highest degradation values were obtained using the consortium C2 and biostimulation with 5% corn stover at 30% relative humidity, reaching degradation values of 96.25% Phe, 80.47% Pyr and 71.15% BaP after 30 days. Consortium C1 also reached high PAH degradation values, comparable with those of consortium C2. PCR-DGGE revealed that most of the microorganisms composing microbial consortia adapted and prevailed until day 30.

Finally in **Chapter 7,** a metagenomic approach was used to analyze some of the effects (e.g. displacement, permanence, disappearance) produced between soil microbiota and consortium C1 during the bioremediation of a PAH-contaminated soil, providing insights regarding the metabolic pathways and specific enzymes involved in the bioremediation process. Bioaugmentation and biostimulation treatments produced appreciable changes on the microbial diversity of hydrocarbon contaminated soils, shifting native microbial communities in favor of degrading specific populations. Functional metagenomics suggested a bias towards aromatic hydrocarbon and intermediary degradation pathways, greatly favoring PAH mineralization. In contrast, cytochrome P450-mediated pathways were found to be significantly reduced in inoculated soils. PAH biodegradation was faster and showed higher degradation values when compared with other mixed microbial consortia in soil as a result of an increased co-metabolic degradation. The main differences between inoculated and non-inoculated soils were observed in aromatic ring-hydroxylating dioxygenases, laccase, protocatechuate, chloroaromatic compounds, salicylate and benzoate-degrading enzyme genes. Taking into account the results from the functional analysis, we proposed several concurrent metabolic pathways taking place in soils during Phe, Pyr and BaP degradation.

RESUMEN

Esta tesis se enfoca en el uso de consorcios microbianos mixtos conformados por microorganismos nativos y genéticamente modificados (GEMs) en la biorremediación de suelos contaminados con Hidrocarburos Aromáticos Policíclicos (PAHs). Para ello se evaluaron las condiciones óptimas para la biorremediación de suelos mediante las técnicas de bioestimulación y bioaumentación, teniendo en cuenta las interacciones y efectos producidos entre la microbiota propia del suelo y los consorcios inoculados por medio de análisis metagenómicos.

En el **Capítulo 2** se describe la obtención, identificación y selección de aislados bacterianos y fúngicos altamente tolerantes a PAHs, capaces de degradar PAHs de bajo (LMW) y alto peso molecular (HMW). Un total de 50 aislados fueron identificados y evaluados para conocer su potencial de tolerar concentraciones elevadas de fenantreno (Phe), pireno (Pyr) y benzo[a]pireno (BaP). *Trichoderma asperellum* H15, *Aspergillus nomius* H7, *Aspergillus flavus* H6, *Pseudomonas aeruginosa* B7, *Klebsiella* sp. B10 y *Stenotrophomonas maltophilia* B14 se mostraron capaces de utilizar PAHs como única fuente de carbono y presentaron niveles notablemente altos de tolerancia, de hasta 6,000 mg L^{-1}. Se construyó un consorcio microbiano conformado por 12 cepas fúngicas/bacterianas tolerantes para la biorremediación de un suelo contaminado con PAHs, consiguiendo una degradación de 87.76% de Phe, 48.18% de Pyr y 56.55 % de BaP. Este fue el primer estudio en donde se evaluó la tolerancia microbiana a concentraciones extremas de PAHs, resultando en un consorcio degradador y altamente tolerante con la capacidad de utilizar PAHs como única fuente de carbono.

En el **Capítulo 3** se evaluó la capacidad de *Trichoderma asperellum* H15, la cepa que mostró los niveles más altos de tolerancia, para degradar PAHs en suelos junto con un tratamiento de bioestimulación utilizando bagazo de caña. *T. asperellum* H15 se adaptó rápidamente a los suelos contaminados con PAHs, degradando hasta 74% de Phe, 63% de Pyr y 81% de BaP después de 14 días. Se encontró que las actividades enzimáticas de tipo catecol 1,2-dioxigenasa, lacasa y peroxidasa están directamente involucradas en el proceso de degradación por este microorganismo. Los resultados demostraron el alto potencial de *T. asperellum* para ser usado por si solo en un proceso de biorremediación de suelos. Este es el primer estudio en el que se describe el papel de *T. asperellum* en la degradación de LMW y HMW PAHs.

En el **Capítulo 4** se evaluó el efecto de los PAHs sobre el crecimiento, morfología y capacidad de esporulación de dos cepas fúngicas degradadoras. La presencia de altas concentraciones de PAHs produjeron efectos significativamente perjudiciales sobre el crecimiento radial de *A. nomius* H7, así como cambios en la pigmentación del micelio y la abundancia y capacidad de esporulación. En contraste, el crecimiento de *T. asperellum* H15 no se vio afectado aunque la capacidad de esporular se presentó únicamente hasta 4000 mg L^{-1} y se presentaron cambios en los patrones de esporulación y pigmentación del micelio. Los resultados sugieren que los PAHs podrían desencadenar modificaciones importantes de la membrana celular y mecanismos de

defensa involucrados en la producción de pigmentos, así como afectar las vías centrales de esporulación y producir un retraso en el proceso de esporulación.

En el **Capítulo 5** se construyeron consorcios microbianos degradadores mejorados utilizando un método de selección inducida. Para la construcción de los consorcios se utilizaron cepas bacterianas y fúngicas nativas, además de dos cepas GEM de *A. niger* expresando los genes LiP y MnP de *P. chrysosporium*. Después de una selección inicial utilizando pruebas de antagonismo microbiano, el proceso de selección fue llevado a cabo a nivel microcosmos utilizando un suelo estéril contaminado con una mezcla de PAHs y monitoreando la supervivencia o desaparición de cada cepa por PCR-DGGE. Como resultado se conformaron dos consorcios degradadores denominados C1 (conformado por cepas nativas) y C2 (conformado por cepas nativas y GEM), capaces de degradar hasta 91% de Phe, 64.2% de Pyr y 64.9% de BaP después de dos semanas de tratamiento. Los resultados mostraron que ambos consorcios poseen un alto potencial para la biodegradación de PAHs debido a su alta tolerancia hacia compuestos aromáticos, su capacidad de utilizar los contaminantes como fuente de carbono y al proceso de selección utilizado.

Posteriormente en el **Capítulo 6** se establecieron las condiciones óptimas para la degradación de PAHs en suelos mediante la bioaumentación con los dos consorcios y bioestimulación con bagazo de caña y residuos de maíz. Los ensayos se llevaron a cabo utilizando suelos estériles contaminados con 2000 mg Kg^{-1} de una mezcla de Phe, Pyr y BaP. Las condiciones óptimas para la degradación consistieron en 30% de humedad relativa y una relación de suelo/residuo agroindustrial de 95:5. Los valores más altos de degradación se consiguieron con el consorcio C2, residuos de maíz al 5% y una humedad relativa de 30%, alcanzando una degradación de 96.25% de Phe, 80.47% de Pyr y 71.15% de BaP después de 30 días. El consorcio C1 produjo resultados comparables con aquellos del consorcio C2. Los perfiles de DGGE mostraron que la mayoría de los microorganismos permanecieron hasta el final del proceso.

Por último, en el **Capítulo 7** se realizó un estudio metagenómico para analizar algunos de los efectos producidos entre la microbiota nativa y los consorcios degradadores durante la biorremediación de un suelo contaminado con PAHs, proporcionando información con respecto a las vías metabólicas y enzimas específicas involucradas en el proceso. La bioaumentación y bioestimulación produjo cambios apreciables en la diversidad microbiana de los suelos, desplazando algunas comunidades microbianas en favor de las poblaciones degradadoras de PAHs. El análisis funcional sugirió un direccionamiento del metabolismo hacia las vías de degradación de compuestos aromáticos y de intermediarios tales como el salicilato, protocatecuato, gentisato y benzoato, favoreciendo la mineralización de los PAHs. La abundancia de genes implicados en el catabolismo de PAHs vía citocromo P450 se encontró significativamente reducida en los suelos inoculados, indicando una probable reducción de la formación de intermediarios tóxicos. La degradación de los PAHs se produjo de manera más rápida y alcanzó niveles más elevados que reportes previos utilizando condiciones similares, como consecuencia de una degradación cometabólica y sinérgica. Se encontraron diferencias significativas en la abundancia de diversos genes de dioxigenasas, lacasa y descarboxilasas. Con base en los resultados fue posible proponer varias de las vías de degradación de PAHs que potencialmente tienen lugar en el suelo durante el proceso de biorremediación.

INTRODUCTION

For decades, many places around the world have been the scene of industrial activities which have produced contamination of soils and groundwater with persistent organic pollutants. Often such pollutants represent serious risks to human health and the environment. The expansion and development of the petrochemical industry in several countries, including Mexico, has led to serious environmental issues impacting the ecological balance of ecosystems and human health. As a result, large amounts of hazardous wastes have been produced from petrochemical activities and oil refining. The occurrence of accidents and environmental emergencies such as leaks, spills and fires during the storage and transport of oil derivatives and the uncontrolled disposal of waste, greatly contributes to the contamination of soils and groundwater by petroleum hydrocarbons.

Polycyclic Aromatic Hydrocarbons (PAHs) are a class of environmental recalcitrant pollutant that arise from petrochemical operations and the incomplete combustion of fossil organic matter. PAHs are organic compounds consisting of two or more fused aromatic rings in a linear or grouped arrangement. They are recalcitrant compounds in soils, being highly hydrophobic and therefore readily absorbed in the gastrointestinal tract of mammals and rapidly distributed to a variety of tissues with a marked tendency to fatty deposits. Because of their high toxicity and persistence, PAHs are considered persistent environmental pollutants that may have a detrimental effect on the flora and fauna of affected habitats, resulting in the absorption and accumulation of toxic chemicals in food chains and in some cases, serious health problems or genetic defects in humans.

There are several technologies and strategies to eliminate or reduce pollution with PAHs in soils, whose optimal operating conditions are mainly determined by the physical and chemical properties of the pollutant, its concentration and the prevailing environmental conditions. However, microbial degradation is considered the main natural degradation form of PAHs in soils; bioremediation, based on the use of microorganisms to degrade the contaminants, is a promising technology because of its high efficiency and cost-effectiveness. For over three decades it has been shown that microorganisms such as bacteria, fungi and algae possess specific catabolic activities that can be exploited for the remediation of soils and water impacted with low and high molecular weight PAHs. Bacteria belonging to genus *Sphingomonas, Burkholderia, Pseudomonas, Acinetobacter, Rhodococcus, Mycobacterium* and *Streptomyces*, among others, are well known PAH-degraders; on the other hand, white-rot fungi such as *Phanerochaete chrysosporium, Trametes versicolor* and *Pleurotus ostreatus* and non-ligninolytic fungi commonly found in soil, as some of the genus *Aspergillus, Fusarium, Cladosporium* and *Penicillium*. However, current knowledge about the microbial diversity, metabolic profiles and the impact produced by inoculated microbial populations on native communities during the degradation of PAHs is still limited.

This study focuses on the use of fungal-bacterial consortia composed of native and genetically engineered organisms in the bioremediation of soils polluted with PAHs. To that end, the optimal conditions for the bioremediation of soils by combining biostimulation bioaugmentation were evaluated, using also a metagenomic approach to analyze some of the interactions and effects produced between soil native microbiota and the introduced organisms, providing insights regarding the metabolic pathways and specific enzymes involved in the PAH-degradation process.

CHAPTER 1

LITERATURE REVIEW AND RESEARCH DIRECTIONS

1.1 HYDROCARBON CONTAMINATION OF SOILS: MÉXICO

In Mexico, the expansion and development of several industrial activities, particularly in the petrochemical industry and oil refining, has resulted in large quantities of hazardous wastes. The unappropriated handling and disposal of these contaminants could have serious effects on the health of human populations and the ecological balance of the affected ecosystems. Environmental emergencies and accidents such as leaks, spills and fires during storage and transportation of crude oil and its derivatives, as well as the uncontrolled disposal of generated wastes greatly contribute to the contamination of soils. Historically, the number of sites with soil contamination in México is elevated. According to the National Institute of Statistics and Geography (INEGI), the surface of contaminated or degraded soil by chemical causes was over 2.5 million hectares until 2000 (INEGI, 2000). Regarding the hydrocarbon-polluted areas, only in 2006 more than 3000 tons of crude oil were spilled in soils due to accidents, emergencies and robbery from pipelines (PEMEX, 2006). By the end of 2013 the final inventory of environmental passives of soils contaminated with hydrocarbons was 1,020 hectares, along with 1533 events of leaks and spills of crude oil and derivatives. In total, hydrocarbon spills in soils are estimated in 7276 tons only during 2013 (PEMEX, 2014)

Continuous contamination with crude oil and its derivatives favor the deposition and accumulation of xenobiotics and potentially toxic compounds in soils. One of the environmental pollutants considered as a priority in Mexico because of its high toxicity and persistence are the Polycyclic Aromatic Hydrocarbons (PAHs). PAHs are molecules with physical and chemical characteristics that greatly contributes for its persistence in the soils, having toxic, mutagenic and teratogenic properties (Haritash and Kaushik, 2009). Therefore, PAHs are considered recalcitrant environmental pollutants that have a detrimental impact on the flora and fauna of affected habitats. During the last century there has been an increase in the amount of PAHs released into the environment from anthropogenic sources and atmospheric deposition from natural sources (Juhasz and Naidu, 2000). It is believed that accumulation of anthropogenic PAHs in soil is a consequence of a continuous process of deposition that exceeds the natural mechanisms of environmental elimination. Because of its toxicity, mutagenicity and carcinogenicity, many

PAHs have been identified as priority pollutants by numerous regulatory authorities including the Environmental Protection Agency of the United States (US-EPA, 2008).

1.2 POLYCYCLIC AROMATIC HYDROCARBONS

Polycyclic Aromatic Hydrocarbons (PAHs) are a group of chemically similar organic compounds containing two or more fused aromatic rings, produced as a result of the incomplete combustion and pyrolysis of organic matter. PAH compounds possess low volatility at room temperature, high molecular weights and low solubility in water. Several natural and anthropogenic sources contribute to the release of PAHs to the environment, such as forest fires, volcanic eruptions, vehicle emissions, oil refining and industrial combustion of fossil fuels. Because of its toxicity, mutagenicity and carcinogenicity, many PAHs have been identified as priority pollutants by numerous regulatory authorities including the Environmental Protection Agency of the United States (US-EPA, 2008) (Table 1). It is possible to classify the different PAHs into alternant PAHs, composed only of six-membered rings (e.g. phenanthrene) and non-alternant PAHs, that may also contain rings other than those with six carbon atoms (e.g. acenaphthene). PAHs are highly hydrophobic compounds, and as the molecular weight of PAHs increases their solubility in water and volatility decreases (Haritash and Kaushik, 2009).

Chemical and photochemical reactivity, as well as many physical and biological characteristics, are greatly influenced by structural aspects like the degree of saturation, number of rings and spatial configuration of PAHs (Mukherji and Ghosh, 2012). Moreover, as the association of a contaminant with the soil organic matter is directly related with its hydrophobicity, PAHs are highly recalcitrant compounds having high octanol-water partitioning coeficients (K_{ow}) (Accardi-Dey and Gschwend, 2003).

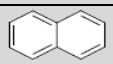
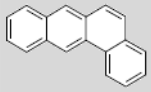
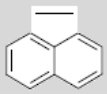
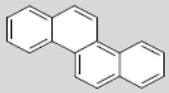
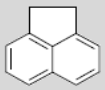
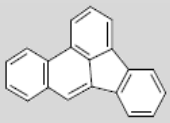
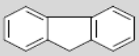
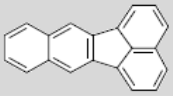
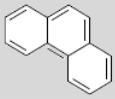
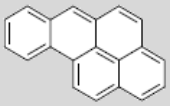
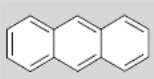
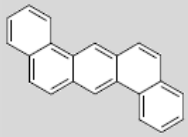
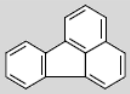
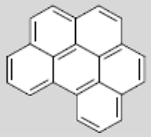
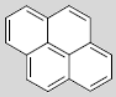
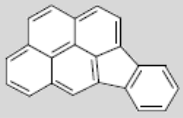
1.2.1 TOXICITY OF PAHS

There are more than 100 PAHs identified, but only 16 of these compounds have been classified as priority pollutants by the US-EPA (US-EPA, 2008). A large number of PAHs possess toxic, mutagenic and/or carcinogenic properties (Goldman *et al.*, 2001; Mastrangelo *et al.*, 1996). PAHs are poorly hydrosoluble and therefore are readily absorbed in the gastrointestinal tract of mammals, being rapidly distributed in a variety of tissues with a marked tendency towards fatty tissues. That is why PAHs are considered persistent environmental contaminants that can have a detrimental effect on the flora and fauna of affected habitats, resulting in the absorption and accumulation of toxic chemicals in food chains and, in some cases, serious health problems or genetic defects in humans. PAH absorption in humans can take place by inhalation, ingestion or contact.

As mentioned above, PAH toxic and mutagenic properties are favored by mainly by its chemical structure. PAHs having a “bay” or a “K” region possess different toxic potential due to the site of

metabolic activation (Fig. 1). In some cases a metabolic activation by monooxygenase enzymes (mainly cytochrome P450) to form toxic intermediates as phenols, trans-dihydrodiols, quinones, and occasionally diol epoxides (Wattiau *et al.*, 2002). Production of diol epoxides are directly associated with carcinogenicity. Diol epoxides generated in the bay region of a PAH molecule are highly reactive and mutagenic and can produce covalent interactions with DNA, forming PAH-DNA adducts (Weis *et al.*, 1998).

Table 1. Name and structure of 16 priority PAHs (US-EPA, 2008).

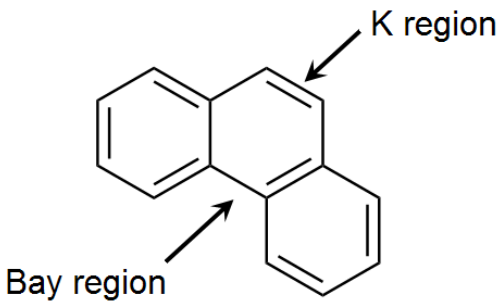
PAH COMPOUND	RINGS	STRUCTURE	PAH COMPOUND	RINGS	STRUCTURE
Naphthalene	2		Benzo[*a*]anthracene	4	
Acenaphthylene	3		Chrysene	4	
Acenaphthene	3		Benzo[*b*]fluoranthene	5	
Fluorene	3		Benzo[*k*]fluoranthene	5	
Phenanthrene	3		Benzo[*a*]pyrene	5	
Anthracene	3		Dibenzo[*a,h*]anthracene	5	
Fluoranthene	4		Benzo[*ghi*]perylene	6	
Pyrene	4		Indene[1,2,3-*cd*]pyrene	6	

The formation of PAH-DNA adducts plays a key role in the initiation of cancer, especially when they occurs at a site critical for the growth or differentiation of cells. Thus, DNA adducts can be measured and used as markers of exposure and PAH-specific genetic damage. While some of 2 and 3-ringed PAHs has not been found to be carcinogens for mammals, genotoxicity tests have revealed a great amount of 3-6 rings PAHs having probable toxic, teratogenic and mutagenic effects in exposed humans (WHO-IARC, 2010). PAH genotoxicity increases as molecule size augment to 4 or 5 rings (Kanaly and Harayama, 2000). According to the World Health Organization (WHO) International Agency for Research of Cancer (IARC) classification, benzo[a]pyrene, dibenzo[a,h]anthracene and benzo[a]anthracene are probable human carcinogens (2A category) while benzo[a]fluoranthene, benzo[k]fluoranthene and Indene[a,2,3-cd]pyrene are possible carcinogens (2B category). These compounds are also included in the US-EPA list of 16 priority PAHs.

1.2.2 ENVIRONMENTAL FATE OF PAHS

Pollutants such as PAHs can be adsorbed in soil material or even in dissolution in small amounts, but most of them are adsorbed to soil particles. Once in soil PAHs can be transformed by physical or chemical agents, sediment, bioaccumulate or can eventually be degraded by soil microbial communities (Juhasz and Naidu, 2000). Because of their low vapor pressures, PAHs remain in solid form and thus volatilization is limited to only few low molecular compounds (e.g. naphthalene, fluorene). In addition, a high content of organic matter negatively affects the volatilization and increases the adsorption extent (Alexander, 1999). PAHs can also be oxidized by absorbing electromagnetic radiations from sunlight, a process dependent on variables such as soil texture, temperature, salinity and pH (Kong and Ferry, 2003). On mangrove contamination with oil, photochemical oxidation was seen to have important role in PAH degradation as reported by Ke *et al.* (2002). However, microbial degradation is considered the main natural form of degradation of PAHs (Juhasz and Naidu, 2000).

Figure 1. "Bay" and "K" regions in a phenanthrene molecule



1.3 PAH REMEDIATION TECHNOLOGIES IN SOIL

In response to PAH contamination problematic, several physical, chemical, thermal and biologic technologies are available to remove or reduce to its maximum the degree of contamination in soils (Table 2), each one having its own advantages and disadvantages. The selection of the method of remediation greatly depends on factors such as the extent of the contamination, type and concentration of the contaminants, physicochemical characteristics of the soil, soil availability and cost, among others. A known fact is that a single remediation technology may not be the solution for all types of soils contaminated with PAHs. Integrated soil remediation technologies, which combine separation and destruction of PAHs appear to be a good choice in the technical field, allowing PAH removal with improved efficiency. However, it is not a secret that the cost of implementing a remediation technology plays a crucial and decisive role in choosing a method. In this sense, bioremediation has large advantages over other technologies as well as being environmentally friendly and produce good results in terms of removal.

1.3.1 PHYSICOCHEMICAL METHODS

The most commonly used physicochemical methods for removing PAHs in soil are the solvent extraction and washing, and extraction with supercritical and subcritical fluids. Solvent extraction does not destroy the pollutants, but concentrates it to make it easier to recycle or destroy using another technique. Washing can use individual solvents or mixtures, such as acetone, hexane, ethanol, methanol, ethyl acetate and mixtures thereof with water (Rababah and Matsuzawa, 2002). It is also common to use mixtures of water and surfactant compounds (e.g. Tween 40, Tween 80, Brij 30, Brij 35, SDS) (Ahn *et al.*, 2008). The rate at which PAHs are dissolved in solvents depends on several factors, such as temperature, moisture content and degree of pollution, among others. Supercritical fluid extraction uses a substance subjected to temperature and pressure conditions above its critical point, exhibiting "hybrid" properties between liquid and gas phases (supercritic fluid). PAHs adsorbed in soils are extracted by dissolution or adsorption/desorption, commonly using methylene chloride and CO_2 as supercritical fluids (Miege *et al.*, 2003). On the other hand, subcritical fluid extraction uses substances maintained at liquid state and below its critical point under high temperature and pressure conditions. Extraction uses water heated at 100-274°C under high pressure to keep it liquid. As temperature raise its polarity decreases, being more hydrophobic than water and being able to dissolve PAHs (Lagadec *et al.*, 2000).

1.3.2 CHEMICAL METHODS

Chemical oxidation: The oxidation reactions can be used to remediate soils contaminated with PAHs. Different types of oxidants have been investigated, since the most commonly studied ozone and Fenton reagents to less common oxidants such as potassium permanganate, hydrogen peroxide and activated sodium persulfate.

Table 2. Overview of PAH remediation technologies in soil (modified from Gan *et al.* (2009))

TECHNOLOGIES	METHODS
Physicochemical	Soil washing with water, organic solvents, surfactants and cyclodextrins Supercritical fluid extraction (SFE) Subcritical fluid extraction
Chemical	Chemical oxidation (Fenton´s reagent, ozone, $KMnO_4$, H_2O_2, activated sodium persulfate) Photocatalytic degradation Electrokinetic remediation
Thermal	Incineration Thermal desorption
Biological	In situ/ex situ bioremediation (bioaugmentation, biostimulation, land farming, composting, bioventing, phytoremediation)

Fenton reactive (Fe(II)–H_2O_2) decomposes hydrogen peroxide (H_2O_2) to form hydroxyl radicals (OH), which in turn are able to degrade organic compounds (RH or R) by subtracting hydrogen or adding hydroxyl molecules. Fenton oxidation have been successfully used to degrade fluoranthene, benzo[a]pyrene and benzo[a]fluoranthene in contaminated soils, although some toxic sub products were seen to form during the process (Flotron *et al.*, 2005). On the other hand, ozone (O_3) can trigger indirect oxidation reactions by the decomposition of OH radicals. Ozone oxidation has also been used to remediate soils contaminated with phenanthrene, but similar to Fenton oxidation, polar and presumably toxic products are formed during the treatment (O'Mahony *et al.*, 2006).

Photocatalytic degradation: Photocatalytic degradation use photocatalyzers, promoting oxidation reactions that destroy organic contaminants in the presence of light radiation. This technology has been widely used for wastewater treatment, and has recently extended its application to the treatment of contaminated soils with PAHs. Phenanthrene, pyrene and benzo[a]pyrene have been degraded in soils using titanium dioxide (TiO_2) as catalyzer in presence of UV radiation (Zhang *et al.*, 2008). Photocatalytic degradation showed to be efficient for PAH degradation, particularly for benzo[a]pyrene at acid pH.

1.3.3 BIOLOGICAL METHODS – BIOREMEDIATION

Bioremediation uses the metabolic versatility of microorganisms to degrade different hazardous pollutants, including PAHs. The main objective of bioremediation is to transform organic pollutants into non-toxic metabolites or mineralize them to carbon dioxide (CO_2) and water (Haritash and Kaushik, 2009). A viable soil remediation technology requires microorganisms capable of a rapid adaptation. Many factors influence the microorganisms for using pollutants as substrates; therefore, understanding the mechanisms, catabolic pathways and responsible

enzymes for pollutant degradation is a key point to define the most relevant factors in for implementing a bioremediation technology at field scale.

Two main strategies for soil bioremediation are commonly used: (1) biostimulation consists in the addition of fertilizers, texturizing agents and aeration to improving the growth conditions of soil native degrading populations, and (2) the addition of specific pollutant-degrading microorganisms, or so called bioaugmentation. These two approaches can be applied through *in-situ* techniques such as land farming, composting and biopile for the degradation of PAHs and other hydrocarbons in soil, while more advanced methods *ex-situ*, as the use of bioreactors, provide better control temperature and pressure to improve the degradation processes but lacks in versatility (Alexander, 1999).

Bioaugmentation: Bioaugmentation is an effective strategy in sites highly contaminated with PAHs and native microbial populations with low proportion of PAH-degrading microorganisms ($<10^5$ CFU/g), which results in lower biodegradation rates. PAH and hydrocarbon biodegradation through bioaugmentation with autochthonous microorganisms have been successfully used (El Fantroussi and Agathos, 2005; Mrozik and Piotrowska-Seget, 2010; Sayara *et al.*, 2011), although there is controversy about reproducibility, operating conditions and scaling efficiency.

Autochthonous populations conforming soil microbial communities are responsible for a biotic balance that acts to prevent the establishment of foreign species. This "homeostasis" allows a community to resist biotic and abiotic environmental changes, although important abiotic disturbances (such as an episode of extensive contamination) can produce appreciable changes in the community. Native soil microbial populations established in a polluted environment possess a high degree of stability and resilience towards introduced microorganisms, being an extremely important factor in the long-term success of a bioaugmentation process (Atlas and Bartha, 1998). Introducing different exogenous species is rarely enough to permanently alter the soil microbial community. Because of this, bioaugmentation should not only face the possibility to degrade a pollutant, but also to address the main factors associated with the homeostasis of the microbial community. Therefore, the microorganism(s) selected for bioaugmentation should ideally be one common member of the native community, but if that is not possible, should at least have a great adaptation and growth capability under conditions of competition, predation and parasitism (Alexander, 1999). The isolation and reintroduction of organisms from a contaminated site increases the survival of the inoculum, their growth and degradation capability (Gentry *et al.*, 2004a; Mishra *et al.*, 2001; Silva *et al.*, 2009).

Recent advances in the molecular genetics of bioremediation processes and methods of genetic modification enable the development of genetically modified microorganisms degrade different PAHs. There are native microorganisms with high metabolic and physiological versatility, which can be used to degrade PAHs. However, there are also microorganisms in soil without degrading abilities, but with other interesting features such as survive in extreme conditions. It is possible to

increase the remediation potential of these microorganisms by genetic engineering. While current regulations in Mexico restrict the release of Genetically Engineered Microorganism (GEMs) to environment (SEMARNAT, 2005), there is several reports of GEMs especially designed to degrade PAHs (Cortés-Espinosa and Absalón, 2013; Cortes-Espinosa *et al.*, 2011; Layton *et al.*, 2012; Paul *et al.*, 2005). In general, there is a positive correlation between the relative abundance of genes involved in bioremediation and the ability to degrade pollutants. However, the genes required for bioremediation could be present, but not expressed (Lovley, 2003). Genetic engineering is an interesting way to improve the degradation of PAHs by soil microorganisms by means of two alternative strategies: introduction or the alteration of a specific gene (Gentry *et al.*, 2004b). Perhaps one of the biggest challenges is to generate microorganisms that, when applied to contaminated sites, can degrade the contaminant with high efficiency and limited risks. The risks associated with the uncontrolled growth and spread of GEMs and horizontal gene transfer can be dramatically reduced by inducing a rapid death after the degradation of PAHs. Therefore, it is better to consider the use of GEMs with poor survival, as obtained by introducing suicide systems, instead of persistent strains (Garbisu and Alkorta, 1999).

Biostimulation: Biostimulation consist in the addition of nutrients, texturizing agents and the improvement of aeration of soils in order to increase the amount of substrates used by the microorganisms and promote cometabolism between native soil microorganisms. PAH-contaminated sites often possess low-diversity native microbiota, but have adapted organisms capable of degrading hydrocarbons of different molecular weight (Cunliffe and Kertesz, 2006). Since these microorganisms exist in very low amounts (in the order of $1x10^3$ CFU/gram soil) is necessary to increase their number and improve conditions to accelerate degradation processes. Thus, biostimulation involves the addition of aqueous solutions (containing nutrients and/or oxygen) and vegetal residues to contaminated soils to stimulate the activity of indigenous degrading microorganisms (cometabolism) and accelerate the biodegradation of organic pollutants as PAHs, favored also by the increase of compounds such as N, P, K, S and Cu (de Lorenzo, 2008). Substrates such as sugarcane bagasse, corn, wheat and oat residues, sludges, dried blood, vermicompost and compost have been used to accelerate PAH degradation in soils (Fernandez-Luqueno *et al.*, 2011; Liebeg and Cutright, 1999). Biostimulation possess limitations, particularly in clay, highly stratified or too heterogeneous soils as a consequence of poor oxygen transfer rates. Other factors restricting its application, includes soils with extreme conditions limiting microbial growth or an increase in contaminant mobility (Volke-Sepulveda, 2002).

While nutritional requirements of microorganisms are approximately the same as the composition of their cells, the exception to this is carbon, which is required in high amounts (Suthersan, 1999). Based on this, the optimum ratio of Carbon/Nitrogen/Phosphorous recommended for bioremediation processes is approximately of 100:10:1. If carbon source is easily and quickly converted into CO_2, then more carbon is required to support microbial soil communities. In addition to carbon, nitrogen and phosphorous nutrients are commonly added in the biostimulation process. Since each supplemental nutrient can have a different redox potential depending on the

terminal electron acceptor, especial care must be taken to determine the type and amount of nutrients to be added in the process of biostimulation, in order to maintain an optimal redox potential. The specific ratio will depend on the rate and extent of degradation of PAHs, bioavailability of nutrients and soil type, among others.

1.4 MICROBIAL DEGRADATION OF PAHS

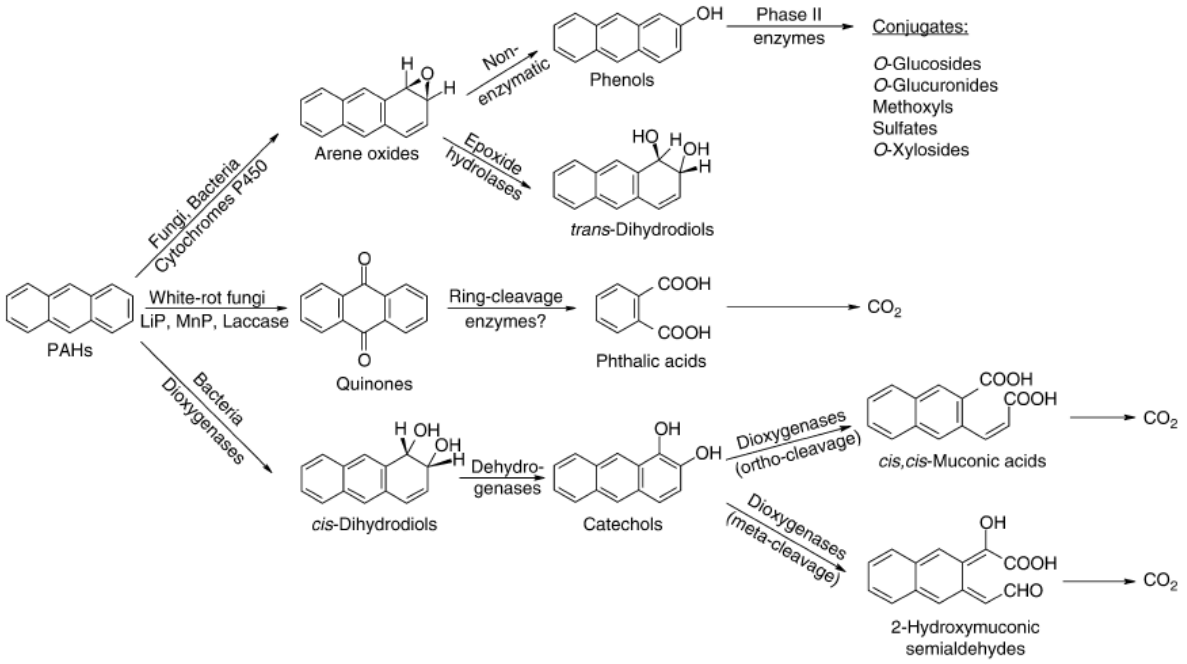
PAH degraders are common members of the native soil microbial communities. Microorganisms such as bacteria, fungi and algae possess specific catabolic activities that can be exploited for the remediation of soil and water impacted with low and high molecular weight PAHs. Bacteria belonging to genus *Sphingomonas, Burkholderia, Pseudomonas, Acinetobacter, Rhodococcus, Mycobacterium* and *Streptomyces*, among others, are well known PAH-degraders as well white-rot fungi including *Phanerochaete chrysosporium, Trametes versicolor* and *Pleurotus ostreatus* and non-ligninolytic fungi commonly found in soil, as some of the genus *Aspergillus, Fusarium, Cladosporium* and *Penicillium*, among others (Cerniglia and Sutherland, 2010; Seo *et al.*, 2009; Todd *et al.*, 2002). In uncontaminated soils, fungi and bacteria capable of degrading PAHs obtain the carbon and energy needed for its growth from the degradation of organic compounds (Juhasz and Naidu, 2000). PAH microbial degradation pathways tend to exhibit a broad substrate specificity, being driven by different oxidoreductase enzymes including monooxygenases, dioxygenases, peroxidases and laccases (Fig. 2), and occur either aerobically or anaerobically (Haritash and Kaushik, 2009). Many of these organisms are able to degrade lignin, since it is the most abundant form of aromatic-derived carbon in nature, whose function is to protect the plant polysaccharides from enzymatic attacks. In consequence, lignin degraders are widely distributed in nature and have been isolated from various sources including soil and feces from animals consuming wood. Lignin degradation is achieved through the production of relatively non-specific enzymes and can be exploited for the degradation of organic pollutants as PAHs (Juhasz and Naidu, 2000).

1.4.1 BACTERIAL DEGRADATION

Many bacterial species capable of degrading PAHs have been described (Table 3). The ability of bacteria to degrade PAHs is mostly determined by:

- The ability of bacteria to tolerate PAH concentrations in soil
- The ability of bacteria to transport PAHs inside the cell
- The PAH being an inductor of the degrading enzymes
- The PAH being a substrate for available degrading enzymes
- The PAH being an inductor of transport system and degrading enzymes

Figure 2. General scheme of PAH microbial degradation pathways (reproduced from Cerniglia and Sutherland (2010) with permission from Springer-Verlag).



Bacterial degradation of PAHs can occur either by aerobic or anaerobic pathway, although aerobic mechanisms are the most studied. The metabolism of PAHs by aerobic bacteria is usually initiated by dioxygenases, which incorporate two atoms of O_2 into the PAH molecule to form one or more isomeric *cis*-dihydrodiol metabolites (Fig. 2) (Seo *et al.*, 2009). Commonly, bacterial dioxygenases are multicomponent enzyme systems (e.g., naphthalene 1,2-dioxygenase consists of a ferredoxin reductase, a ferredoxin and a terminal iron-sulfur protein with alpha and beta subunits). *Cis*-dihydrodiols are reduced by dihydrodiol dehydrogenases to form dihydroxylated aromatic intermediates (catechols), which then may serve as substrates for *ortho* and *meta* ring-fission dioxygenases. The ring-cleavage products are further metabolized to tricarboxylic acid cycle intermediates and eventually mineralized to CO_2 (Seo *et al.*, 2009). In addition, some Actinobacteria members can produce monooxygenases that oxidize PAHs to *trans*-dihydrodiols (Coleman *et al.*, 2012), molecules more frequently found among the metabolites produced by fungi. On the other hand, anaerobic pathways are much less known. Anaerobic mechanisms use nitrate as electron acceptor and are believed to be slower (Pothuluri and Cerniglia, 1997). Both aerobic and anaerobic pathways produces modifications on intermediary products followed by ring cleavage. Degradation of PAHs by aerobic bacteria under controlled conditions has been successfully applied in *ex-situ* treatments of PAHs (Eriksson *et al.*, 2003; Quantin *et al.*, 2005).

Table 3. PAH degradation by different bacterial species (modified from Juhasz and Naidu (2000))

PAH COMPOUND	**ORGANISM**
Naphthalene	*Acinetobacter calcoaceticus, Alcaligenes denitrificans, Mycobacterium sp., Pseudomonas sp., Pseudomonas putida, Pseudomonas fluorescens, Sphingomonas paucimobilis, Brevundimonas vesicularis, Burkholderia cepacia, Comamonas testosteroni, Rhodococcus sp., Corynebacterium renale, Moraxella sp., Streptomyces sp., Bacillus cereus, Pseudomonas marginalis, Pseudomonas stutzeri, Pseudomonas saccharophila, Neptunomonas naphthovorans, Cycloclasticus sp.*
Acenaphthene	*Beijernickia sp., Pseudomonas putida, Pseudomonas fluorescens, Burkholderia cepacia, Pseudomonas sp., Cycloclasticus sp., Neptunomonas naphthovorans, Alcaligenes eutrophus, Alcaligenes paradoxus*
Phenanthrene	*Aeromonas sp., A. faecalis, A. denitrificans, Arthrobacter polychromogenes, Beijernickia sp., Micrococcus sp., Mycobacterium sp., Pseudomonas putida, Sphingomonas paucimobilis, Rhodococcus sp., Vibrio sp., Nocardia sp., Flavobacterium sp., Streptomyces sp., Streptomyces griseus, Acinetobacter sp., Pseudomonas aeruginosa, Pseudomonas stutzeri, Pseudomonas saccharophila, Stenotrophomonas maltophilia, Cycloclasticus sp., Pseudomonas fluorescens, Acinetobacter calcoaceticus, Gordona sp., Sphingomonas sp., Comamonas testosteroni, Cycloclasticus pugetii, Sphingomonas yanoikuyae, Agrobacterium sp., Bacillus sp., Burkholderia sp., Sphingomonas sp., Pseudomonas sp., Rhodotorula glutinis, Nocardioides sp., Flavobacterium gondwanense*
Anthracene	*Beijernickia sp., Mycobacterium sp., Pseudomonas putida, Sphingomonas paucimobilis, Burkholderia cepacia, Rhodococcus sp., Flavobacterium sp., Arthrobacter sp., Pseudomonas marginalis, Cycloclasticus sp., Pseudomonas fluorescens, Sphingomonas yanoikuyae, Acinetobacter calcoaceticus, Gordona sp., Sphingomonas sp., Comamonas testosteroni, Cycloclasticus pugetii*
Pyrene	*Alcaligenes denitrificans, Mycobacterium sp., Rhodococcus sp., Sphingomonas paucimobilis, Stenotrophomonas maltophilia, Acinetobacter calcoaceticus, Gordona sp., Sphingomonas sp., Pseudomonas putida, Burkholderia cepacia, Pseudomonas saccharophilia*
Chrysene	*Rhodococcus sp., Pseudomonas marginalis, Sphingomonas paucimobilis, Stenotrophomonas maltophilia, Acinetobacter calcoaceticus, Agrobacterium sp., Bacillus sp., Burkholderia sp., Sphingomonas sp., Pseudomonas sp., Pseudomonas saccharophilia*
Benzo[*a*]anthracene	*Alcaligenes denitrificans, Beijernickia sp., Pseudomonas putida, Sphingomonas paucimobilis, Stenotrophomonas maltophilia, Agrobacterium sp., Bacillus sp., Burkholderia sp., Sphingomonas sp., Pseudomonas sp., Pseudomonas saccharophilia*
Benzo[*a*]pyrene	*Mycobacterium sp. Rhodanobacter* sp., *Sphingomonas yanoikuyae, Stenotrophomonas maltophilia, Xanthamonas* sp.

1.4.2 FUNGAL DEGRADATION

Two fungal groups are directly involved in PAH degradation: (1) ligninolytic fungi, a group composed mainly of basidiomycetes that produce enzymes to degrade the lignin in wood but does not adapt well to soils; and (2) non-ligninolytic fungi that do not produce ligninolytic enzymes, but in contrast to basidiomycetes, are common members of soil microbiota and hence presents good adaption to soil. Most of the major groups of non-ligninolytic fungi, including zygomycetes, ascomycetes, hyphomycetes, and others, include PAH degraders (Cerniglia and Sutherland, 2010) (Table 4).

Metabolism of PAHs by non-ligninolytic fungi usually starts with an initial oxidation by cytochrome P450 monooxygenases and O_2 to produce an arene oxide and water. This unstable intermediate can be hydrated by an epoxide hydrolase to form a *trans*-dihydrodiol, or rearranged non-enzymatically to form a phenol. PAH *trans*-dihydrodiols and phenols may be methylated or converted to sulfates, glucosides, glucuronides, or xylosides (Capotorti *et al.*, 2005; Cerniglia, 1997). Similarly as in mammals, fungal degradation of PAHs via cytochrome P450 can also result in the metabolic activation of PAHs leading to genotoxicity. White-rot fungi, a large group of ligninolytic basidiomycetes that includes species of *Phanerochaete*, *Pleurotus*, and *Trametes* may also produce a cytochrome P450 that functions in the initial oxidation of PAHs (van den Brink *et al.*, 1998). However, white-rot fungi also produce extracellular ligninolytic enzymes, including lignin peroxidases (LiP), manganese peroxidases (MnP), and laccases also involved in PAH degradation. These enzymes normally break down lignin molecules but may also oxidize PAHs to form PAH diphenols, which are easily autoxidized to quinones.

LiP, in the presence of H_2O_2, oxidizes PAHs with oxidation potentials $\leq$7.55 eV (Bogan and Lamar, 1996). LiP from *Phanerochaete chrysosporium* has been shown to oxidize pyrene, benzo[a]pyrene and several other PAHs (in der Wiesche *et al.*, 1996); Hiratsuka *et al.*, 2005), while LiP from *Bjerkandera adusta* and *Nemalotoma frowardi* oxidize anthracene and pyrene and a purified LiP from Nematoloma frowardi oxidizes anthracene and pyrene (Grünther *et al.*, 1998; Gramss *et al.*, 1999). Manganese peroxidase (MnP) oxidizes PAHs by means of the Mn-dependent peroxidation of unsaturated lipids. MnP from *Trametes trogii* and *Phanerochaete chrysosporium* oxidizes PAHs in the presence of unsaturated fatty acids, including phenanthrene, pyrene and benzo[a]pyrene (Cortés-Espinosa and Absalón, 2013; Cortes-Espinosa *et al.*, 2011; Levin *et al.*, 2003). Laccase, a copper-containing enzyme, oxidizes PAHs in the presence of mediator compounds, such as phenol, aniline, 4-hydroxybenzoic acid, 4-hydroxybenzyl alcohol, methionine, cysteine, or reduced glutathione (Johannes and Majcherczyk, 2000).

Table 4. PAH degradation by different fungal species (modified from Juhasz and Naidu (2000))

PAH COMPOUND	**ORGANISM**
Naphthalene	*Aspergillus niger, Basidiobolus ranarum, Candida utilis, Choanephora campincta, Circinella sp., Claviceps paspali, Cokeromyces poitrassi, Conidiobolus gonimodes, Cunninghamella bainieri, Cunninghamella elegans, Cunninghamella japonica, Emericellopsis sp., Epicoccum nigrum, Gilbertella persicaria, Gliocladium sp., Helicostylum piriforme, Hyphochytrium catenoides, Linderina pennispora, Mucor hiemalis, Neurospora crassa, Panaeolus cambodginensis, Panaeolus subbalteatus, Penicillium chrysogenum, Pestalotia sp., Phycomyes blakesleeanus, Psilocybe cubensis, Psilocybe strictipes, Psilocybe stuntzii, Psilocybe subaeruginascens, Rhizophlyctis harderi, Rhizophlyctis rosea, Rhizopus oryzae, Smittium culisetae, Smittium simulii, Syncephalastrum racemosum, Thamnidium anomalum, Zygorhynchus moelleri*
Phenanthrene	*Aspergillus sp., Cunninghamella elegans, Phanerochaete chrysosporium, Phanerochaete laevis, Pleurotus ostreatus, Trametes versicolor, Bjerkandera adjusta, Pleurotus ostreatus, Cylindrocladium simplex, Monosporium olivaceum, Curvularia lunata, Curvularia tuberculata, Laetiporus sulphureus, Daedaela quercina, Flamulina velutipes, marasmiellus sp., Penicullium sp., Kuehneromyces mutabilis, Laetiporus sulphureus, Agrocybe aegerita, Aspergillus niger, Syncephalastrum racemosum, Trichoderma sp.*
Anthracene	*Bjerkandera sp., Cunninghamella elegans, Phanerochaete chrysosporium, Phanerochaete laevis, Ramaria sp., R. solani, Trametes versicolor, Pleurotus ostreatus, Cylindrocladium simplex, Monosporium olivaceum, Curvularia lunata, Curvularia tuberculata, Cryphonectria parasitica, Ceriporiopsis subvermispora, Oxysporus sp., Cladosporium herbarum, Drechslera spicifera, Verticillium lecanii, Fusarium moniliforme, Rhizopus arrizus, Coriolopsis polyzona, Laetiporus sulphureus, Daedaela quercina, Penicillium sp*
Fluoranthene	*Cunninghamella elegans, Cunninghamella blackesleeana, Cunninghamella echinulata, Bjerkandera adjusta, Pleurotus ostreatus, Sporormiella australis, Cryptococcus albidus, Cicinobolus cesatii, Pestalotia palmarum, Beauveria alba, Aspergillus terreus, Mortierella ramanniana, Rhizopus arrhizus, Laetiporus sulphureus, Flamulina velutipes, marasmiellus sp., Penicillium sp.*
Pyrene	*Cunninghamella elegans, Phanerochaete chrysosporium, Penicillium sp., Penicillium janthinellum, Penicillium glabrum, Pleurotus ostreatus, Syncephalastrum racemosum, Bjerkandera adjusta, Pleurotus sp., Dichomitus squalens, Flammulina velutipe, Trammetes versicolor, Kuehneromyces mutabilis, Laetiporus sulphureus, Agrocybe aegerita*
Benzo[*a*] anthracene	*Cunninghamella elegans, Trametes versicolor, Phanerochaete laevis, Phanerochaete janthinellum*
Chrysene	*Penicillium janthinellum, Syncephalastrum racemosus, Penicillium sp.*

Laccases from *Trametes versicolor*, *Coriolopsis rigida*, and *Rigidoporus lignosus* oxidize PAHs *in vitro* in the presence of artificial mediator compounds, such as 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 1-hydroxybenzotriazole (HBT) (Cambria *et al.*, 2008; Gómez *et al.*, 2006; Majcherczyk and Johannes, 2000). PAH oxidation by laccase/mediator systems can occur without direct contact between the PAH and the enzyme. Laccase first oxidizes the mediator, producing free radicals or radical cations that then oxidize the PAH by abstracting single electrons (Johannes *et al.*, 1998). Laccase oxidation of high molecular weight PAHs typically produces quinones and polymers but little CO_2 (Collins *et al.*, 1996; Rama *et al.*, 1998). The degradation of PAHs by ligninolytic fungi may also involve intracellular enzymes, including cytochrome P450 monooxygenase and epoxide hydrolase (Masaphy *et al.*, 1996, Bezalel *et al.*, 1997).

1.4.3 DEGRADATION BY BACTERIAL-FUNGAL CONSORTIA

One way to overcome the numerous barriers in the degradation of PAHs in soil, especially those of high molecular weight, is the use of defined co-cultures (consortia) of fungi and bacteria (Boonchan *et al.*, 2000). These barriers include the inability of bacteria to transport high molecular weight (HMW) PAHs inside the cell due to the molecular size, the PAH not being a substrate for the available enzymes or not being a suitable inductor for transport or degrading enzymes (Juhasz and Naidu, 2000).

The process of PAH degradation by fungal/bacterial consortia is initiated by the release of extracellular fungal (LiP, MnP, laccase) or some bacterial (ring cleavage dioxygenases) enzymes that break molecules that are too large to pass through most bacterial cell walls, producing a partial oxidative degradation of PAH (Hammel *et al.*, 1995). This initial oxidation increases the chances for bacterial degradation and mineralization as the oxidized metabolites usually have increased water solubility and reactivity, eliminating the initial oxidation of the ring as a limiting step for bacterial metabolism (Cerniglia, 1997). This process not only overcomes the inability of the several bacterial species to transport PAHs into the cell, but also prevents the accumulation of fungal metabolites. There is evidence describing an inhibitory effect of metabolite accumulation on PAH degradation (Barclay *et al.*, 1995). There is also evidence for a cooperative degradation of HMW-PAHs by fungal/bacterial consortia (Anastasi *et al.*, 2009; Dries and Smets, 2002). The study of Boonchan *et al.* (2000) was one of the first studies using a defined coculture of bacteria and fungi to improve the degradation of high molecular weight PAHs.

1.5 TREATABILITY STUDIES OF PAHS

1.5.1 SOLID STATE FERMENTATION

Solid-State Fermentation (SFF), or solid culture, is the growth of microorganisms in solid or semi-solid medium in the absence of free water, with relative humidities ranging from 30 to 70%. Under these conditions the growth of microorganisms such as fungi is favored due to its ability to

grow in media with more than 0.80 water activity (Mitchell *et al.*, 2006). SFF is a complex heterogeneous system where solid, liquid and gaseous phases coexist. This technique has been applied since ancient times in the preparation of various fermented foods and has been used for the production of several metabolites of commercial interest, such as antibiotics, enzymes, alcohol, methane and citric acid (Solis-Pereira *et al.*, 1993).

Culture process involves oxygen transportation through air injection, as well as oxygen consumption and CO_2 generation through microbial respiration. These gases are transported from the inner solid phase towards the surrounding gas phase. The oxygen transfer depends on the thin layer of water surrounding the substrate, which is the site where the microorganisms grow, highlighting the importance of a good oxygen diffusion into the medium. Therefore, the availability of oxygen becomes one limiting factor in the growth of microorganisms in SFF. The presence of a high content of humidity causes the substrate to be compacted, and in addition, anoxic microniches are created (van de Lagemaat and Pyle, 2004).

In recent years there has been a growing interest in the process of SSF to develop bioprocesses, such as treatments and biodegradation of hazardous compounds, decaying wood, food production, and secondary metabolite production, including antibiotics, alkaloids, enzymes, organic acids and biopesticides. Furthermore, the use of "waste" materials in solid state fermentation systems such as agroindustrial wastes, represents both economic and metabolic advantages. These wastes often contain high amounts of sugars utilizable by microorganisms (e.g., sugarcane bagasse), which allow rapid microbial growth and more efficient biosynthesis or removal processes.

1.5.2 BIODEGRADATION STUDIES AT MICROCOSM LEVEL

Soil microcosm testing is a useful tool to evaluate the biodegradation potential of PAHs and the development of models to predict the environmental fate of these molecules in soil. While there are different definitions of 'microcosm', a typical one is that of "an intact and minimally disturbed ecosystem taken to the laboratory for study in its natural state" (Wimpenny, 1997). Microcosm can vary in complexity from simple bottles to highly sophisticated systems allowing variations of environmental parameters for a more accurate simulation. The design of microcosm systems closely related with the real environmental conditions are more likely to produce relevant results. Concentrations of PAHs and its subproducts can be subsequently monitored in several compartiments of the microcosm and at different times, being possible to obtain relevant information as kinetic behavior, partition equilibria, and overall behavior of biodegradation. In addition, these systems can be used for the selection and definition of appropriate conditions for large-scale bioremediation. During treatability studies, microcosm tests can be periodically monitored for degradation of PAHs, molecular testing, microbial counts, pH, relative humidity content and nutrient concentration by subsampling.

1.5.3 MONITORING OF MICROCOSMS SYSTEMS

To demonstrate that a bioremediation technology is potentially useful, it is necessary to monitor the most relevant variables of PAH degradation under controlled conditions. For practical reasons this cannot be easily performed *in-situ*, and therefore must be performed in a laboratory at microcosm level. Monitoring tests provide relevant information of microcosms systems and usually include the application of laboratory methods (either microbiological, analytical or molecular) to measure the effectiveness of bioremediation under certain conditions.

Microbial enumeration: Initial enumeration of total heterotrophic microorganisms and PAH degraders in contaminated soils may provide useful information on background soil metabolic activity and the extent to which microbial populations have adapted to site conditions. In addition to the initial microbial contaminated soil assessment, monitoring of microbial populations during bioremediation of soils is a useful tool for monitoring changes and differentiate the active microorganisms in the degradation of PAHs Results may also indicate whether soil contains an "active" microbial population, able to contribute to the bioremediation process. Good correlations have been observed between microbial counts and hydrocarbon degradation (Al-Awadhi *et al.*, 2012; Song *et al.*, 1990).

CO_2 evolution: Measurements of total CO_2 production can provide excellent information about the potential biodegradability of PAHs in contaminated soils. The approach, which is considered a preliminary step in the study of the feasibility of a remediation technology, provides quick and relatively unambiguous data over time, suitable for testing different options for biological treatment such as the effect of biostimulation and bioaugmentation. Tests may also be useful to confirm the active degradation of PAHs during the entire bioremediation process. During respirometric tests, rates of oxygen consumption and CO_2 evolution can be monitored by automated equipment that can handle a large number of samples simultaneously, or can also be analyzed with simple titration methods (Pritchard *et al.*, 1992).

Molecular monitoring: Characterization of microbial communities presents limitations due to the lack of sensitive detection methods. Traditional microbiological approaches, such as isolation of pure cultures and further study of their physiological and biochemical properties are not always well suited to study microbial communities and their behavior. In fact, about 99% of the microorganisms present in nature cannot be isolated due to a lack of knowledge of their physiological needs. Therefore, molecular techniques have been developed to compensate the disadvantages inherent to traditional culture methods. The validation and performance of a bioremediation strategy should be based not only on the effect of the microorganisms in soil (biodegradation of the contaminant), but also in the detection and monitoring of the inoculated microorganisms. Molecular techniques based on the Polymerase Chain Reaction (PCR) of 16S rRNA, RT-PCR and real-time PCR are becoming standard tools for the detection and quantification of microorganisms previously added to soils. Similarly, other less conventional methods such as FISH (Fluorescent In Situ Hybridization), DGGE (Denaturing Gradient Gel

Electrophoresis), TGGE (Temperature Gradient Gel Electrophoresis), DNA microarrays and metagenomics are being tested and implemented to study and better comprehend the microbial degradation of PAHs (Lendvay *et al.*, 2003).

1.5.4 METAGENOMICS AND HYDROCARBON BIOREMEDIATION

As previously mentioned, most of the microorganisms present in nature cannot be cultivated under standard laboratory conditions, which is a serious limitation to studying the diversity and function of environmental microbial communities. This is particularly relevant in soil, which is probably one of the most complex environments due to its extremely high microbial diversity and heterogeneous nature. Metagenomics refers to the study of all the genomes contained in an environmental or biological matrix directly from DNA samples without isolating or culturing microorganisms (Tringe and Rubin, 2005), thus allowing the study not only of the taxonomic diversity (e.g. species richness, microbial structure and distribution) of the soil, but also of the real metabolic potential of soil microorganisms.

The power of metagenomic studies relies on the use of high-throughput automated sequencing methods, introduced during the last decade into the study of microbial ecology and capable of revealing the taxonomic and functional aspects of microbial communities at a new level of resolution (Sogin *et al.*, 2006). Traditional automated Sanger sequencing is considered a 'first-generation' technology, while Next-Generation Sequencing (NGS) are newer methods broadly grouped as template preparation, sequencing, imaging, and data analysis. NGS technologies are also very sensitive compared with Sanger sequencing, with approximately 99.9% accuracy in <200 bp amplicons and 99% for <400 bp amplicons (Metzker, 2010). One of the major advantages of NGS is the production of massive quantities of data quickly and cheaply, which has opened the possibility to perform large-scale comparative studies. Commercially available technologies include Roche 454 platforms, Illumina/Solexa, Life Technologies/Ion platforms, Applied Biosystems/SOLiD and Helicos BioSciences systems, among others.

Metagenomics may provide valuable information about the entire functional gene composition of microbial communities, overcoming limitations associated with the use of only one gene (e.g. the 16s rRNA gene), as well as genetic information on novel enzymes, functional profiles and potential metabolic pathways (Thomas *et al.*, 2012). With sequencing prices falling and a large list of bioinformatic tools available for the sequence assembly, gene prediction and taxonomic binning of metagenomic data (Prakash and Taylor, 2012), metagenomics is one of the most valuable and fastest growing scientific disciplines applicable to the study of microbial ecology.

Recent studies using NGS during hydrocarbon bioremediation processes have shown the great potential of these technologies to identify, monitor and estimate proportions of crude oil (Coulon *et al.*, 2012; dos Santos *et al.*, 2011) and diesel degrading populations (Yergeau *et al.*, 2012b) present in soils and bioreactors, making possible the discovery of novel degradation pathways

(Sierra-García *et al.*, 2014). Important evidence about the regulation of PAH degradation processes has also been found by the metagenomic study of pure bacterial strains (Uchiyama and Miyazaki, 2013), increasing the understanding of the function of important transcriptional regulators. However, there is still scarce information about the metagenomics aspects of microbial communities involved in PAH degradation in soils. As PAH-degrading populations are highly diverse and their taxonomic and functional variation depends on both biotic and abiotic interactions, the metagenomic analysis of soil microbial communities directly involved in PAH degradation can provide important insights regarding the metabolic pathways and specific enzymes involved in the bioremediation process. In this study we used NGS technologies to compare the taxonomic composition and functional profiles of PAH-contaminated soils during a bioremediation process by bioaugmentation and biostimulation.

1.6 RESEARCH DIRECTIONS

1.6.1 RATIONALE

PAHs are persistent pollutants that negatively impacts soil flora and fauna and may produce serious health issues and genetic defects in humans. Although microorganisms capable of PAH degradation may already be present in PAH-contaminated soils, the environmental conditions in these areas are frequently adverse to them. In some cases, native microbiota at polluted sites are not able to show an adequate metabolic potential for the degradation or complete mineralization of PAHs due to the very low number of microorganisms capable of metabolize them. In addition, the main disadvantage of bioremediation systems lies in the prolonged periods of time needed to obtain positive results. This is a key factor responsible of the low efficiency of some *in situ* bioremediation processes, such as natural attenuation processes or biopile treatments. In response to this problem, the implementation of faster and more efficient technologies for the degradation of PAHs in soils becomes necessary.

Biological methods possess advantages over physicochemical and thermal methods, such as its cost-effectiveness, the possibility of fully mineralize pollutants and the little need for further treatment; however, they are characterized for being slow processes which require prolonged periods of time. Therefore, the use of biological methods as biostimulation and bioaugmentation constitutes an excellent alternative for the treatment of soils polluted with PAHs. The combined use of bioaugmentation and biostimulation may improve the rate of oxidation and degradation of PAHs by increasing specific microbial populations with high degradative potential, and at the same time stimulating native microbial populations through the addition of nutrients and texturizers. The use of degrading microbial consortia is beneficial as some filamentous fungi possess enzymatic machinery capable of break aromatic rings and lead to oxidation of the PAHs, generating more soluble intermediates which can be used by other organisms, such as bacteria. Furthermore, the use of genetically engineered microorganisms (GEMs) could contribute to increase degradation and mineralization rates. Previous studies have shown that both biotic and

abiotic factors have a great influence on the efficiency of bioaugmentation and biostimulation processes (Mrozik and Piotrowska-Seget, 2010). Thus, a key factor in the implementation of those processes is to establish ideal conditions for the growth and degradation by inoculated microbial consortia, taking into account the effects and interactions between native microbiota and introduced degrading consortia during the bioremediation process, as well as its adaptation and survival, especially for GEMs.

For all the above, this study aims to establish the optimal conditions for the degradation of PAHs in soils by microbial consortia composed by native fungal-bacterial strains and GEM strains, as well to analyze the interactions and effects with native microbiota during a bioremediation process by biostimulation and bioaugmentation.

1.6.2 HYPOTHESIS

The combined use of biostimulation and bioaugmentation using degrading native consortia and genetically modified microorganisms will enhance PAH degradation rates in soil, causing positive effects on the taxonomic and functional diversity of soil native microbial communities.

1.6.3 RESEARCH OBJECTIVES

GENERAL OBJECTIVE

To evaluate the growth conditions, degradative ability and metagenomic diversity at microcosm level of microbial consortia composed by native and genetically modified microorganisms for the bioremediation of PAH-contaminated soils by biostimulation and bioaugmentation.

SPECIFIC OBJECTIVES

To isolate native microorganisms from heavy crude oil-contaminated soils with the ability to degrade PAHs

To construct fungal-bacterial microbial consortia able to degrade PAHs composed by native and genetically modified strains

To establish the optimal conditions for the degradation of PAHs at microcosm using the microbial consortia

To perform treatability tests at microcosm using field-polluted and artificially contaminated soils to evaluate the PAH-degradative ability of consortia in presence of microbial native populations by biostimulation and bioaugmentation

To analyze by a metagenomic approach the microbial taxonomic composition, displacement effects and functional profiles during the bioremediation of a PAH-contaminated soil.

CHAPTER 2

ISOLATION AND SELECTION OF A HIGHLY TOLERANT MICROBIAL CONSORTIUM WITH POTENTIAL FOR PAH BIODEGRADATION FROM HEAVY CRUDE OIL-CONTAMINATED SOILS

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Isolation and Selection of a Highly Tolerant Microbial Consortium with Potential for PAH Biodegradation from Heavy Crude Oil-Contaminated Soils

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Abstract A degrading microbial consortium highly tolerant to three-, four- and five-ring polycyclic aromatic hydrocarbons (PAHs) was selected from 50 fungal and bacterial isolates obtained from crude oil-contaminated soils. Morphological and molecular studies indicated that isolated fungi belonged to genera *Aspergillus*, *Penicillium*, *Fusarium*, *Trichoderma*, *Scedosporium*, and *Acremonium* and bacteria to *Pseudomonas*, *Klebsiella*, *Bacillus*, *Enterobacter*, *Streptomyces*, *Stenotrophomonas*, *Kocuria*, and *Delftia* genera. Individual fungal and bacterial isolates were evaluated for their potential to tolerate high concentrations of different molecular weight PAHs, as phenantrene (Phe), pyrene (Pyr), and benzo[*a*]pyrene (BaP) by surface plate assays, showing significant differences in extension rates for fungi and inhibition ratios for bacteria when both were exposed to 0–6,000 mg of PAHs per liter. *Trichoderma asperellum* H15, *Aspergillus nomius* H7, *Aspergillus flavus* H6, *Pseudomonas aeruginosa* B7, *Klebsiella* sp. B10, and *Stenotrophomonas maltophilia* B14 grew using PAHs as sole carbon source and presented a remarkably high tolerance to PAHs, up to 6,000 mg l^{-1}. The consortium composed of 12 fungal and bacterial PAH-tolerant isolates for the bioremediation of a PAH-contaminated soiled to a removal of 87.76 % Phe, 48.18 % Pyr, and 56.55 % BaP after 14 days. The degrading microbial consortium presented high potential for bioremediation and may be useful for the treatment of sites polluted with PAHs due to their elevated tolerance to high molecular weight (HMW) PAHs and their capacity to utilize them as energy source. This is the first study which evaluated the microbial tolerance to extreme concentrations of PAHs, resulting in a degrading consortium and highly tolerant consortium compared with those reported in other studies, where the concentrations tested are low.

Keywords Polycyclic aromatic hydrocarbons (PAHs) · Microbial tolerance · Bioremediation · Degrading consortium · Microbial diversity · Heavy crude oil

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1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are an important group of organic pollutants containing two or more fused aromatic rings, mainly produced as the result of thermal decomposition, incomplete combustion, and pyrolysis of diverse organic molecules (Mrozik and Piotrowska-Seget 2010). Several natural and anthropogenic sources contribute to the release of PAHs into the environment, particularly, petrochemical activities, and their related residues exert a strong negative impact on the environment and account for the majority of PAHs and other hydrocarbons released into soils and water

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bodies. PAHs are considered persistent pollutants with potential harmful effects on the flora and fauna of affected habitats, resulting in the absorption and accumulation of several toxic products and active intermediaries of their metabolism in diverse organisms. Because of their persistence and potential cytotoxic, carcinogenic, genotoxic, and mutagenic effects, the removal of PAHs and other hydrocarbon compounds from contaminated soils has become an increasing environmental priority. There is a variety of mechanisms by which PAHs are removed naturally from the environment, including several forms of oxidation, adsorption, volatilization, bioaccumulation, and biodegradation. Although chemical, physicochemical, and thermal technologies are available for remediation of impacted soils, microbial degradation is considered the main natural degradation form of PAHs in soils. More than 50 genera of bacteria and fungi contain species capable of degrading low molecular weight (LMW) or high molecular weight (HMW) PAHs under aerobic or anaerobic conditions (Seo et al. 2009; Cerniglia and Sutherland 2010). The main catalytic pathways for the microbial oxidation of PAH rings have been well described in diverse species of bacteria, fungi, and algae, involving the action of mono- and dioxygenase, laccase, and peroxidase enzymes (Haritash and Kaushik 2009).

Although many PAH-degrading microorganisms have been identified, as well as the degradation mechanisms, enzymes, and genes implied in this process (Cerniglia and Sutherland 2010; Haritash and Kaushik 2009), there are only a few reports on microbial organotolerance, especially to extreme doses of PAHs (Argumedo-Delira et al. 2012; Hughes and Bridge 2009; Hughes et al. 2007). The evaluation of microbial tolerance to PAHs is important because the ability of microorganisms to metabolize PAHs can be strongly affected by the lack of tolerance to PAHs, constituting one of the causes for PAH persistence in contaminated soils as a result of an inhibited PAH metabolism and poor microbial growth, especially in soils with considerably high amounts of toxic compounds. Moreover, the physicochemical and biological complexity of impacted soils significantly affects the survival and activity of inoculated microbial populations, e.g., antagonistic relationships exerted by indigenous populations from the soils, abiotic factors that trigger bacterial stress, and the concentration and bioavailability of PAHs (Luthy 2004). In this context, the use of native microorganisms not only capable of degrading hydrocarbons but also having high levels of tolerance to LMW and HMW PAHs would reduce the problems associated with adaptation, survival, and degrading activity on soils containing high amounts of heavy hydrocarbon fractions. Heavy crude oil-contaminated soils constitute an excellent source of microorganisms having high tolerance and potential for the partial or complete mineralization of PAHs. This could be due to the large amounts of aromatics, resins, and asphaltenes present in these soils and the fact that microorganisms exposed to these latter during long periods may have an improved ability to degrade them (Premuzic and Lin 1999; Margesin and Schinner 2001). Thus, the aim of this work was to isolate, identify, and select bacterial and fungal isolates with high levels of PAH tolerance from heavy crude oil-contaminated soils in order to build a highly tolerant microbial consortium able to degrade LMW and HMW PAHs in soils.

2 Materials and Methods

2.1 Soil Samples

Three 1-year-old soils contaminated with Maya heavy crude oil, collected from a refinery located in Veracruz, Mexico, (17°59′18″N 94°30′30″W, 26 °C annual average temperature) were used in this study. Uncontaminated soil samples were obtained from the Xalostoc region in Tlaxcala, Mexico (19°24′08″N 98°02′54″W, 18 °C annual average temperature). Homogeneous samples were obtained in a simple random strategy at 30 cm depth, following the procedures described by US-EPA (1996). Soil samples were dried, homogenized, sieved with a 2-mm test sieve, and conserved at 4 °C until physicochemical analyses were conducted. Table 1 shows the main physicochemical characteristics of each soil according to the Official Mexican Standard NOM-021-RECNAT-2000 (SEMARNAT 2000).

2.2 Isolation and Selection of Native Microorganisms

Native fungal isolates were obtained by diluting 1 g of soil in 14-ml of Czapek medium containing (grams per liter): $NaNO_3$, 3; K_2HPO_4, 1; $MgSO_4$, 0.5; KCl, 0.5; $FeSO_4$, 0.01; sucrose, 30; supplemented with ampicillin (80 μg ml^{-1}). For solid Czapek medium, 15 g l^{-1} of

Table 1 Physicochemical characteristics of soils

Parameter	Soil 1		Soil 2		Soil 3		Uncontaminated soil	
	Value	Classification[a]	Value	Classification[a]	Value	Classification[a]	Value	Classification[a]
pH	6.9	Neutral	6.2	Moderately acid	5	Moderately acid	6.8	Neutral
Organic matter content (%)	8.5	Very high	10.2	Very high	10.3	Very high	2.19	Medium
Carbon (%)	3.285	Very high	3.525	Very high	4.585	Very high	0.73	Low
Nitrogen (%)	0.035	Very low	0.015	Very low	0.01	Very low	0.16	High
Phosphorous (%)	0.00011	Low	0.00201	High	0.00195	High	0.000242	Low
C/N/P ratio	100/1.06/0.0033		100/0.42/0.057		100/0.22/0.0425		100/21.9/0.033	
Total petroleum hydrocarbons (mg kg^{-1})	60,000		60,000		60,000		0	
Total PAHs content (mg kg^{-1})	5,000		5,000		5,000		0	
Texture	Sandy loam		Sandy loam		Sandy loam		Sandy loam	

[a] According to Official Mexican Standard NOM-021-RECNAT-2000 [60]

bacteriological agar was added. Tubes were incubated at 30 °C for 5 days with constant agitation at 200 rev min^{-1}. One hundred microliters of supernatant was used to inoculate plates of solid Czapek medium, and after 48 h of incubation at 30 °C, 15 ml of melted, sterile noble agar was added over the surface. After solidifying, 2 ml of Maya crude oil dissolved in 8 ml of acetone was added onto the surface, allowing the acetone to evaporate under sterile conditions. Plates were incubated at 30 °C and after 7 days, colonies showing visible mycelium and sporulation on crude oil layer were picked, inoculated onto potato dextrose agar plates and maintained at 30 °C. Bacterial populations were isolated from the contaminated soil by diluting 1 g of soil in 14 ml of M9 minimal salt medium (MSM) containing (grams per liter): Na_2HPO_4, 12.8; KH_2PO_4, 3; NaCl, 0.5; NH_4Cl, 1; $MgSO_4$, 0.5; $CaCl_2 \cdot 2H_2O$, 0.0152; $FeSO_4$, 0.01; glucose, 20; supplemented with cyclohexymide (100 μg ml^{-1}) (Sambrook and Russell 2001). Cultures were incubated at 30 °C for 5 days with constant agitation at 200 rev min^{-1}. One hundred microliters of the culture supernatant was used to inoculate plates of basal saline medium (BSM) containing (grams per liter): NaCl, 0.3; $(NH_4)_2SO_4$, 0.6; K_2HPO_4, 0.75; KH_2PO_4,0.25; $MgSO_4 \cdot 7H_2O$, 0.15; KNO_3, 0.6; yeast extract, 0.125 using 0.1 % Maya crude oil as sole carbon source. Plates were incubated at 30 °C for 5 days and individual colonies were picked and transferred to new plates of BSM with Maya crude oil as carbon source.

2.3 Molecular Identification of Isolates

Genomic DNA extraction from fungal and bacterial isolates was performed by using the ZR Fungal/Bacterial DNA Kit (Zymo Research, USA). Fungal genomic DNA was extracted from lyophilized mycelium. A 1,450-bp fragment of the bacterial 16 s rRNA gene was PCR amplified by using universal primers P27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1525R (5′-AAGGAGGTGWTCCARCC-3′) as described previously (Lane 1991). ITS1, 5.8s ribosomal RNA, and ITS2 fungal sequences were amplified using primers ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS4B (5′-TCCTCCGCTTATTGATATGC-3′) using conditions described by White et al. (1990). PCR products were analyzed by agarose gel electrophoresis and, subsequently, purified using the QIAquick PCR purification kit (QIAgen). DNA sequencing was performed by using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Invitrogen, USA) in a PerkinElmer/Applied Biosystems sequencer Model 3730, using oligonucleotides P27F/P1525R and ITS5/ITS4B as sequencing primers for bacterial and fungal PCR products, respectively. Resulting sequences were compared with the GenBank database using the BLAST platform to identify the microorganisms (Altschul et al. 1990). Phylogenetic analysis was performed using the MEGA 5 software package (Tamura et al. 2011). The sequences were aligned with the ClustalW function and maximum likelihood trees were constructed with the Kimura two-

parameter model (Kimura 1980). The robustness of the phylogeny was tested by bootstrap analysis with 500 iterations.

2.4 Tolerance Tests to Mixed PAHs

Tolerance of microbial isolates to several doses of analytical grade phenantrene (Phe), pyrene (Pyr), and benzo[*a*]pyrene (BaP) (Sigma Chemicals, USA) was tested by surface plate assays. Bacterial isolates were tested by inhibition surface assays using 6 mm diameter polyvinylidene fluoride discs (Millipore, USA), each one impregnated with a PAH mixture of Phe, Pyr, and BaP (1:1:1) containing final concentrations of 500, 1,000, 2,000, 3,000, 4,000, and 5,000 mg l^{-1}. Bacterial isolates were grown in 5 ml of liquid MSM at 30 °C with agitation until cultures reached an optical density of 0.14 at 600 nm (comparable to a McFarland standard no. 0.5). One hundred microliters of each culture (approximately 1.5×10^7 colony forming units (CFU)) were spread over the surface of MSM plates with 2 % glucose as carbon source, and discs corresponding to each concentration of PAHs were placed onto the surface. Ten microliters of water diluted acetone (1:1) was used as PAH carrier from impregnated discs to inoculated culture medium. Plates were incubated at 30 °C and the inhibition halos were measured every 24 h for 5 days. Discs without PAHs were included as controls. For fungal tolerance, 2 ml of the PAH mixture dissolved in pure acetone was spread on Petri dishes with Toyama's Medium (grams per liter): $(NH_4)_2SO_4$, 1; $MgSO_4\cdot 7H_2O$, 0.5; KH_2PO_4, 3; $CaCl_2\cdot 2H_2O$, 0.5; NaCl, 0.1; $FeSO_4$, 0.001; glucose, 10 (Wunder et al. 1994) and evaporated under sterile conditions to yield final superficial concentrations of 1,000, 2,000, 4,000, and 6,000 mg l^{-1}. Plates were inoculated in the center with 5×10^4 spores and incubated at 30 °C. Radial growth rate measurements were made every 24 h for 10 days. Plates without PAHs and inoculated with each of the fungal isolates were used as controls.

2.5 Solid Culture for Biodegradation of PAHs in Soil by Microbial Consortium

The construction of a microbial consortium able to degrade PAHs in contaminated soils was performed by selecting fungal and bacterial isolates based on: PAH tolerance levels, ability to use them as sole carbon source, and reported capacity to metabolize PAHs. Removal ability of a mixture of Phe, Pyr, and BaP was evaluated in microcosm solid culture systems using sterile sugarcane bagasse (34.34 % carbon, 0.18 % nitrogen, 0.00343 % phosphorous) as fungal growth support. Sterile sugarcane bagasse (dry weight) was placed in 50-ml glass flasks moistened with Czapek medium to reach 30 % moisture content, inoculated with each fungal isolate at final concentration of 2×10^6 spores g^{-1} and incubated for 5 days at 30 °C. Sterile soil was then contaminated with 1,000 mg of PAH mixture per kilogram of soil (1:1:1 ratio) was subsequently mixed with preinoculated sugarcane bagasse. In addition, 2×10^6 CFU g^{-1} of each bacterial isolate was inoculated and incubated at 30 °C for 14 days. Samples of noncontaminated soil were inoculated to determine the effect of PAHs on the growth of microorganisms. Abiotic controls were included to assure that the disappearance of PAHs was caused by biodegradation as well as to compensate for adsorption losses. Assays were carried out in triplicate.

2.6 Heterotrophic Activity

CO_2 evolution in microcosm was measured every 48 h using an Agilent 6890 series gas chromatograph equipped with a thermal conductivity detector and a GS-CarbonPLOT column. CO_2 was reported as milligrams of CO_2 per grams of initial dry matter (IDM).

2.7 PAH Analysis

Residual PAHs were extracted from 1 g of IDM (soil plus sugarcane bagasse) with the addition of 25 ml of a dichloromethane–acetone solution (7:3 ratio) using an Anton Paar Multiwave 3000 SOLV apparatus for 20 min, according to EPA 3546 method. The resulting extracts were evaporated, suspended in 2 ml of acetonitrile and analyzed in an HP Agilent 1100 HPLC system equipped with a C18 reverse-phase column, with an UV absorbance detector set at 245–360 nm under isocratic conditions in acetonitrile:water (90:10) and a flow rate of 1 ml/min.

2.8 Statistical Analysis

Data were analyzed by analysis of variance followed by a multiple comparison test (LSD) with SPSS Statistics Software version 19 (IBM), considering statistically significant differences those with a p value <0.05.

3 Results

3.1 Microbial Isolation from Soil

A total of 29 bacterial and 21 fungal isolates were obtained from crude oil-contaminated soils, based on their capacity to tolerate or use Maya crude oil as sole source of carbon and energy. Morphological analysis of fungal isolates showed diversity in macroscopic and microscopic morphologies with typical structures of *Aspergillus*, *Penicillium*, *Fusarium*, *Rhizopus*, *Scedosporium*, *Acremonium*, *Trichoderma*, and *Beauveria* genera. BLAST analysis of ITS1, 5.8 s ribosomal RNA, and ITS2 sequences indicated that isolates corresponded to eight different fungal genera and 11 species with genetic similarity values close to 100 % with those reported in GenBank (Table 2). Isolates belonging to a same species (*Aspergillus flavus*, *Aspergillus nomius*, *Trichoderma asperellum*) were found to have 100 % similarity in their ITS sequences. For bacterial isolates, Gram-positive and Gram-negative organisms with bacillary, filamentous, and cocoid morphologies were observed and, as with fungal isolates, isolates of the same species showed 100 % similarity between their sequences (e.g., *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Pseudomonas nitroreducens*, *Delftia* sp., *Corynebacterium glutamicum*, *Streptomyces* sp.) (Table 3). To determine the phylogenetic position of the microbial isolates, ITS1 and 16S rRNA sequences were aligned with the corresponding sequences of several known PAH-degrading and non-PAH-degrading organisms. Fungal native isolates were grouped into Ascomycota (20 isolates) and Zygomycota (one isolate) phyla (Fig. 1). The tree based on bacterial 16S rRNA gene sequences (Fig. 2) indicated that these isolates were grouped into Actinobacteria (six isolates), Firmicutes (three isolates), and Proteobacteria (20) phyla; Gammaproteobacteria members (16) of the latter were the most frequent organisms (Table 3). The 16S rRNA-based phylogenetic tree showed that bacterial isolates, especially corresponding to Proteobacteria are closely related between them and include previously reported PAH-degrading organisms.

Table 2 BLAST identification of fungal native isolated from heavy crude oil-contaminated soil

Isolate	Closest related species (GenBank accession number)	Identity	Phylum	Class
H1	*Aspergillus fumigatus* strain ASH (JX006238.1)	99 %	Ascomycota	Eurotiomycetes
H2	*Aspergillus fumigatus* strain ASH (JX006238.1)	99 %	Ascomycota	Eurotiomycetes
H3	*Fusarium equiseti* (FR669193.1)	99 %	Ascomycota	Sordariomycetes
H4	*Aspergillus flavus* isolate RF1 (JQ975004.1)	100 %	Ascomycota	Eurotiomycetes
H5	*Aspergillus flavus* isolate RF1 (JQ975004.1)	100 %	Ascomycota	Eurotiomycetes
H6	*Aspergillus flavus* isolate RF1 (JQ975004.1)	100 %	Ascomycota	Eurotiomycetes
H7	*Aspergillus nomius* strain NRRL 6552 (AF338647.1)	100 %	Ascomycota	Eurotiomycetes
H8	*Aspergillus nomius* strain NRRL 6552 (AF338647.1)	100 %	Ascomycota	Eurotiomycetes
H9	*Rhizomucor variabilis* strain SGE39 (JQ776538.1)	100 %	Zygomycota	Zygomycetes
H10	*Scedosporium apiospermum* strain IFM 55501 (AB489081.1)	99 %	Ascomycota	Sordariomycetes
H11	*Acremonium* sp. YX (FJ770373.1)	100 %	Ascomycota	Sordariomycetes
H12	*Penicillium commune* isolate NJP11 (Q710540.1)	100 %	Ascomycota	Eurotiomycetes
H13	*Penicillium commune* strain 0109CI37K3 (FR799456.1)	100 %	Ascomycota	Eurotiomycetes
H14	*Beauveria bassiana* strain BLe-06 (JX149538.1)	100 %	Ascomycota	Sordariomycetes
H15	*Trichoderma asperellum* isolate T20 (JF501661.1)	100 %	Ascomycota	Sordariomycetes
H16	*Aspergillus fumigatus* strain SGE57 (JQ776545.1)	100 %	Ascomycota	Eurotiomycetes
H17	*Trichoderma asperellum* isolate T20 (JF501661.1)	100 %	Ascomycota	Sordariomycetes
H18	*Trichoderma asperellum* isolate T20 (JF501661.1)	100 %	Ascomycota	Sordariomycetes
H19	*Aspergillus fumigatus* strain SGE57 (JQ776545.1)	100 %	Ascomycota	Eurotiomycetes
H20	*Aspergillus niger* isolate F7-02 (JN561274.1)	100 %	Ascomycota	Eurotiomycetes
H21	*Acremonium* sp. YX (FJ770373.1)	100 %	Ascomycota	Sordariomycetes

Table 3 BLAST identification of bacterial native isolated from heavy crude oil-contaminated soils

Isolate	Closest related sequence (GenBank accession number)	Identity	Phylum	Class
B1	*Klebsiella pneumoniae* strain AIMST (JN835542.1)	100 %	Proteobacteria	Gammaproteobacteria
B2	*Klebsiella pneumoniae* strain AIMST (JN835542.1)	100 %	Proteobacteria	Gammaproteobacteria
B3	*Enterobacter* sp. NISOC_03 (HQ419280.1)	99 %	Proteobacteria	Gammaproteobacteria
B4	*Bacillus cereus* strain CCMM (JN208091.1)	99 %	Firmicutes	Bacilli
B5	*Enterobacter aerogenes* strain KNUC5012 (JQ682638.1)	98 %	Proteobacteria	Gammaproteobacteria
B6	*Pseudomonas aeruginosa* strain DKH-3 (JQ773433.1)	100 %	Proteobacteria	Gammaproteobacteria
B7	*Pseudomonas aeruginosa* strain DKH-3 (JQ773433.1)	100 %	Proteobacteria	Gammaproteobacteria
B8	*Streptomyces* sp. YIM 75358 (JN188956.1)	100 %	Actinobacteria	Actinobacteria
B9	*Stenotrophomonas* sp. p22(2011) (HQ652605.1)	100 %	Proteobacteria	Gammaproteobacteria
B10	*Klebsiella* sp. L1.111 16S ribosomal RNA gene (JQ811532.1)	100 %	Proteobacteria	Gammaproteobacteria
B11	*Corynebacterium glutamicum* strain TCCC27018 (EU231607.1)	99 %	Actinobacteria	Actinobacteria
B12	*Pseudomonas nitroreducens* strain YEMCu23 (JQ582968.1)	100 %	Proteobacteria	Gammaproteobacteria
B13	*Delftia* sp. T3-6 (JN595858.1)	99 %	Proteobacteria	Betaproteobacteria
B14	*Stenotrophomonas maltophilia* strain BBE11-1 (JQ619623.1)	100 %	Proteobacteria	Gammaproteobacteria
B15	*Delftia* sp. BCA19 (HE716888.1)	99 %	Proteobacteria	Gammaproteobacteria
B16	*Comamonas* sp. XJ-L79 (EU817495.1)	98 %	Proteobacteria	Betaproteobacteria
B17	*Pseudomonas nitroreducens* strain UAM-Ps2 (JQ586349.1)	99 %	Proteobacteria	Gammaproteobacteria
B18	*Pseudomonas nitroreducens* strain R5-791 (JQ659791.1)	100 %	Proteobacteria	Gammaproteobacteria
B19	*Pseudomonas nitroreducens* strain R5-791 (JQ659791.1)	99 %	Proteobacteria	Gammaproteobacteria
B20	*Enterobacter asburiae* strain KNUC5007 (JQ682630.1)	99 %	Proteobacteria	Gammaproteobacteria
B21	*Delftia* sp. T3-6 (JN595858.1)	100 %	Proteobacteria	Betaproteobacteria
B22	*Streptomyces* sp. 13658E (EU741184.1)	99 %	Actinobacteria	Actinobacteria
B23	*Citrobacter freundii* strain F1 (FJ608234.1)	100 %	Proteobacteria	Gammaproteobacteria
B24	*Corynebacterium glutamicum* strain TCCC27018 (EU231607.1)	99 %	Actinobacteria	Actinobacteria
B25	*Corynebacterium glutamicum* strain TCCC27018 (EU231607.1)	99 %	Actinobacteria	Actinobacteria
B26	*Kocuria* sp. SGb392 (HQ224638.1)	100 %	Actinobacteria	Actinobacteria
B27	*Delftia* sp. L2128 (JQ419620.1)	100 %	Proteobacteria	Betaproteobacteria
B28	*Bacillus flexus* strain WY2 (JQ936679.1)	99 %	Firmicutes	Bacilli
B29	*Bacillus simplex* strain Z8B-50 (HQ238704.1)	98 %	Firmicutes	Bacilli

3.2 Tolerance of Individual Isolates to Mixed PAHs

Due to the very slow growth exhibited during isolation and plate purification (<1 cm in 10 days without PAHs), fungal isolates H10 (*Scedosporium apiospermum*), H11, and H21 (*Acremonium* sp.) were excluded from tolerance tests. The ability of the remaining 18 fungal isolates to grow in the presence of Phe, Pyr, and BaP in surface plate assays is shown in Table 4. Fungal radial extension rates exhibited significant differences when growing in the presence of 1,000, 2,000, 4,000, and 6,000 mg of the PAH mixture per liter. *A. flavus* and *A. nomius* isolates did not show a significant inhibitory effect at 1,000 and 2,000 mg l^{-1}, although they exhibited slow growth even without PAHs (0.22 and 0.21 cm day^{-1}, respectively). *Rhizomucor variabilis* showed no significant inhibition up to 1,000 mg l^{-1}, but at higher concentrations of PAHs, its growth was severely affected. *Aspergillus fumigatus* H1/H2/H16, *Fusarium equiseti* H3, *Penicillium commune* H12/H13, and *Beauveria bassiana* H14 proved to be the most PAH-sensitive isolates since the rate of growth notably decreased in the presence of the lowest concentration (1,000 mg l^{-1}) of PAHs. In contrast, there were no apparent negative effects of PAH presence on the radial extension rates of any of the three *T. asperellum*

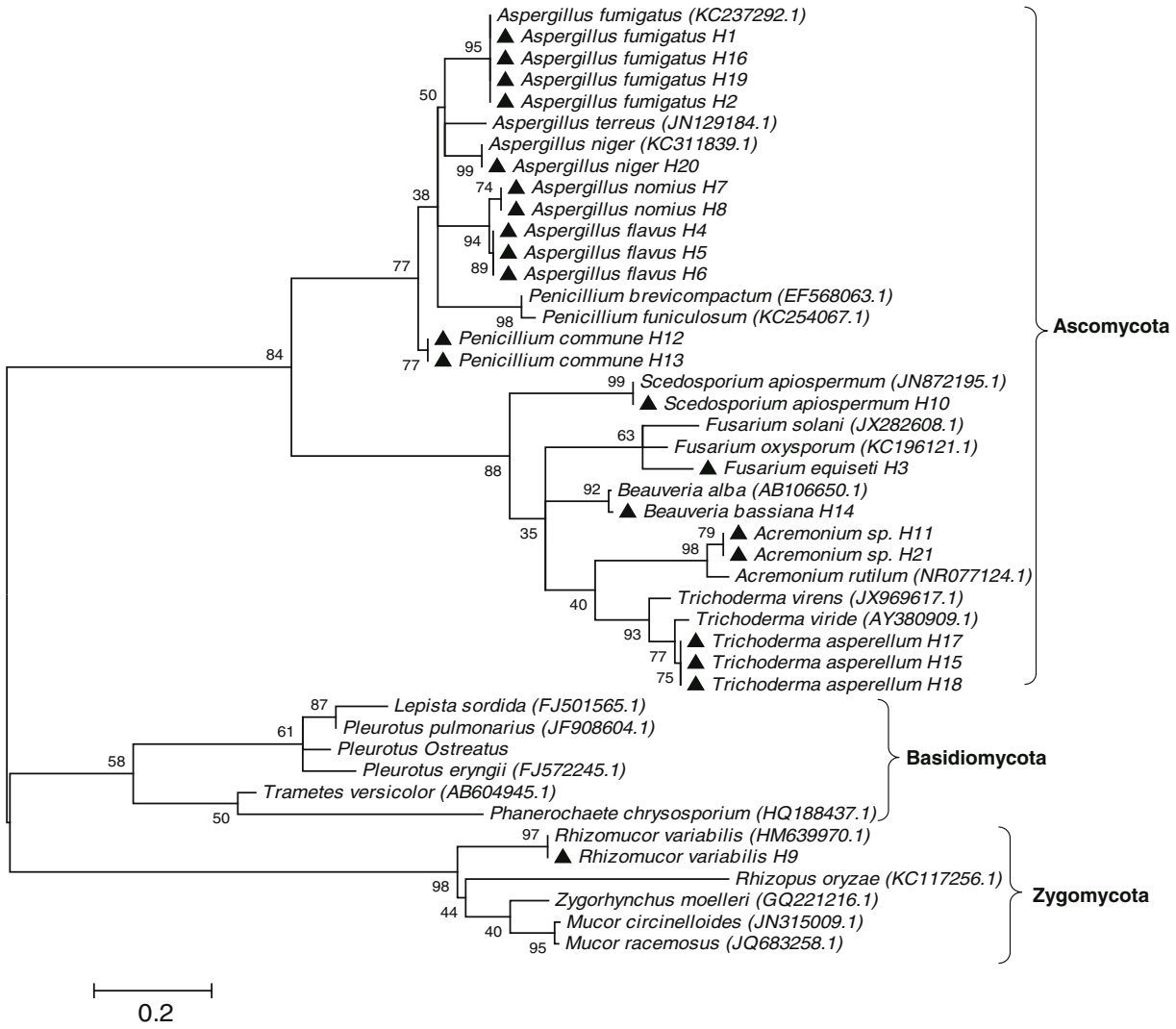


Fig 1 Consensus phylogenetic tree based on partial fungal ITS1, 5,8 s rRNA, and ITS2 sequences (600 bp). The evolutionary history was inferred by using the maximum likelihood method based on the Kimura two-parameter model. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. *Black triangles* indicate the native organisms isolated in this study. GenBank accession numbers of reference sequences are indicated

isolates, since the rates did not decrease notoriously compared to control after 10 days of growth at 6,000 mg of PAHs per liter (0.47 cm day^{-1}), although sporulation capacity was inhibited at that concentration. A similar situation was observed for *A. fumigatus*, H19 being able to sporulate at high concentrations of PAHs, although showing a significant reduction in the growth rate. *Aspergillus niger* H20 was able to sporulate at 1,000 mg l^{-1} but its growth was severely inhibited in the presence of higher concentrations, with visible changes in mycelium pigmentation (Fig. 3). Regardless of their growth rate, only isolates of *R. variabilis* (H9), *T. asperellum* (H15, H17, H18), and *A. fumigatus* H19 were able to sporulate in the presence of 4,000 mg of PAHs per liter (Fig. 3). With the exception of the *Trichoderma* isolates, all the fungal isolates significantly decreased their extension rate at 6,000 mg l^{-1} compared to their respective controls. In addition, PAH-tolerant isolates of *A. flavus* (H6), *A. nomius* (H7), and *T. asperellum* (H15) were capable of growth by using 200 mg of PAH mixture per liter as sole carbon source (data not shown).

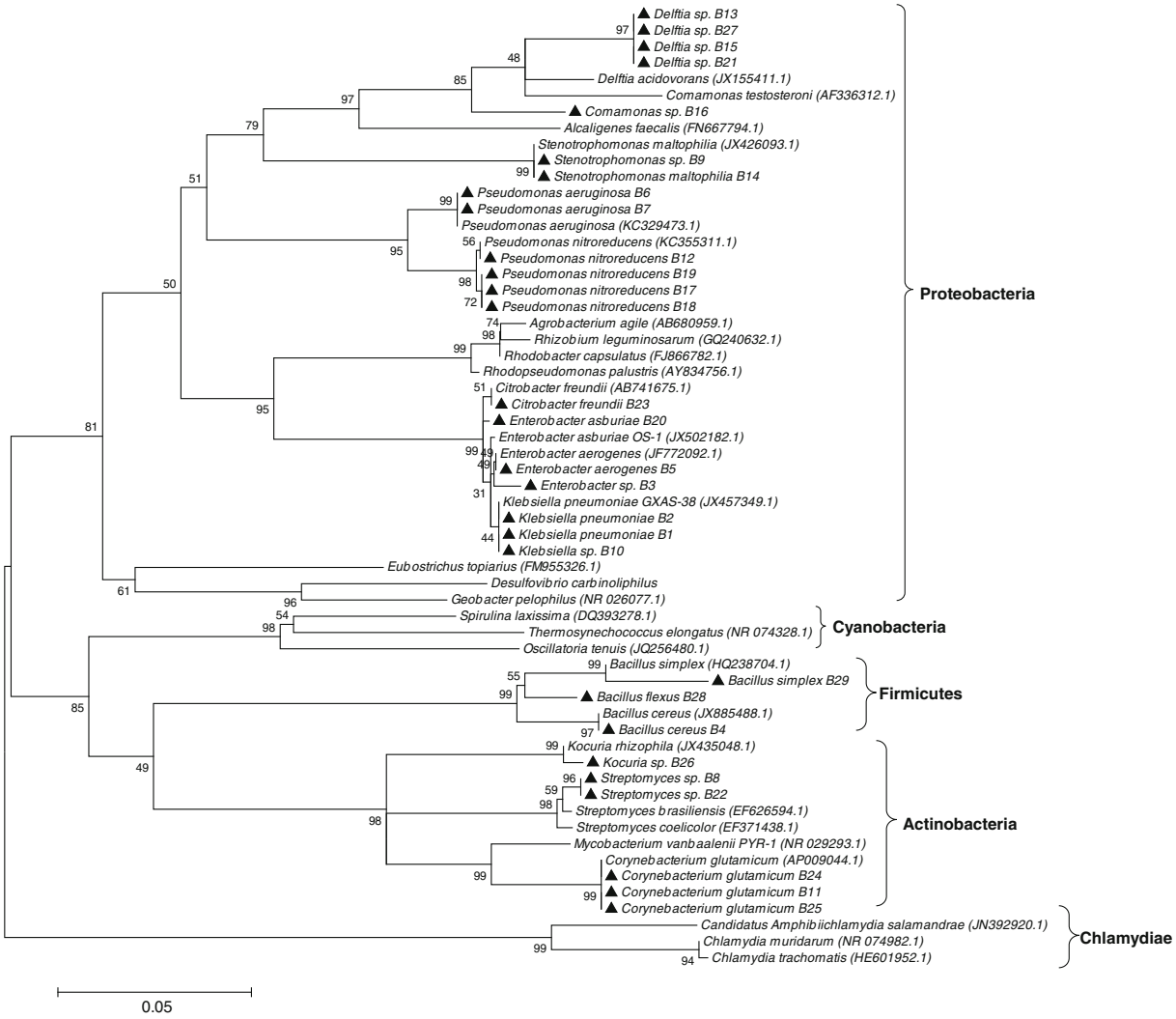


Fig 2 Consensus phylogenetic tree based on partial bacterial 16 s rRNA sequences (700 bp). The evolutionary history was inferred by using the maximum likelihood method based on the Kimura two-parameter model. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. *Black triangles* indicate the native organisms isolated in this study. GenBank accession numbers of reference sequences are indicated

The radius of the inhibition halos (millimeters) of bacterial isolates in the presence of different concentrations of a mixture of Phe, Pyr, and BaP are shown in Table 5. As expected, inhibition halos were greater for discs with higher concentrations of PAHs. Gram-positive isolates (*Bacillus* sp., *Bacillus cereus*, *Bacillus flexus*, *Bacillus simplex*, *Streptomyces* sp., *Corynebacterium* sp., *Kocuria* sp.) were less tolerant to higher concentrations of PAHs, showing inhibition halos even at 500 mg l^{-1}. On the other hand, *P. aeruginosa* B7, *Klebsiella* sp. B10, and *Stenotrophomonas maltophilia* B14 isolates showed the highest tolerance having no visible growth inhibition when exposed to concentrations up to 2,000 mg of the PAH mixture per liter. Enterobacteriaceae members were the most tolerant organisms isolated. The use of acetone solution as PAH carrier did not seem to have an adverse effect on the growth of isolates, since inhibition halos were not observed in the control discs without PAH treated only with acetone. Only seven out of 29 isolates (B4, B17, B25, B26, B27, B28, B29) were incapable of growing on the PAH mixture as the sole carbon source (data not shown).

Table 4 Radial extension rate (cm d^{-1}) of fungal isolates in presence of different concentrations of a mixture of phenantrene, pyrene, and benzo[*a*]pyrene (1:1:1). Standard deviation values are shown in parentheses

Isolate	PAH mixture concentration in medium (mg l^{-1})				
	0 (control)	1,000	2,000	4,000	6,000
Aspergillus fumigatus H1	0.342 (±0.03)	0.062* (±0.001)	0.047* (±0.004)	0.03* (±0.003)	0.020* (±0.003)
Aspergillus fumigatus H2	0.336 (±0.02)	0.063* (±0.004)	0.047* (±0.004)	0.031* (±0.003)	0.015* (±0.001)
Fusarium equiseti H3	0.245 (±0.01)	0.058* (±0.009)	0.046* (±0.004)	0.047* (±0.005)	0.048* (±0.003)
Aspergillus flavus H4	0.228 (±0.009)	0.216 (±0.009)	0.180 (±0.014)	0.129* (±0.018)	0.097* (±0.013)
Aspergillus flavus H5	0.225 (±0.02)	0.213 (±0.02)	0.184 (±0.03)	0.128* (±0.03)	0.109* (±0.009)
Aspergillus flavus H6	0.209 (±0.02)	0.188 (±0.09)	0.155 (±0.01)	0.152* (±0.02)	0.135* (±0.01)
Aspergillus nomius H7	0.222 (±0.03)	0.215 (±0.01)	0.198 (±0.01)	0.186* (±0.03)	0.173* (±0.02)
Aspergillus nomius H8	0.243 (±0.03)	0.223 (±0.02)	0.199 (±0.01)	0.184* (±0.02)	0.170* (±0.02)
Rhizomucor variabilis H9	0.340 (±0.03)	0.316 (±0.01)	0.095* (±0.03)	0.085* (±0.02)	0.075* (±0.01)
Penicillium commune H12	0.265 (±0.05)	0.064* (±0.02)	0.055* (±0.02)	0.047* (±0.005)	0.047* (±0.004)
Penicillium commune H13	0.110 (±0.03)	0.066 (±0.02)	0.054 (±0.03)	0.045* (±0.004)	0.046* (±0.005)
Beauveria bassiana H14	0.376 (±0.02)	0.193 (±0.02)	0.139* (±0.01)	0.090* (±0.02)	0.090* (±0.01)
Trichoderma asperellum H15	0.472 (±0.04)	0.476 (±0.03)	0.469 (±0.02)	0.456 (±0.03)	0.473 (±0.04)
Aspergillus fumigatus H16	0.253 (±0.03)	0.139 (±0.02)	0.054* (±0.007)	0.052* (±0.003)	0.052* (±0.01)
Trichoderma asperellum H17	0.449 (±0.008)	0.429 (±0.02)	0.447 (±0.003)	0.454 (±0.02)	0.455 (±0.01)
Trichoderma asperellum H18	0.415 (±0.02)	0.419 (±0.02)	0.415 (±0.04)	0.413 (±0.01)	0.410 (±0.02)
Aspergillus fumigatus H19	0.425 (±0.04)	0.133* (±0.02)	0.133* (±0.06)	0.129* (±0.01)	0.105* (±0.01)
Aspergillus niger H20	0.409 (±0.03)	0.147* (±0.006)	0.141* (±0.01)	0.102* (±0.01)	0.077* (±0.01)

* Statistically significant differences ($p \leq 0.05$) in radial extension rate at different concentrations with respect to the control (0 mg l^{-1})

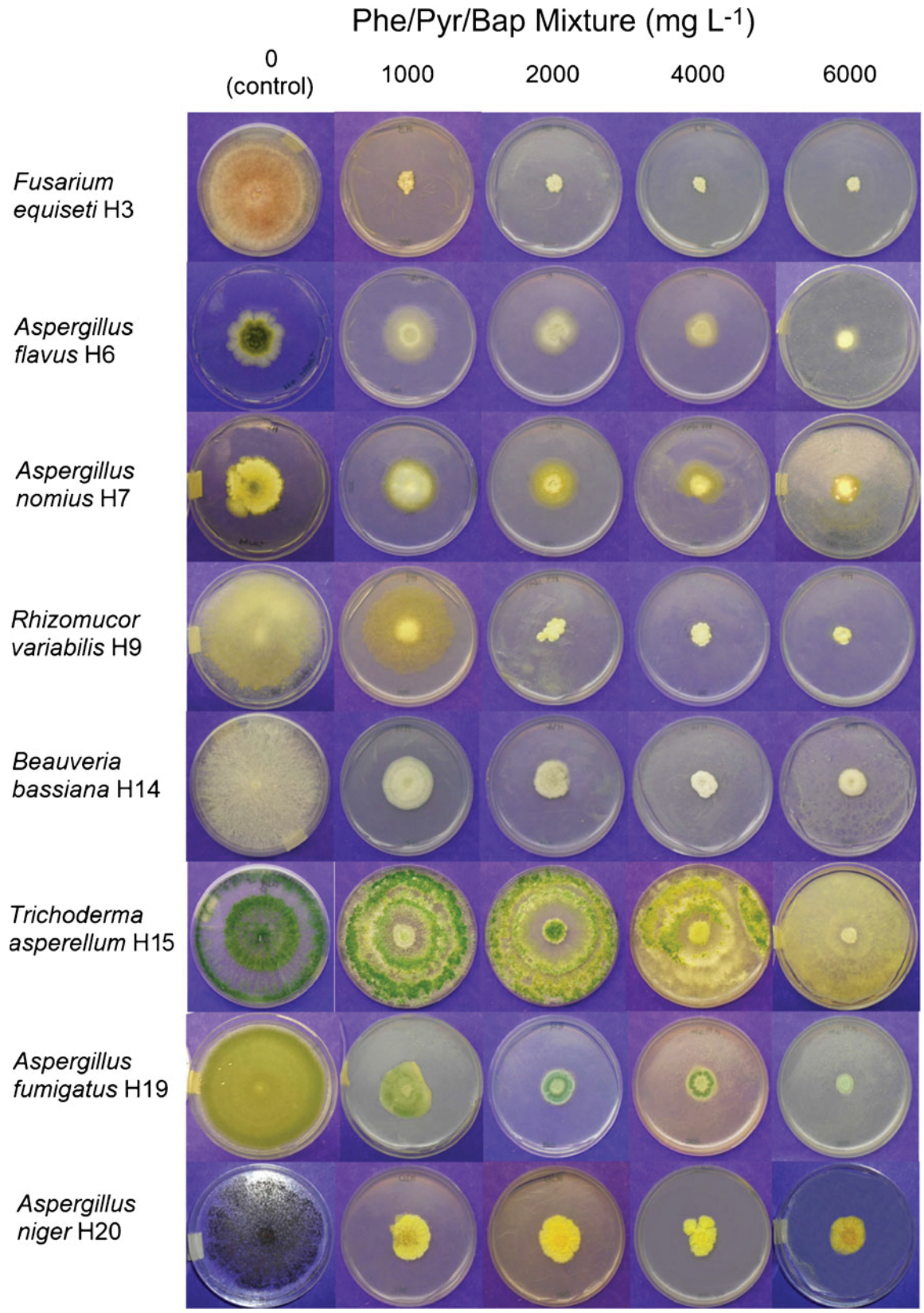
3.3 Degradation of PAHs in Soil by a Tolerant Microbial Consortium

Five fungal (*A. flavus* H6, *A. nomius* H7, *R. variabilis* H9, *T. asperellum* H15, *A. fumigatus* H19) and seven bacterial isolates (*K. pneumoniae* B1, *Enterobacter* sp. B3, *B. cereus* B4, *P. aeruginosa* B6, *Streptomyces* sp. B8, *Klebsiella* sp. B10, *S. maltophilia* B14) were selected to form the degrading microbial consortium. The results showed that the presence of PAHs in soil microcosms produced a rapid initial increase in CO_2 values by microbial-degrading populations. CO_2 production in microcosms systems increased gradually over the 14 days of growth, showing higher respiratory levels during the first week and a rapid decrease on day 8. However, microcosms containing soil with 1,000 mg of PAHs per kilogram produced significantly higher amounts of CO_2 at day 14 than the noncontaminated microcosms ($p=0.001$) (Fig. 4). During the initial days, Phe was degraded at a higher rate that Pyr and BaP. Removal percentages of Phe, Pyr, and BaP after 8 days of growth in microcosms were 28.21, 15.54, and 11.57 %, respectively; by day 14, removal values increased to 87.76 % for Phe, 48.18 % for Pyr, and 56.55 % for BaP (Fig. 5). By day 14, abiotic losses were negligible, being 5.32 % for Phe, 1.33 % for Pyr, and 0.41 % for BaP.

4 Discussion

PAH-degrading microbial populations in soils are faced with organic compounds that are potentially toxic to them as a source of carbon and energy (Ramos et al. 2002). In this way, the lack of organotolerance of PAH-degrading populations is a factor that could contribute to the persistence of PAHs in contaminated soils as a consequence of poor growth and inhibited metabolism, especially in those with considerable amounts of toxic compounds.

In this study, 50 microbial isolates were obtained from Maya crude oil-contaminated soils and a tolerant-degrading microbial consortium was built from them. Bacterial isolates were considered to be hydrocarbon-


Phe/Pyr/Bap Mixture (mg L-1)
0 (control)
1000
2000
4000
6000
Fusarium equiseti H3
Aspergillus flavus H6
Aspergillus nomius H7
Rhizomucor variabilis H9
Beauveria bassiana H14
Trichoderma asperellum H15
Aspergillus fumigatus H19
Aspergillus niger H20

◀ **Fig 3** Radial growth of isolated fungal strains in Petri dishes with different concentrations of a PAH mixture (0–6,000 mg l^{-1}). Results showed the effect of PAH concentration of the morphology and growth of the isolates after 10 days of growth

utilizing microorganisms based on the use of crude oil as sole carbon and energy source, while fungal isolates were selected on the basis of their capacity to grow over a layer of the same crude oil. As expected, several known species of PAH degraders were isolated, including *P. aeruginosa* (S. J. Kim et al. 2008), *Pseudomonas* sp. (Avramova et al. 2008), *Enterobacter* sp. (Bautista et al. 2009), *Stenothrophomonas* sp. (Bautista et al. 2009), *S. maltophilia* (Boonchan et al. 2000), *Bacillus* sp. (Wong et al. 2004), *B. cereus* (Kazunga and Aitken 2000), *Streptomyces* sp. (Chaudhary et al. 2011), *C. glutamicum* (Shen et al. 2012), *Delftia* sp. (Chen and

Table 5 Inhibition halos radius (mm) of bacterial isolates in presence of different concentrations of a mixture of phenantrene, pyrene, and benzo[*a*]pyrene (1:1:1)

Isolate	PAH disc concentration (mg l^{-1})						
	0 (control)	500	1,000	2,000	3,000	4,000	5,000
Klebsiella pneumoniae B1	0	0.78 (±0.21)	0.95 (±0.26)	1.5 (±0.15)	2.37 (±0.14)	2.675 (±0.04)	2.97 (±0.21)
Klebsiella pneumoniae B2	0	1.5 (±0.24)	1.96 (±0.32)	2.1 (±0.11)	2.35 (±0.12)	2.5 (±0.1)	3.275 (±0.08)
Enterobacter sp. B3	0	0	1.125 (±0.25)	1.15 (±0.27)	3.105 (±0.21)	3.255 (±0.07)	4.12 (±0.1)
Bacillus cereus B4	0	0.5 (±0.15)	0.785 (±0.36)	1.2 (±0.04)	1.62 (±0.23)	2 (±0.14)	2.335 (±0.04)
Enterobacter aerogenes B5	0	0	0.405 (±0.22)	0.53 (±0.09)	0.995 (±0.15)	2.5 (±0.15)	3.715 (±0.04)
Pseudomonas aeruginosa B6	0	0	0	0.735 (±0.08)	1.24 (±0.11)	2 (±0.25)	2.845 (±0.18)
Pseudomonas aeruginosa B7	0	0	0	0	0.7 (±0.1)	2 (±0.11)	2.6 (±0.04)
Streptomyces sp. B8	0	1 (±0.1)	1.4 (±0.2)	2 (±0.1)	2.6 (±0.22)	3.3 (±0.12)	4.3 (±0.1)
Stenotrophomonas sp. B9	0	0.605 (±0.15)	0.87 (±0.24)	1.47 (±0.2)	2.09 (±0.1)	3.7 (±0.17)	4.71 (±0.15)
Klebsiella sp. B10	0	0	0	0	2.515 (±0.1)	2.5 (±0.08)	3.385 (±0.19)
Corynebacterium glutamicum B11	0	0.855 (±0.23)	1.395 (±0.14)	1.8 (±0.3)	3.7 (±0.05)	4.17 (±0.16)	5.42 (±0.16)
Pseudomonas nitroreducens B12	0	0.95 (±0.1)	1.5 (±0.08)	2.1 (±0.24)	4.05 (±0.35)	4.05 (±0.27)	4.835 (±0.13)
Delftia sp. B13	0	0.35 (±0.11)	1 (±0.11)	1.7 (±0.26)	2.5 (±0.17)	2.5 (±0.15)	3.6 (±0.16)
Stenotrophomonas maltophilia B14	0	0	0	0	1.15 (±0.07)	2 (±0.1)	2.45 (±0.2)
Delftia sp. B15	0	0.6 (±0.28)	1.05 (±0.12)	1 (±0.2)	1.42 (±0.14)	3.23 (±0.12)	4.865 (±0.28)
Comamonas sp. B16	0	0.25 (±0.06)	0.16 (±0.08)	0.81 (±0.14)	2.29 (±0.15)	2.545 (±0.11)	2.505 (±0.15)
Pseudomonas nitroreducens B17	0	0.26 (±0.12)	2.4 (±0.22)	3.1 (±0.16)	2.57 (±0.26)	3.71 (±0.26)	4.9 (±0.2)
Pseudomonas nitroreducens B18	0	0.45 (±0.14)	2 (±0.25)	3 (±0.11)	2.8 (±0.23)	4 (±0.19)	5.27 (±0.15)
Pseudomonas nitroreducens B19	0	0.5 (±0.13)	2.5 (±0.26)	3 (±0.15)	3.05 (±0.35)	3.95 (±0.12)	5.45 (±0.28)
Enterobacter asburiae B20	0	0	0.38 (±0.11)	0.79 (±0.21)	0.84 (±0.11)	0.92 (±0.12)	2.34 (±0.17)
Delftia sp. B21	0	0.5 (±0.08)	0.86 (±0.21)	0.95 (±0.18)	1.82 (±0.27)	2.88 (±0.11)	4.8 (±0.11)
Streptomyces sp. B22	0	1 (±0.14)	1.4 (±0.28)	2 (±0.12)	2.1 (±0.26)	3 (±0.13)	4 (±0.1)
Citrobacter freundii B23	0	1.5 (±0.33)	3.3 (±0.36)	3.305 (±0.21)	2.5 (±0.16)	2.95 (±0.16)	2.83 (±0.13)
Corynebacterium glutamicum B24	0	0.215 (±0.09)	1 (±0.14)	2.25 (±0.07)	3.49 (±0.12)	3.85 (±0.24)	5.25 (±0.42)
Corynebacterium glutamicum B25	0	0.5 (±0.13)	1 (±0.1)	1.81 (±0.2)	2.915 (±0.15)	3.95 (±0.14)	3.945 (±0.35)
Kocuria sp. B26	0	0.5 (±0.11)	1 (±0.17)	1.78 (±0.24)	2.65 (±0.11)	3.465 (±0.1)	2 (±0.15)
Delftia sp. B27	0	1.125 (±0.31)	1.32 (±0.23)	2.27 (±0.24)	3.575 (±0.22)	4.385 (±0.22)	4.78 (±0.14)
Bacillus flexus B28	0	0.34 (±0.07)	1.26 (±0.12)	1.855 (±0.2)	2.42 (±0.1)	3.785 (±0.21)	5.305 (±0.31)
Bacillus simplex B29	0	1.31 (±0.14)	1.64 (±0.11)	3.37 (±0.33)	3.5 (±0.25)	3.935 (±0.15)	6.195 (±0.56)

Standard deviation values are shown in parentheses

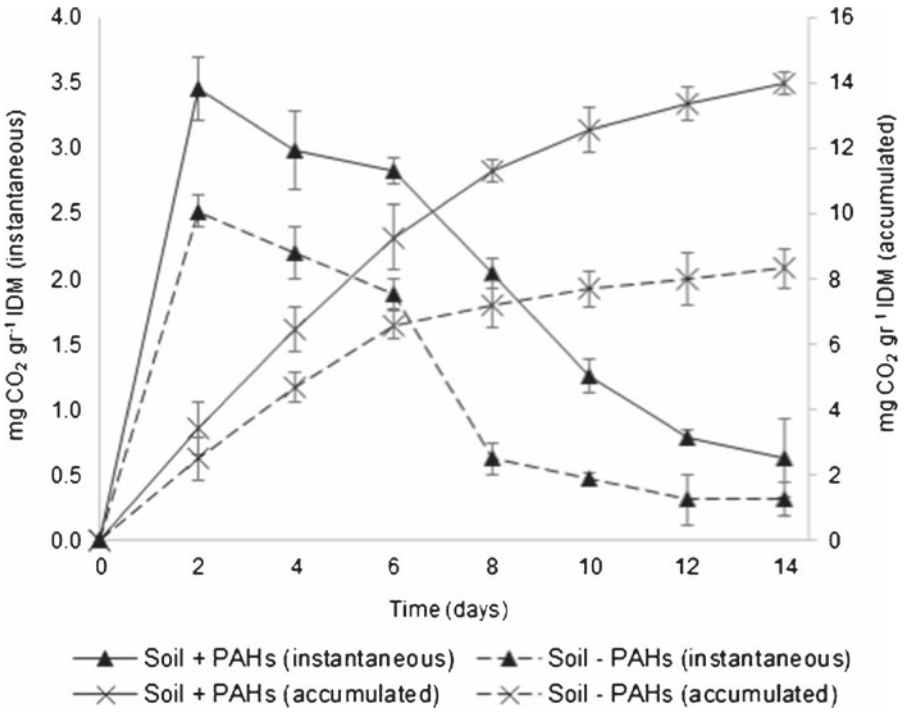


Fig 4 Microbial activity in solid culture by microbial consortium in a soil contaminated with 1,000 mg of PAHs per kilogram of soil. CO_2 production in noninoculated controls was negligible

Hickey 2011), and *Kocuria* sp. (Diaz-Ramirez et al. 2003) as well as the fungal species *A. fumigatus* (Okoro and Amund 2010), *A. niger* (Cortes-Espinosa et al. 2006), *A. flavus* (Romero et al. 2010), *Trichoderma* sp. (Bhandari et al. 2007), *Acremonium* sp. (Hughes and Bridge 2009), and *Scedosporium* sp. (Bhandari et al. 2007). Most of the microorganisms described in this study have already been described as hydrocarbon degraders; however, *T. asperellum*, are not reported for hydrocarbon degradation and also might

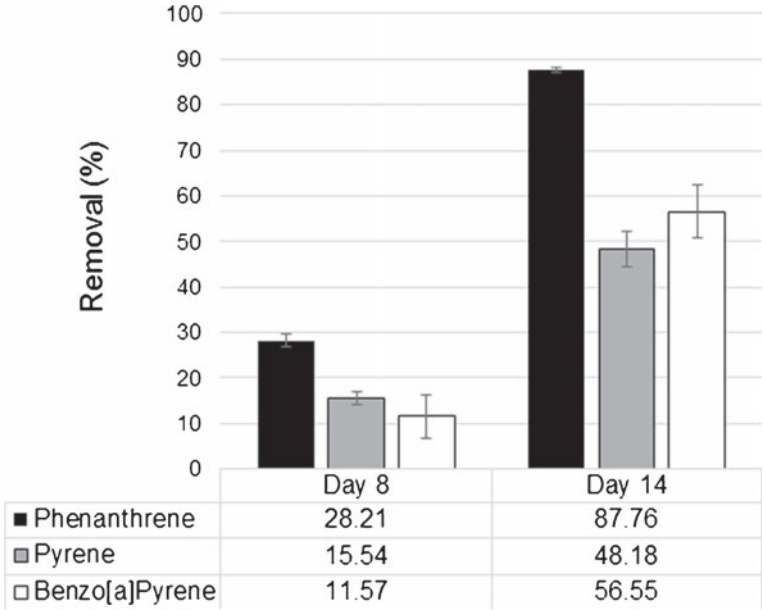


Fig. 5 PAH removal in solid culture by microbial consortium in a soil contaminated with 1,000 mg of PAHs per kilogram of soil

have advantages over other microorganisms used for the bioremediation of soils contaminated with PAHs as discussed below.

The high diversity of isolates able to use and tolerate heavy crude oil could be associated with the elevated degree of contamination of the soil (60,000 mg total petroleum hydrocarbons kg^{-1}), the high levels of organic matter, and the fact that Maya crude has very high levels of aromatic compounds (36 %), asphaltenes (30 %), sulfur (3.4 %), heavy metals (>300 mg kg^{-1}), resins, and other toxic compounds (PEMEX 2002). This increases the chances of isolating microbial populations able to tolerate and degrade high concentrations of these compounds. Previous exposure to aromatic hydrocarbons strongly influences the type and number of hydrocarbon-degrading organisms found in soils, which, in turn, largely determines the ability of a native microbial community to degrade PAHs (Hinga and Batchellor 2005). Previous studies have shown a significantly improved microbial degradation of pollutants by isolates cultivated from samples with high levels of pollution (Yu et al. 2005; Johnsen et al. 2007).

Since hydrocarbon-contaminated soils usually contain complex mixtures of low molecular weight (LMW) and high molecular weight (HMW) PAHs, mixtures of three-, four-, and five-ring PAHs were used in this study to better address the inhibitory effect caused on microbial isolates in a hypothetical hydrocarbon-impacted soil. Results showed that fungal tolerance significantly decreased when exposed to high doses of a PAH mixture, *Aspergillus*, *Rhizomucor*, and *Trichoderma* the being the most tolerant genera. Taking into account previous reports regarding PAH degradation and tolerance in several species of *Aspergillus*, *Penicillium*, and other non-ligninolytic fungi showing significant growth inhibition from 200 to 400 mg of Phe per liter under similar conditions (Cortes-Espinosa et al. 2006), isolates from this study exhibit a remarkably high tolerance to PAHs (up to 6,000 mg l^{-1}) and the ability to utilize them as carbon source.

T. asperellum isolates were, by far, the most tolerant fungal organisms isolated. Extension growth rates showed no differences between the control without PAH and the highest concentration tested (6,000 mg l^{-1}), although sporulation ability was detected only up to 4,000 mg l^{-1} and some visible changes in sporulation patterns and pigmentation were seen. Recently, moderate levels of tolerance for crude oil, Phe, naphthalene (Nap) (>250 mg l^{-1}), and BaP (<100 mg l^{-1}) have been reported for several *Trichoderma* sp. strains (Argumedo-Delira et al. 2012). The high tolerance found in this study and the previous reports showing the ability of several species of *Trichoderma* to remove Phe (Hadibarata et al. 2007), Nap (Argumedo-Delira et al. 2012), pyrene (Saraswathy and Hallberg 2002), and other HMW PAHs even in the presence of heavy metals (Atagana 2009; Verdin et al. 2004), make these PAH-tolerant isolates of particular interest for bioremediation purposes. In fact, this is the first report involving *T. asperellum* as a possible bioremediation agent due to its elevated tolerance to LMW and HMW PAHs and its ability to use them as sole carbon source. Although laccase and peroxidase activities have been described for *Trichoderma* species (Cazares-Garcia et al. 2013; Cristica et al. 2010), it has been suggested that, similar to bacteria, the ability of *Trichoderma* to degrade Phe lies in the release of broad substrate specificity enzymes other than cytochrome P450 oxidases (1,2 and 2,3 dioxygenases). *Trichoderma* dioxygenases are involved in the initial breakdown of aromatic rings, forming unstable *cis*-dihydrodiols that can be subsequently degraded by the catechol pathway (Hadibarata et al. 2007). White-rot fungi such as *Phanerochaete chrysosporium*, *Anthracophyllum discolor*, *Irpex lateus*, *Pleurotus ostreatus*, and *Trametes versicolor* possess laccase and peroxidase enzymes that increase their PAH removal capacity and presumably their tolerance to aromatic compounds (Field et al. 1996). However, only intermediate doses of PAHs (<700 mg l^{-1}) have been tested with white-rot fungi in solid media (Byss et al. 2008; Borras et al. 2010; Acevedo et al. 2011), and even if they could tolerate higher concentrations, they are easily displaced by soil-indigenous populations if used in a bioremediation process. Non-ligninolytic fungi such as those isolated in this study have the advantage of being common soil organisms, avoiding this drawback.

On the other hand and similar to fungal isolates, bacterial tolerance also showed differences when exposed to different doses of PAHs. In general, it was found that bacteria tolerated higher concentrations of the PAH mixture to a lesser extent than fungi. Only three isolates (*P. aeruginosa* B7, *Klebsiella* sp. B10, and *S. maltophilia* B14) showed no growth inhibition at 2,000 mg l^{-1}, all being Gram-negative and belonging to the Gammaproteobacteria class. Previous studies have shown dominance and an improved tolerance of Gammaproteobacteria in bacterial populations present

in soils contaminated with hydrocarbons (Bordenave et al. 2007). *Pseudomonas* genus, as well as *S. maltophilia*, has been found to be highly resistant to organic solvents and possess the ability to degrade a variety of PAHs (Nap, Phe, Pyr) (Lazaroaie 2010; Boonchan et al. 1998). The resistance mechanisms of bacteria to organic solvents and aromatic hydrocarbons include transforming them into nontoxic compounds, rigidity of the cell wall, impermeabilization of the cell surface, presence of constitutive, and inducible efflux pumps related to resistance, nodulation, cell division, changes in protein patterns, and synthesis of specific tolerance proteins, among others (Lazaroaie 2009; Torres et al. 2011; Ramos et al. 2002). These mechanisms are believed to be more effective in Gram-negative bacteria mainly due to the presence of an additional outer membrane, allowing quick modifications in the composition of lipopolysaccharides and fatty acids in Gram-negative organisms (Lazaroaie 2010).

Although the observed bacterial tolerance was lower than that of several fungal isolates, this was not totally unexpected. One feature of PAH-contaminated soils is the presence of numerous LMW and HMW PAH mixtures, BaP being one of the most carcinogenic. Although some bacterial species can solubilize HMW PAHs by producing surfactants and extracellular products, BaP is toxic to the vast majority of degrading bacteria, except for some reported species of *Burkholderia* (A. Juhasz et al. 1997), *Stenothrophomonas* (Boonchan et al. 1998), *Sphingomonas* (Rentz et al. 2008), *Xantamonas*, and *Mycobacterium* (Grosser et al. 1991). As we used a mixture of PAHs including BaP in this work, we could expect a lesser tolerance in bacteria than those observed in fungi since single populations of bacteria barely tolerate and degrade this compound. However, the fact that bacterial isolates tolerant to 2,000 mg of a PAH mixture per liter (approximately 667 mg BaP kg^{-1}) were found opens the possibility of finding organisms with high biotechnological potential for bioremediation of HMW PAHs. Since BaP is hardly utilized by bacteria as sole energy source, they rely on cometabolic degradation to utilize it (Seo et al. 2009).

The use of a mixed consortium composed of highly PAH-tolerant degrading isolates was showed to be suitable for removing considerable amounts of LMW and HMW PAHs in a contaminated soil. The final concentration of PAHs in contaminated soil (1,000 mg kg^{-1} soil) did not appear to produce an inhibitory effect over the growth of the consortium, since instantaneous CO_2 production reached its maximum at 2 days and compared to the soil without PAHs, CO_2 evolution remained higher along every day of the incubation period. Moreover, antagonism tests between all selected isolates did not show considerable inhibitory effects between them (data not shown).

Phe degradation by the consortium was relatively low by day 8 (28.21 %), but by day 14 increased importantly (87.76 %). A similar situation was observed for HMW PAHs, although little Pyr and BaP biodegradation took place in the first week of growth (15.54 and 11.57 %), an evident increase in the rate of biodegradation was observed during week 2 (48.18 and 56.55 %). A possible explanation for this is that BaP can barely be used as a carbon and energy source, so an alternative substrate must be present to initiate microbial growth and to induce the expression of catabolic enzymes. Since some PAHs are poor inducers of their own degradative enzymes (Bouchez et al. 1995), this substrate could, in fact, be a LMW PAH (A. L. Juhasz and Naidu 2000) as well as sugarcane bagasse. Therefore, an increased cometabolic degradation of HMW PAHs in a mixture with LMW PAHs can be achieved in response to the presence and the degradation pathways of LMW PAHs, as demonstrated previously with a five PAH mixture (A. L. Juhasz and Naidu 2000; Yuan et al. 2003). Our results are consistent with previous reports showing an enhanced degradation of HMW PAHs and LMW PAHs by fungi–bacteria cocultures in soil (Chavez-Gomez et al. 2003), as well as elevated HMW PAH mineralization using co-cultured *Penicillium janthinellum* and *Stenothrophomonas malthophilia* (Boonchan et al. 2000).

It is noteworthy that PAH soil concentrations used in this study were higher than those used in many of previous works evaluating fungi–bacteria, fungi–fungi, and bacteria–bacteria consortia, where the maximum average values of individual PAHs are approximately 100–200 mg kg (Chavez-Gomez et al. 2003; Kim and Lee 2007; Li et al. 2008; Wu et al. 2013). Furthermore, PAH biodegradation occurred faster and reached higher values when compared with other mixed consortia (Kim and Lee 2007; Li et al. 2009). In general, CO_2 production through the biodegradation process in soil was lower when compared with reported values of similar mixed consortia in microcosm, since maximum instantaneous and accumulated production only reached 3.45 mg $CO_2 g^{-1}$ IDM for day 2 and 14 mg $CO_2 g^{-1}$

IDM by day 14, respectively (Chavez-Gomez et al. 2003; Jacques et al. 2008). A lower growth rate along with a high PAH biodegradation rate could indicate that microorganisms rapidly reach stationary phase and, thus, secondary metabolism, producing enzymes (e.g., laccases and peroxidases) that could be positively influencing the oxidation of PAHs (Conesa et al. 2002; Gochev and Krastanov 2007). This also agrees with the fact that most of the degradation occurred after the first week of treatment (Fig. 5), just when CO_2 production decreased (Fig. 4).

Taking into account that the adaptation and competition of introduced microorganisms with the native flora of the soil can be a limiting factor in the strategies of bioaugmentation (Cheung and Kinkle 2001), the origin of the consortium, obtained from soils extensively polluted with heavy crude oil, and its ability to grow with PAHs as sole carbon source confer a great adaptive advantage greatly favoring its permanence in contaminated soils. The use of sugar cane bagasse positively influences the degradation process of PAHs, serving as fungal growth support, soil texturizing agent, and alternative carbon source; increasing the number of usable substrates; stimulating growth; and promoting microbial cometabolism. (Fernandez-Luqueno et al. 2011). Although microbial tolerance to hydrocarbons does not appear to be directly related to their degradation capability (Montgomery et al. 2010), the use of highly tolerant isolates could improve the ability of mixed microbial consortia (bacteria–bacteria, fungi–bacteria) to degrade LMW and HMW PAHs by cometabolism through the synergistic effect of fungal and bacterial metabolic activities. Furthermore, in the presence of mixed consortia, the adaptation of microorganisms and degradation of PAHs in soils has been shown to improve (Y. M. Kim et al. 2009; Chavez-Gomez et al. 2003).

5 Conclusion

To our knowledge, this represents the first study in which the bacterial and fungal tolerance to extreme concentrations of LMW and HMW PAHs is evaluated, resulting in a highly tolerant degrading microbial consortium. In this study, microorganisms exhibiting remarkably high tolerance levels to LMW and HMW PAHs and a consortium derived from them were isolated and identified. The main advantages of this mixed consortium are (1) the highly polluted site where strains come from and (2) its high tolerance to LMW and HMW PAHs, both of them facilitating their adaptation and competition in polluted soils and improving the rate of removal/degradation of PAHs by cometabolism. This highlights the potential use of the identified microorganisms for the bioremediation of soils extensively impacted by petroleum hydrocarbons. Further studies testing the degradation capabilities of the individual isolates, intermediary production, and permanence of the consortium in soil are necessary in order to better understand the real biotechnological potential of these tolerant organisms in real contaminated soils.

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CHAPTER 3

DEGRADATION OF POLYCYCLIC AROMATIC HYDROCARBONS IN SOIL BY A TOLERANT STRAIN OF *TRICHODERMA ASPERELLUM*

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RESEARCH ARTICLE

Degradation of polycyclic aromatic hydrocarbons in soil by a tolerant strain of *Trichoderma asperellum*

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Abstract *Trichoderma asperellum* H15, a previously isolated strain characterized by its high tolerance to low (LMW) and high molecular weight (HMW) PAHs, was tested for its ability to degrade 3–5 ring PAHs (phenanthrene, pyrene, and benzo[a]pyrene) in soil microcosms along with a biostimulation treatment with sugarcane bagasse. *T. asperellum* H15 rapidly adapted to PAH-contaminated soils, producing more CO_2 than uncontaminated microcosms and achieving up to 78 % of phenanthrene degradation in soils contaminated with 1,000 mg Kg^{-1} after 14 days. In soils contaminated with 1,000 mg Kg^{-1} of a three-PAH mixture, strain H15 was shown to degrade 74 % phenanthrene, 63 % pyrene, and 81 % of benzo[a]pyrene. Fungal catechol 1,2 dioxygenase, laccase, and peroxidase enzyme activities were found to be involved in the degradation of PAHs by *T. asperellum*. The results demonstrated the potential of *T. asperellum* H15 to be used in a bioremediation process. This is the first report describing the involvement of *T. asperellum* in LMW and HMW-PAH degradation in soils. These findings, along with the ability to remove large amounts of PAHs in soil found in the present work provide enough evidence to consider *T. asperellum* as a promising and efficient PAH-degrading microorganism.

Keywords *Trichoderma asperellum* · Polycyclic aromatic hydrocarbons (PAHs) · Soil bioremediation · Laccase · Peroxidase · Dioxygenase

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Introduction

Degradation of polycyclic aromatic hydrocarbons (PAHs) in soils has become an environmental priority, mainly because of their elevated persistence and potential harmful effects on human and animal health. PAHs are recalcitrant organic compounds with potential cytotoxic, carcinogenic, genotoxic, and mutagenic effects, characterized by a high hydrophobicity and low aqueous solubility (US-EPA 2008). Low molecular weight (LMW), high molecular weight (HMW) PAHs as well as their toxic intermediary products can be absorbed and accumulated in diverse organisms. Microbial degradation is thought to be the main natural method of degradation of PAHs in soils and biochemical degradation pathways are well documented; several fungal, bacterial, and algal species have been reported as PAH-degrading organisms (Cerniglia and Sutherland 2010; Seo et al. 2009; Todd et al. 2002), making bioremediation an effective and promising technology to remove pollutants from soils.

Fungi belonging to *Trichoderma* genus are worldwide ubiquitous organisms commonly found in soils, known to possess a versatile and powerful enzymatic machinery (e.g. cellulases, hemicellulases, chitinases, proteases, glucanases) useful for the degradation of a wide range of substrates in soils, but specially, cellulosic material (Jaklitsch 2009). *Trichoderma* is one of the biological control agents more commonly used against plant pathogens mainly due to its production of hydrolytic enzymes and secondary metabolites, besides interacting through antibiosis, competing for space and resources, and improving growth and resistance to biotic and abiotic stress (Chernin and Chet 2002). Within the *Trichoderma* genus, *T. asperellum* stands out as a species with a wide range of substrate utilization, high production of antimicrobial compounds and an ability for environmental opportunism through saprotrophic, biotrophic, and mycoparasitic interactions (Chutrakul et al. 2008; Ding et al. 2012;

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Druzhinina et al. 2011). *Trichoderma asperellum* is used as a biological control agent against a wide range of plant pathogens including *Colletotrichum gloeosporioides*, *Phytophthora megakarya*, other pathogenic fungi, and nematodes (de los Santos-Villalobos S et al. 2013; Sharon et al. 2007; Slusarski and Pietr 2009; Tondje et al. 2007).

Although *Trichoderma* species are commonly used for the commercial production of lytic enzymes and as biological control agents, their use in pollutant bioremediation is limited. Several studies have shown the ability of *Trichoderma* to biotransform heavy metals (Atagana 2009; Su et al. 2011) and hydrocarbons (Matsubara et al. 2006). In fact, it is known that several species of the genus *Trichoderma* possess the ability to degrade and metabolize PAHs such as naphthalene, phenanthrene, pyrene, and benzo[a]pyrene, even in the presence of heavy metals (Atagana 2009; Verdin et al. 2004). The species reported as metabolizers include *T. hamatum*, *T. harzianum*, *T. koningii*, *T. viride* and *T. virens* (Argumedo-Delira et al. 2012; Cerniglia and Sutherland 2010). However, there are no reports involving *T. asperellum* as a hydrocarbon or PAH-degrading organism. The use of *T. asperellum* as bioremediation agent on PAH-polluted soils may present additional advantages over the use of other soil microorganisms, such as its high growth rate, wide range of substrates, growth-promoting effects on plants, and the production of oxidizing hydrolytic enzymes including laccases, peroxidises, and dioxygenases (Cazares-Garcia et al. 2013; Hadibarata et al. 2007). Thus, the aim of this work was to evaluate the degradation capability of a strain of *T. asperellum* tolerant to LMW and HMW-PAHs in solid culture, for the bioremediation of PAH-polluted soils.

Material and methods

Fungal strain and inoculum preparation

T. asperellum H15 is a strain previously isolated from a heavy crude oil-contaminated soil, showing increased tolerance levels to 3, 4, and 5-ring PAHs and the ability to use them as sole carbon source (Zafra et al. 2014). This strain has been deposited in the Agricultural Research Service (ARS) patent culture collection with registration number NRRL50869. *T. asperellum* H15 was maintained on potato dextrose agar (PDA) plates at 30 °C. Production of spores was carried out in 250-mL flasks containing 30 mL of PDA, inoculated with strain H15 and incubated at 30 °C. Spores were collected on day 4 with the addition of 20 mL of 0.1 % Tween 80 solution, sterile glass beads and gently shaking the flasks for 2 min. The spore suspension concentration was quantified in a Neubauer haematocytometer chamber using a optical microscope.

Degradation of PAHs by T. asperellum H15 in solid culture

Degradation ability of analytical grade phenanthrene (Phe) and a mixture of Phe, pyrene (Pyr), and benzo[a]pyrene (BaP) by *T. asperellum* H15 was evaluated in microcosm solid culture systems using sugarcane bagasse (34.34 % carbon, 0.18 % nitrogen, 0.00343 % phosphorus) as fungal growth support, texturizing agent and alternative carbon source. Sterile sugarcane bagasse (0.35 g dry weight) was placed in 50-mL glass flasks with Czapeck medium (g L^{-1}: sucrose, 30; sodium nitrate, 3; dipotassium phosphate, 1; magnesium sulfate, 0.5; potassium chloride, 0.5; ferrous sulphate, 0.01; pH 7.3) to reach 30 % moisture content, inoculated with a concentration of 2×10^7 spores of strain H15 per gram of contaminated soil and incubated for 5 days at 30 °C. The inoculated sugarcane bagasse was then mixed with 6.65 g of sterile soil (sandy loam with 2.4 % organic matter, 1.4 % total organic carbon, 0.063 % nitrogen, 0.0023 % phosphorus and pH of 8.41) spiked with 1,000 mg Kg^{-1} of Phe or 1,000 mg Kg^{-1} of a mixture of Phe, Pyr, and BaP (1:1:1 ratio). Soil/sugarcane bagasse mixture was incubated at 30 °C for 14 (Phe-contaminated soil) or 18 days (PAH mixture-contaminated soil). Control samples were prepared by inoculating a non-contaminated soil under the same culture conditions. Abiotic controls, consisting of sterile non-inoculated microcosms treated under the same conditions as those of *Trichoderma*-inoculated systems, were included to confirm that the disappearance of PAHs was caused by biodegradation and not by abiotic factors such as absorption or volatilization. Assays were carried out in triplicate.

Heterotrophic activity measurements

Headspace in each of the microcosms flasks was flushed every 48 h for 10 min with sterile and moistened air, to preserve aerobic conditions and avoid carbon dioxide accumulation. CO_2 evolution in the microcosms was measured every 48 h using an Agilent 6890 Series Gas Chromatograph equipped with a thermal conductivity detector and a GS-CarbonPLOT column. Instantaneous and accumulated CO_2 was reported as milligrams of CO_2 per gram of initial dry matter (IDM).

Enzymatic assays

Activity of *T. asperellum* H15 extracellular laccase and peroxidase enzymes was screened in agar plates by means of the oxidation of chromogenic dyes ABTS (Saparrat et al. 2000), *o*-anisidine (OA) (Conesa et al. 2000) and azure B (AB) (Archibald 1992), respectively, in the presence and absence of 1,000 mg L^{-1} of a mixture of Phe, Pyr, and BaP. Laccase screening was performed in minimal medium plates (g L^{-1}: glucose, 2; $(NH_4)_2\ C_4H_4O_6$, 1; KH_2PO_4, 0.26; $NaHPO_4$, 0.26; $MgSO_4\cdot7H_2O$, 0.5; $CuSO_4\cdot5H_2O$, 0.5; $CaCl_2\cdot5H_2O$, 0.01;

$FeSO_4$, 0.005; $ZnSO_4$ $7H_2O$, 0.005; $NaMoO_4$ $7H_2O$, 0.0002; $MnCl_2 \cdot H_2O$, 0.00009; H_3BO_3, 0.0007; malt extract, 2; ABTS, 0.2; agar, 16. pH 5.5); OA-oxidizing peroxidase activity was evaluated in plates with modified Kirk medium (g L^{-1}: glucose, 10; KH_2PO4, 2; $MgSO_4 \cdot 7H_2O$, 0.5; $CaCl_2$, 0.1; 2,2-dimethylsuccinate, 2,2; $(NH_4)_2$ $C_4H_4O_6$, 0.5; yeast extract, 0.2; *o*-anisidine, 0.3; agar, 16. pH 5.0), and AB-oxidizing peroxidase activity was evaluated in plates with 20 ml of Czapeck medium supplemented with azure B (0.0066 g L^{-1}). Plates were inoculated with PDA discs (5 mm diameter), containing 3-day-old active mycelia. Plates were incubated at 30 °C for 10 days. Determination of specific enzymatic activities was carried out in liquid culture. Glass flasks with 50 mL of minimal medium contaminated with a mixture of Phe (25 mg L^{-1}) and Pyr (25 mg L^{-1}) were inoculated with 1×10^6 spores mL^{-1} of strain H15 and incubated at 30 °C for 10 days. Enzymatic activities were assessed every 48 h from culture supernatants. Laccase extracellular activity was determined spectrophotometrically by the oxidation of ABTS (Nagai et al. 2002). Cationic radical formation was detected by measuring the increase in absorbance at 420 nm (e420=36,000 M^{-1} cm^{-1}). Catechol 1,2 dioxygenase extracellular activity was determined spectrophotometrically by the formation of *cis*, *cis*-muconic acid at 260 nm (e260= 16,800 M^{-1} cm^{-1}) (Wojcieszynska et al. 2011). Catechol 2,3 dioxygenase extracellular activity was determined by the formation of 2-hydroxymuconic semialdehyde at 375 nm (e375=36,000 M^{-1} cm^{-1}) (Wojcieszynska et al. 2011). Phenol red (PSP)-oxidizing peroxidase activity was determined spectrophotometrically at 37 °C by the oxidation of phenol red at 610 nm (Kuwahara et al. 1984). Veratryl alcohol (VA)-oxidizing peroxidase activity was determined spectrophotometrically at 37 °C by the oxidation of veratryl alcohol to verytraldehyde at 310 nm (Tien and Kirk 1988). One unit of enzyme activity (U/l) was defined as the amount of enzyme required to generate 1 μmol of each reaction product in 1 min. Protein concentrations of the culture supernatants were determined by the bicinchoninic acid method (BCA) using bovine serum albumin as standard (Smith et al. 1985).

PAH measurements

Residual PAHs were extracted from 1 g of initial dry matter (for solid culture) with the addition of 25 mL of a dichloromethane-acetone solution (7:3 ratio) using a Multiwave 3000 SOLV apparatus (Anton Paar) for 20 min, according to EPA method 3546. The resulting extracts were evaporated, suspended in 2 mL of acetonitrile and analyzed in an HP Agilent 1100 HPLC system equipped with a C18 reverse-phase column, with an UV absorbance detector set at 245–360 nm under an isocratic ambient in acetonitrile:water (90:10) and a flow rate of 1 ml min^{-1}. For liquid culture, residual PAHs were extracted from mycelium and liquid medium; first the mycelium was filtered from 50 mL medium through cellulose filter paper with medium retention (Whatman grade 1) and resuspended in 10 mL acetone, then, it was sonicated for 10 min, and the organic phase was recovered by filtration with the same type of filter paper. Residual PAHs were extracted from the filtered liquid medium by stirring with 50 mL of ethyl acetate for 30 min, then, this organic phase was mixed with acetone fraction obtained from the mycelium, the mixture were evaporated, resuspended in acetonitrile, and quantified by HPLC as described above.

Statistical analysis

Data were analyzed by analysis of variance (ANOVA) followed by a multiple comparison test (LSD) with SPSS Statistics Software version 19 (IBM), considering statistically significant differences those with a p value <0.05.

Results

Heterotrophic activity of strain H15 in soil microcosms

Figures 1 and 2 show the growth of *T. asperellum* H15 during the PAH biodegradation process in microcosms in the presence of Phe and a mixture of Phe, Pyr and BaP, respectively. CO_2 production by strain H15 in uncontaminated microcosms increased gradually throughout the process, reaching the highest value on day 2 (6.914 mg CO_2 g^{-1} IDM) and a marked and constant decrease from day 4 to 6, with a lower but relatively constant production of CO_2 from day 8 to the end of the process. The presence of a single hydrocarbon (Phe) in soil initially delayed the growth of strain H15 but after day 4, Phe-contaminated microcosms produced CO_2 levels higher than those in uncontaminated microcosms (Fig. 1) with an accumulated CO_2 production of 27.615 versus 22.063 mg CO_2 g^{-1} IDM, respectively. On the other hand and as observed with Phe, the presence of a three-PAH mixture in soil also delayed the initial growth of strain H15, although from day 4, it produced accumulated CO_2 levels higher than those obtained in uncontaminated microcosms (Fig. 2). CO_2 production differences between PAH-contaminated and PAH-uncontaminated microcosms were not significant.

PAH biodegradation in solid culture

The biodegradation efficiency of Phe, Pyr, and BaP by *T. asperellum* H15 in solid culture after 8, 14, and 18 days of incubation is shown in Fig. 3. A high degradation was obtained when Phe was added individually to microcosms, reaching a degradation efficiency of 78.3 % after 14 days of incubation. When a mixture of thee PAHs was added, the degradation efficiency of Phe by strain H15 was relatively

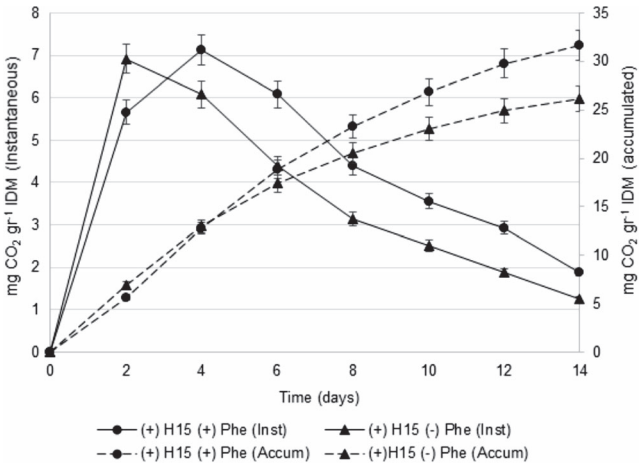


Fig. 1 Microbial activity of *T. asperellum* H15 in microcosms with soil contaminated with 1,000 mg Kg^{-1} of phenanthrene

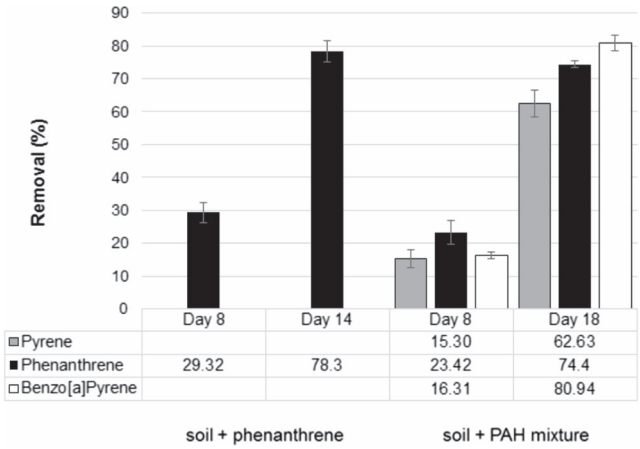


Fig. 3 PAH removal by *T. asperellum* H15 in microcosms with sterile soil contaminated with 1,000 mg Kg^{-1} of Phe or 1,000 mg Kg^{-1} of a mixture of PAHs

low for day 8 (23.4 %), but for day 18 had increased substantially (74.4 %). The same situation was observed for Pyr and BaP; even though there was a lower degradation of Pyr and BaP during the first week of growth (15.3 and 16.31 %, respectively), there was a marked increase by day 18 (62.63 and 80.94 %, respectively). The abiotic losses of PAHs during the biodegradation experiments were similar and negligible.

Enzymatic assays and PAH removal in liquid culture

Plate-based screening of laccase and peroxidase activity by strain H15 is shown in Fig. 4. Extracellular laccase activity was observed from day 2 of incubation, when there was an evident increase in ABTS oxidation in plates containing 1,000 mg L^{-1} of the PAH mixture compared with those without PAHs (Fig. 4a). By day 10, plates with and without PAHs showed a complete oxidation of ABTS. Peroxidase activity, assessed by the oxidation of OA and AB, was detected only from day 7. Strain H15 was able to oxidize OA and AB in plates with no substantially appreciable differences regarding the presence or absence of PAHs in medium, although AB showed a lower intensity of the halo coloration in comparison with OA plates (Fig. 4b, c). By day 10 of incubation, the oxidation of both dyes was clear, but the overall oxidation rate was slower, and the effect of the presence of PAHs was less evident than ABTS plates.

Figures 5 and 6 show the specific activities of five PAH-oxidizing enzymes, as well as Phe and Pyr degradation in liquid medium. The presence of PAHs in the liquid cultures led to an increase in the specific activity of laccase from day 4 to 8 in comparison to controls without PAHs, maintaining relatively constant values from day 4 to 8. VA-oxidizing peroxidase activity was also higher by day 6 in comparison to controls without PAHs, reaching its highest value (23.94 U mg^{-1}), although by day 8, enzyme activity decreased (20.4 U mg^{-1}) and controls without PAHs reached their maximum (25.29 U

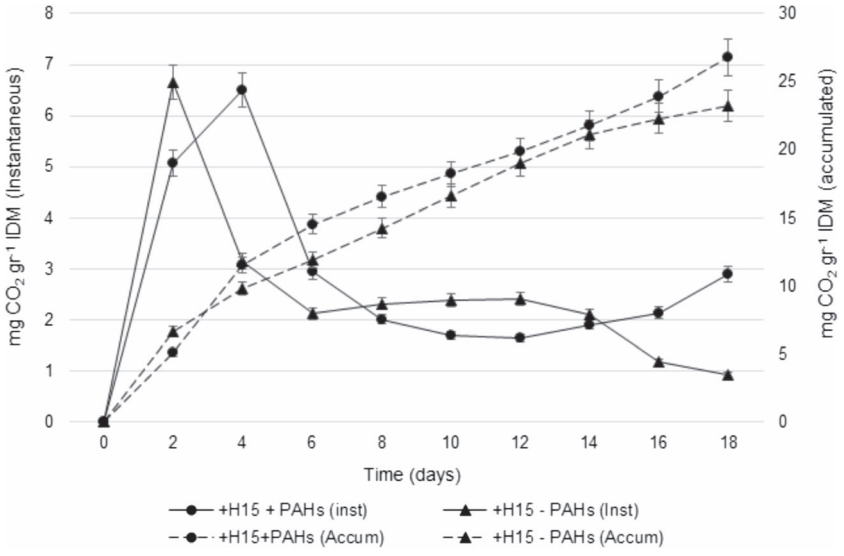


Fig. 2 Microbial activity of *T. asperellum* H15 in microcosms with soil contaminated with 1,000 mg Kg^{-1} of a mixture of three PAHs

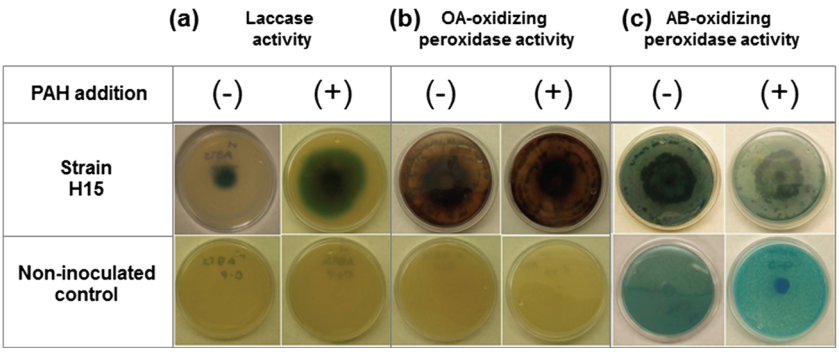


Fig. 4 Plate-based enzymatic screening of *T. asperellum* H15. **a** ABTS oxidation after 2 days of growth in minimal medium; **b** *o*-anisidine oxidation after 6 days of growth in modified Kirk medium; **c** azure B decoloration after 10 days of growth in Czapeck medium

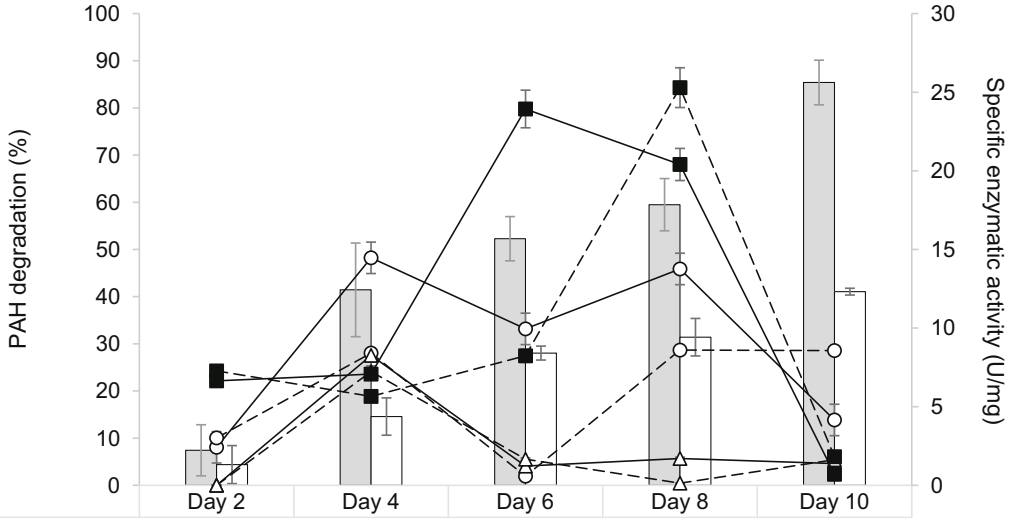
mg^{-1}). In contrast, PSP-oxidizing peroxidase activity was identical in cultures with and without PAHs, showing low or basal activity except at day 4 (Fig. 5). The presence of PAHs in the medium also produced an evident initial increase in the specific activity of catechol 1,2 dioxygenase (from day 2 to 6) and catechol 2,3 dioxygenase (from day 2 to 6) in comparison with controls without PAHs, although the enzyme activity in the latter was higher in medium without PAHs from day 6 (Fig. 6). Maximum degradation values of PAHs were 85.39 % for Phe and 41.06 % for Pyr at day 10. Phe degradation was significantly higher than Pyr degradation from day 2 until the last day of evaluation.

Discussion

In this study, we investigated the ability of *T. asperellum* H15, a strain previously isolated from a heavy crude oil-contaminated soil, to degrade several LMW and HMW PAHs in soil as well as to produce several PAH-oxidizing enzymes in the presence of PAHs associated with the degradation in liquid culture. Few data on PAH degradation by *Trichoderma* species are available; however, studies have shown that several *Trichoderma* species are capable of tolerating and metabolizing hydrocarbons including LMW and HMW PAHs (Argumedo-Delira et al. 2012; Atagana 2009; Hadibarata et al. 2007; Ravelet et al. 2000; Saraswathy and Hallberg 2002; Verdin et al. 2004). Previous work in our group showed remarkably high tolerance levels of *T. asperellum* H15 (up to 6,000 mg Kg^{-1}) toward the same LMW and HMW PAHs tested in this work, as well as the ability to use them as sole carbon source (Zafra et al. 2014).

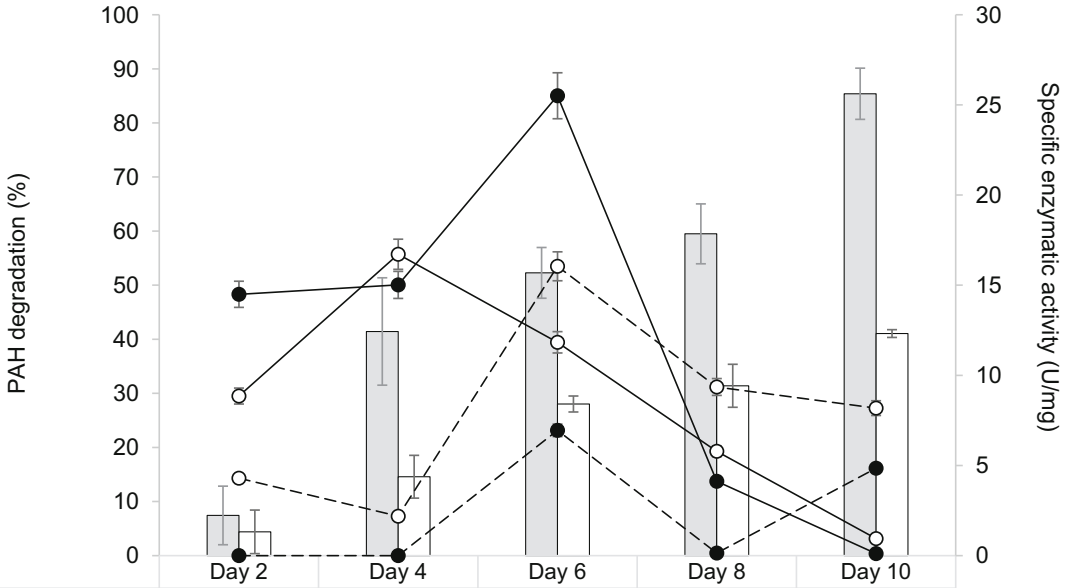
Many fungal species possess the ability to degrade PAHs and the potential to remediate polluted soils. However, one limiting factor in the success of these organisms is the inability to adapt and grow properly on extensively contaminated soils (Tabak et al. 2003). Solid-state fermentation in microcosms showed that although the presence of PAHs initially delayed the growth of strain H15 in soil, CO_2 production remained higher in contaminated microcosms from day 4 when compared to uncontaminated microcosms. This suggests a rapid and successful adaption of strain H15 in PAH-polluted soils, as well as an ability to use the sugarcane bagasse and PAHs for growth. Although CO_2 production does not correlate with PAH degradation levels, strain H15 produced remarkably higher amounts of CO_2, at least twice, in comparison with other reported native and transformant PAH-degrading fungi when growing in microcosm at comparable PAH concentrations (Cortes-Espinosa et al. 2006, 2011; Reyes-Cesar et al. 2014). Unlike some reports indicating low tolerance levels to PAHs in several *Trichoderma* members such as *T. harzianum*, *T. viride* or *Trichoderma* sp. strains (Argumedo-Delira et al. 2012; Matsubara et al. 2006; Verdin et al. 2004), the elevated tolerance levels showed by *T. asperellum* H15 could facilitate its adaptation to polluted soils and thus improve the rate of removal/degradation of PAHs.

Biodegradation assays showed that soils contaminated with *T. asperellum* H15 led to a rapid degradation of considerable amounts of Phe, Pyr, and Bap. Although low Phe biodegradation took place by day 8 (29.32 % for Phe-contaminated soil and 23.42 % for PAH mixture-contaminated soils), by the end of the process, it had increased importantly (78.3 and 74.4 %, respectively). The same situation was observed in microcosms contaminated with a mixture of three PAHs, where scarce Pyr and BaP degradation was observed during the first week of growth (15.3 and 16.31 %) but by the end of the second week also increased substantially (62.63 and 80.94 %). This notorious shift in the PAH degradation rate could be associated with the induction of enzymes involved in the degradation of PAHs at different stages. HMW PAHs, particularly BaP, are barely metabolized substrates or used as a sole carbon and energy source and are poor inducers of their own degradative enzymes (Bouchez et al. 1995). The presence of alternative substrates, including LMW-PAHs, favors microbial growth and induces the expression of PAH catabolic enzymes (Juhasz and Naidu 2000). Our results are consistent with previous reports showing an increased cometabolic degradation of HMW PAHs in response to the presence of LMW PAHs and their corresponding degradative pathways (Juhasz and Naidu 2000; Yuan et al. 2003). PAH degradation by strain H15, particularly of HMW PAHs, showed to be higher and faster than with other reported non-ligninolytic and ligninolytic degrading fungi. For example, *Irpex lacteus*, *Coprinus cinereus*, and *Pleurotus ostreatus* degraded 65–80 % Phe and 30–65 % Pyr out of 400 mg Kg^{-1} after 28 days of incubation (Matsubara et al.



	Day 2	Day 4	Day 6	Day 8	Day 10
Phenanthrene degradation (%)	7.42	41.44	52.28	59.49	85.39
Pyrene degradation (%)	4.39	14.58	28.03	31.40	41.06
Laccase (+) PAHs (U/mg)	2.42	14.47	9.95	13.76	4.16
Laccase (-) PAHs (U/mg)	3.01	8.41	0.58	8.60	8.57
PSP-oxidizing perox (+) PAHs (U/mg)	0.00	8.26	1.22	1.70	1.39
PSP-oxidizing perox (-) PAHs (U/mg)	0.00	7.23	1.66	0.14	1.63
VA-oxidizing perox (+) PAHs (U/mg)	6.64	7.07	23.93	20.40	0.70
VA-oxidizing perox (-) PAHs (U/mg)	7.27	5.65	8.24	25.29	1.82

Fig. 5 Laccase and peroxidase specific activity of *T. asperellum* H15 in liquid culture with 100 mg L^{-1} of a mixture of phenanthrene and pyrene



	Day 2	Day 4	Day 6	Day 8	Day 10
Phenanthrene degradation (%)	7.42	41.44	52.28	59.49	85.39
Pyrene degradation (%)	4.39	14.58	28.03	31.40	41.06
1,2 dioxygenase (+) PAHs (U/mg)	14.49	15.02	25.51	4.12	0.11
1,2 dioxygenase (-) PAHs (U/mg)	0.00	0.00	6.95	0.14	4.85
2,3 dioxygenase (+) PAHs (U/mg)	8.85	16.71	11.83	5.79	0.94
2,3 dioxygenase (-) PAHs (U/mg)	4.29	2.18	16.05	9.35	8.18

Fig. 6 Catechol dioxygenase specific enzymatic activity of *T. asperellum* H15 in liquid culture with 100 mg L^{-1} of a mixture of phenanthrene and pyrene

2006), while *Anthracophyllum discolor* degraded 75 % out of 45 mg Kg^{-1} BaP in soil after 60 days of incubation (Acevedo et al. 2011). The same was observed with strains of *Fusarium* sp., *Monilinia* sp., *Aspergillus terreus* and *Talaromyces spectabilis* strains in soil microcosms and solid culture when using similar LMW and HMW-PAH mixtures, even in the presence of concentrations below 100 mg Kg^{-1} of individual PAHs (Reyes-Cesar et al. 2014; Thion et al. 2013; Wu et al. 2008;) and regarding the degradation of 10 mg Kg^{-1} of Pyr in liquid culture by *Trichoderma harzianum* (Ravelet et al. 2000). This depicts the high potential of strain H15 to remediate PAH in contaminated soils.

The metabolism of PAHs by fungi has been extensively studied, especially in basidiomycetes. Most fungi metabolize PAHs with enzymes that include LiP, MnP, laccases, cytochrome P450 monooxygenases, and epoxide hydrolases (Cerniglia and Sutherland 2010). In contrast, aromatic-ring-hydroxylating dioxygenases are more commonly found in bacteria and greatly contribute to the initial ring cleavage of aromatic compounds, including LMW PAHs (Hadibarata and Tachibana 2010). Probable mechanisms for PAH degradation in *T. asperellum* could include the production of laccases (Cazares-Garcia et al. 2013), peroxidases (Cristica et al. 2010), and dioxygenases (Hadibarata et al. 2007) among others. We observed that the presence of PAHs in liquid cultures led to a significant increase in the activity of catechol 1,2 and 2,3 dioxygenases during the initial 4 days of incubation, reaching a maximum production at day 6 and a subsequent decrease from day 8. This could indicate an involvement of these enzymes in the early stages of degradation, which play a key role in the initial oxidation of aromatic compounds in other microorganisms (Seo et al. 2009). Hadibarata et al. (2007) reported a direct implication of catechol 1,2 and 2,3 dioxygenases in the degradation of Phe by *Trichoderma* sp. 109, a strain similar to *T. asperellum* H15 that possesses the ability to grow with PAHs as sole carbon source. 1-Hydroxy-2-naphthoic acid, salicylic acid, and catechol were identified as major intermediaries, indicating a Phe degradation pathway in *Trichoderma* via dioxygenation at positions 3 and 4 and subsequent meta-cleavage, leading to PAH mineralization. A high production of extracellular catechol 1,2 and 2,3 dioxygenases associated with PAH metabolism have been also observed in the degradation of chrysene by *Fusarium* sp. (Hidayat et al. 2012), as well naphthalene and BaP by ligninolytic fungi *Armillaria* sp. and *Polyporus* sp. (Hadibarata et al. 2012; Hadibarata and Kristanti 2012). In contrast, laccase activity was nearly undetectable during the initial 2 days of degradation but increased notoriously from day 4, with a higher production in response to the presence of PAHs. It is likely that *T. asperellum* laccases are involved in the oxidation of aromatic rings and play a crucial role in the degradation of PAHs by *T. asperellum*, as they are strongly linked to aromatic hydrocarbon degradation in other fungi (Baldrian 2006; Haritash and Kaushik 2009). Three different *T. asperellum* laccase genes have been identified *in silico* (Cazares-Garcia et al. 2013), two of them being extracellular enzymes with probable PAH-oxidizing capacity. On the other hand, there is no evidence of classic fungal peroxidases such as MnP and LiP in *T. asperellum* genome, nor in the secretome of this fungus grown in sugarcane bagasse (Marx et al. 2013). However, we found evidence suggesting that strain H15 produced peroxidases with OA/PSP-oxidizing activity (related with MnP activity) and AB/VA-oxidizing activity (related with LiP activity) in solid and liquid culture. Increased activity of VA-oxidizing peroxidase activity was observed in treatments with soil contaminated with PAHs; this result suggests that this enzyme is involved in the degradation of PAHs; in contrast, PSP-oxidizing peroxidase activity, although detectable during the first days, did not appear to be involved in the degradation of PAHs by *T. asperellum*. Identified *T. asperellum* peroxidases include cytochrome C peroxidases, catalase peroxidases, glutathione peroxidise, and dye decolorizing (DyP-type) peroxidases (Fawal 2014). Although peroxidase activity in *T. asperellum* has been described mainly as a response against oxidative stress (Fawal et al. 2013), VA-oxidizing peroxidase activity detected in strain H15 could in fact be related to DyP-type peroxidase (TaspDyPrx01, PeroxiBase ID 12842). DyP peroxidases possess a broad substrate specificity and have been previously described as LiP in *Actinobacteria*, having a significant role in bacterial lignin degradation (Ahmad et al. 2011) as well as a role in fungal degradation of lignin by basidiomycetes (Liers et al. 2011).

Sugarcane bagasse, in addition to serving as soil texturizer, could have also contributed with carbohydrates that could have been utilized by *T. asperellum* as carbon source. In fact, *T. asperellum* produces a potent lignocellulolytic cocktail when grown on sugarcane bagasse (Marx et al. 2013), favoring the use of alternative carbon sources (including PAHs). This is particularly relevant in soil, where a complex mixture of substrates can be found and greatly favors the use of biostimulation in conjunction with *T. asperellum* for the bioremediation of soils.

The results of this study indicate that *T. asperellum* H15 possesses the ability to degrade high amounts of LMW and HMW PAHs from contaminated soils and has a great potential for use in soil remediation processes. Based on our findings, we suggest that PAH degradation mechanisms in *T. asperellum* H15 could be mediated by dioxygenase enzymes, which could contribute to the initial degradation of LMW-PAHs (Phe) and subsequently, laccase, peroxidise, and dioxygenase enzymes continue the degradation process of the remaining LMW and HMW-PAHs.

Conclusions

Our results show that *T. asperellum* H15 is an efficient PAH degrader in soil and is able to tolerate high amounts of PAHs and that the presence of PAHs induces the production of enzymes involved in the oxidation of PAH aromatic rings at

different stages. This, along with its great ability to grow in soil, make *T. asperellum* a microorganism with great potential for use in the bioremediation of PAH-contaminated soils. To our knowledge, this is the first report on the biodegradation of LMW and HMW PAHs by *T. asperellum*. Further studies testing the bioremediation of impacted soils at field scale, as well as intermediate production are necessary to better address the degradation mechanisms and bioremediation potential of this microorganism.

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CHAPTER 4

MORPHOLOGICAL CHANGES AND GROWTH OF FILAMENTOUS FUNGI IN PRESENCE OF HIGH CONCENTRATIONS OF PAHS

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Morphological changes and growth of filamentous fungi in presence of high concentrations of PAHs

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Abstract

In this study, we evaluated the effect of low and high molecular weight Polycyclic Aromatic hydrocarbons (PAHs) -Phenanthrene, Pyrene and Benzo[a]pyrene- on the radial growth and morphology of PAH-degrading strains *Aspergillus nomius* H7 and *Trichoderma asperellum* H15. The presence of PAHs in solid medium produced significant detrimental effects on the radial growth of *A. nomius* H7 at 4000 and 6000 mg l^{-1} as well changes in mycelium pigmentation, abundance and sporulation ability at 1000-6000 mg L^{-1}. In contrast, radial growth of *T. asperellum* H15 was not affected at any of the doses tested, although sporulation was observed only up to 4000 mg l^{-1} and as with H7 strain, some visible changes in sporulation patterns and mycelium pigmentation were seen. Our results suggest that fungal strains exposed to high doses of PAHs significantly vary its growth rate and sporulation characteristics in response to physiological and defense mechanisms affecting both pigment production and conidiation processes. This is relevant for the better understanding of fungal adaptation in PAH-polluted environments, as well for developing and implementing adequate strategies for the remediation of contaminated soils.

Keywords: Polycyclic Aromatic Hydrocarbons (PAHs), bioremediation, radial growth, sporulation, fungal physiology

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are an important group of persistent organic pollutants mainly produced as the result of thermal decomposition, incomplete combustion and pyrolysis of diverse organic molecules (Mrozik and Piotrowska-Seget, 2010). Due to their physicochemical properties, PAHs are highly hydrophobic persistent compounds with potential cytotoxic, carcinogenic, genotoxic and mutagenic effects on flora and fauna of impacted habitats, resulting in the absorption and accumulation of several toxic products and active intermediaries of their metabolism in diverse organisms. Several natural and anthropogenic sources contribute to the release of PAHs into the environment, but particularly, petrochemical activities and their related residues exert a strong negative impact on the environment and account for the majority of PAHs and other hydrocarbons released into soils and water bodies (Haritash and Kaushik, 2009).

Removal of low (LMW) and high molecular weight (HMW) PAHs from contaminated soils has become an increasing environmental priority (US-EPA, 2008). Although chemical, physicochemical and thermal technologies are available for the remediation of contaminated soils, microbial degradation is considered the main natural degradation form of PAHs. Many bacterial and fungal species are capable of degrading LMW or HMW-PAHs under aerobic or anaerobic conditions (Cerniglia and Sutherland, 2010; Seo *et al.*, 2009), involving the action of mono and dioxygenase, laccase, and peroxidase enzymes among others (Haritash and Kaushik, 2009). Several fungal species possess the ability to degrade PAHs and the potential to remediate polluted soils, however, one limiting factor in the success of these organisms is the inability to adapt and properly grow on extensively contaminated soils (Tabak *et al.*, 2003). The use of native microorganisms capable not only of degrading but also having high levels of tolerance to PAHs would reduce the problems associated with adaptation, survival and activity on soils containing high amounts of heavy hydrocarbon fractions (Margesin and Schinner, 2001). Despite this, the effect of aromatic hydrocarbons on the mycelial growth rate and sporulation of tolerant/degrading fungi could be an important factor not only for the microbial adaptation, but also for the adequate cell development and degradation of PAHs. While in soil, microbial populations are often faced with compounds that could serve as a source of carbon and energy, but are toxic to them (Ramos *et al.*, 2002). In this sense, the impact of PAHs on fungal populations is a key factor influencing its removal or persistence, especially in soils with high amounts of toxic compounds. This is particularly important for the selection of suitable microorganisms for the bioremediation of soils impacted with PAHs. Thus the aim of this work was to evaluate the effect of PAHs on the growth of two PAH-degrading fungal strains, in order to better address the possible inhibitory effects and morphological changes caused on fungi at different PAH doses.

Materials and Methods

PAH-degrading microorganisms

Aspergillus nomius H7 and *Trichoderma asperellum* H15 were previously isolated from a heavy-crude oil contaminated-soil and characterized as hydrocarbon-degrading strains, showing increased tolerance levels to 3 (Phenanthrene), 4 (Pyrene) and 5-ring (Benzo[a]pyrene) PAHs (Zafra *et al.*, 2014b). Fungi were maintained on Potato Dextrose Agar (PDA) plates at 30°C. Fresh spores were produced in 250 ml flasks containing 30 ml of PDA, inoculated with each of the strains and incubated at 30°C. After 3 d of growth, spores were collected with the addition of 20 ml of 0.1% tween 80 solution and sterile glass beads.

Effect of PAHs on radial growth

The effect of analytical grade Phenantrene (Phe), Pyrene (Pyr), and Benzo[a]pyrene (Bap) (Sigma, USA) on the radial growth of *A. nomius* H7 and *T. asperellum* H15 was tested by surface plate assays. 2 ml of a mixture of Phe, Pyr and BaP (1:1:1) dissolved in acetone (Sigma, USA) were sparse in petri dishes containing 20 ml of Toyama´s Medium (Wunder *et al.*, 1994) and evaporated under sterile conditions, to yield final superficial concentrations of 1000, 2000, 4000 and 6000 mg l^{-1}. Plates were centrally inoculated with 1×10^4 spores and incubated at 30°C for 7 d. Plates without PAHs and inoculated with each of the fungal isolates were used as controls. Mycelium radial extension rate measurements (cm d^{-1}) were made every 24 h by using a vernier digital caliper (Mitotuyo, Kawasaki, Japan).

Effect of PAHs on fungal morphology

Effect of PAHs on fungal morphology were monitored by direct macromorphological observations in colony variations as well as microscopic changes in mycelium and spores after 10 d of growth. Stereomicroscopic observations were made by using a Zoom 2000™ Stereozoom Microscope (Leica, Wetzlar, Germany). Plate photographs and photomicrographs were taken with a PowerShot SX500 IS digital camera (Canon, New York, USA).

Statistical Analysis

Fungal growth assays were set by using a 2 x 5 factorial design (two strains and five PAH doses). Data from radial extension rates were analyzed by Analysis of Variance (ANOVA) followed by a multiple comparison test (LSD) with SPSS Statistics Software version 19 (IBM), considering statistically significant differences those with a p value <0,05. All assays were carried out in triplicate.

Results and discussion

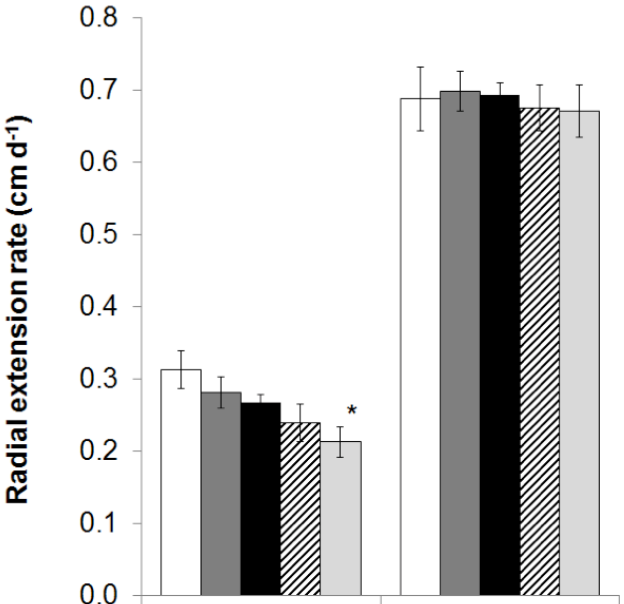
Effect of PAHs on fungal radial growth

Since PAH-contaminated soils typically contain complex mixtures of LMW and HMW PAHs, we used high doses of a mixture of 3, 4 and 5-ring PAH to better address the inhibitory effect caused on fungi in a hypothetical impacted soil. Fig. 1 shows the ability of the fungal strains to grow in the presence of different doses of PAHs. Compared with control plates, *A. nomius* H7 radial extension rates were not significantly different at 1000, 2000 and 4000 mg l^{-1}, but it were when growing in the presence of 6000 mg l^{-1} (p=0,047) of the PAH mixture. Although growth inhibition was observed at higher concentrations, the results showed that *A. nomius* spores could successfully germinate even with 6000 mg l^{-1} of PAHs in medium and reach comparable extension rates at intermediate doses. Besides, H7 strain was isolated from a highly hydrocarbon-polluted soil and have previously showed the ability to grow with the same mixture of PAHs as sole carbon source (Zafra *et al.*, 2014b), features that could confer great adaptive advantage in PAH-contaminated soils (Hinga and Batchellor, 2005). In contrast, there were no apparent detrimental effects of PAHs on the radial growth of *T. asperellum* H15, as growth rates showed no significant differences between controls without PAHs and the highest concentration tested (6000 mg l^{-1}). Unlike *A. nomius*, *T. asperellum* possess a complex enzymatic machinery able to oxidize and cleave aromatic rings, including laccases, peroxidases and dioxygenases among others (Cerniglia and Sutherland, 2010) which could actively contribute for adaptation and proper growth in presence of PAHs. Moreover, *T. asperellum* produces a potent lignocellulolytic cocktail which favors the use of alternative carbon sources (including PAHs) (Marx *et al.*, 2013). This is particularly relevant in soil, where a complex mixture of substrates can be found. The fact of finding mycelium radial extension at extremely high concentrations of PAHs, along with the ability of using PAHs as sole carbon source is a good indicative of the presumptive degradative ability of these strains in soils.

Effect of PAHs on mycelium pigmentation

Previous studies on PAH metabolism indicated that fungal growth could be significantly impaired in presence of PAHs (Brodkorb and Legge, 1992). Our results showed that fungal exposure to increased doses of PAHs caused drastic changes in the morphology of both strains compared with uncontaminated controls, particularly on *A. nomius* H7. PAHs produced evident alterations on mycelium pigmentation of strain H7 even when growing at the lowest dose (Fig. 2), where a slight yellow pigmentation was observed at the periphery of the colonies, being this effect more evident when the contamination levels increased. On the other hand, although radial growth of *T. asperellum* H15 was not affected at any of the PAH doses, mycelium pigmentation was affected particularly at 6000 mg l^{-1} (Fig. 3).

Fig. 1. Radial extension rates of fungal strains in presence of different concentrations of a mixture of Phe, Pyr and BaP



	Aspergillus nomius H7	*Trichoderma asperellum* H15
0 mg L-1	0.314	0.688
1000 mg L-1	0.281	0.698
2000 mg L-1	0.267	0.694
4000 mg L-1	0.240	0.675
6000 mg L-1	0.213	0.671

Our findings are in agreement with previous studies describing yellow pigment formation and mycelial changes in ascomycetes grown on hydrocarbons, including *Aspergillus versicolor*, *Aspergillus ochraceous*, *Aspergillus alliaceous*, *Aspergillus niger* and *Aspergillus terreus* (Nyns *et al.*, 1968; Zajic and Kuehn, 1962). Similar effects have been also observed in *Chrysosporium merdarium* and *Trichoderma harzianum* grown on lubricants, which furthermore caused thick mycelium presence and intense mycelial development (Lugauskas *et al.*, 2008).

Mycelial yellow pigment production have also been linked to aflatoxin production in *A. nomius* and *A. flavus* (Abbas *et al.*, 2004; Shier *et al.*, 2005) as a response against abiotic stress and limiting growth conditions (Yu *et al.*, 1996). The alterations observed in *A. nomius* and *T. asperellum* mycelium could therefore be related with an accumulation of hydrocarbons in the central part of cell membrane, causing dramatic changes in the structure, modifications of membrane fluidity, increase of the mycelium area and eventually a swelling of the bilayer (Sikkema *et al.*, 1995). Furthermore, hydrocarbon induced changes could lead to alteration in membrane function, cellular homeostasis and energy transduction (Pope *et al.*, 1984).

Fig. 2. Effect of different concentrations of Phe, Pyr and BaP on the radial growth and morphology of *Aspergillus nomius* H7

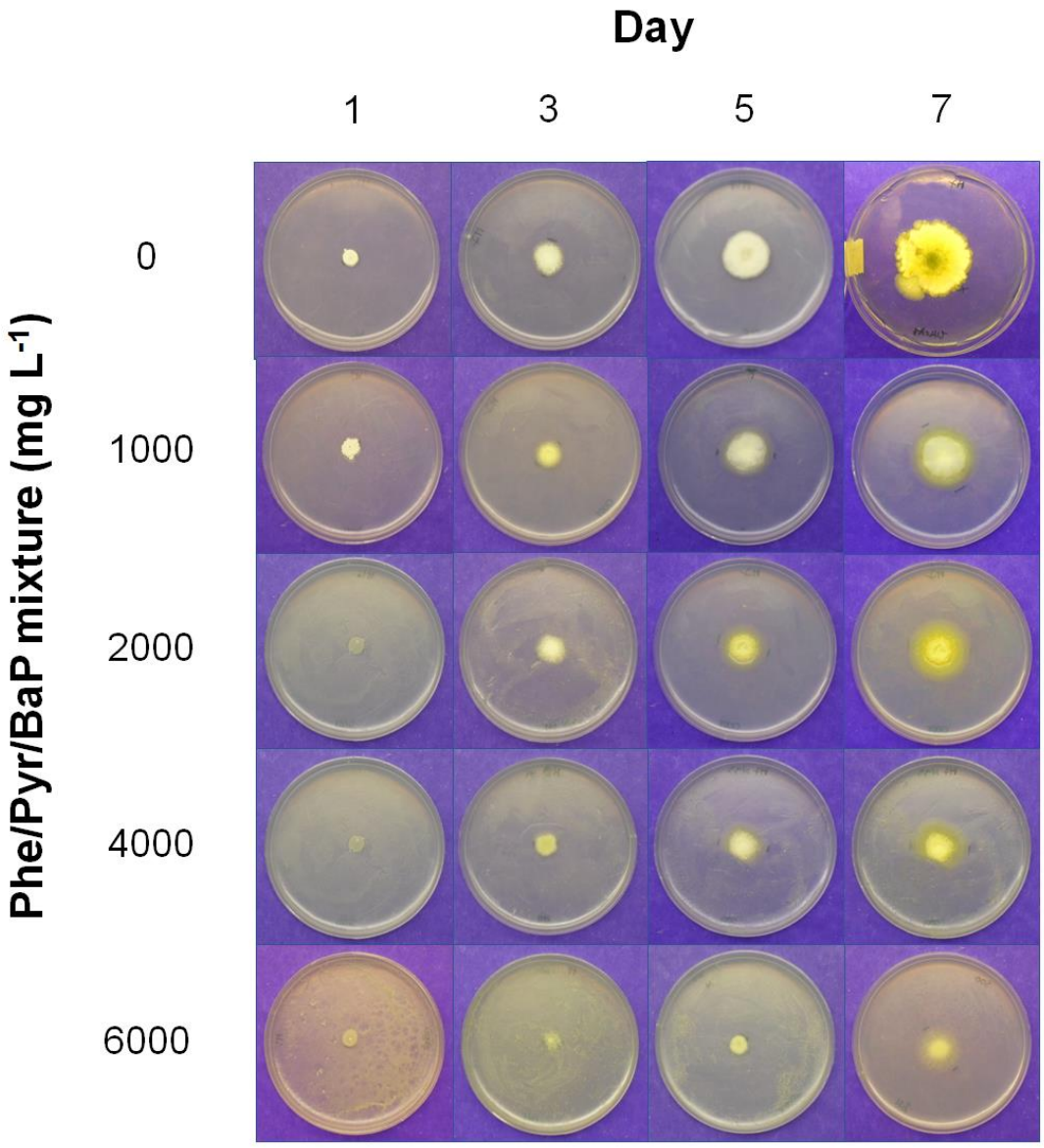
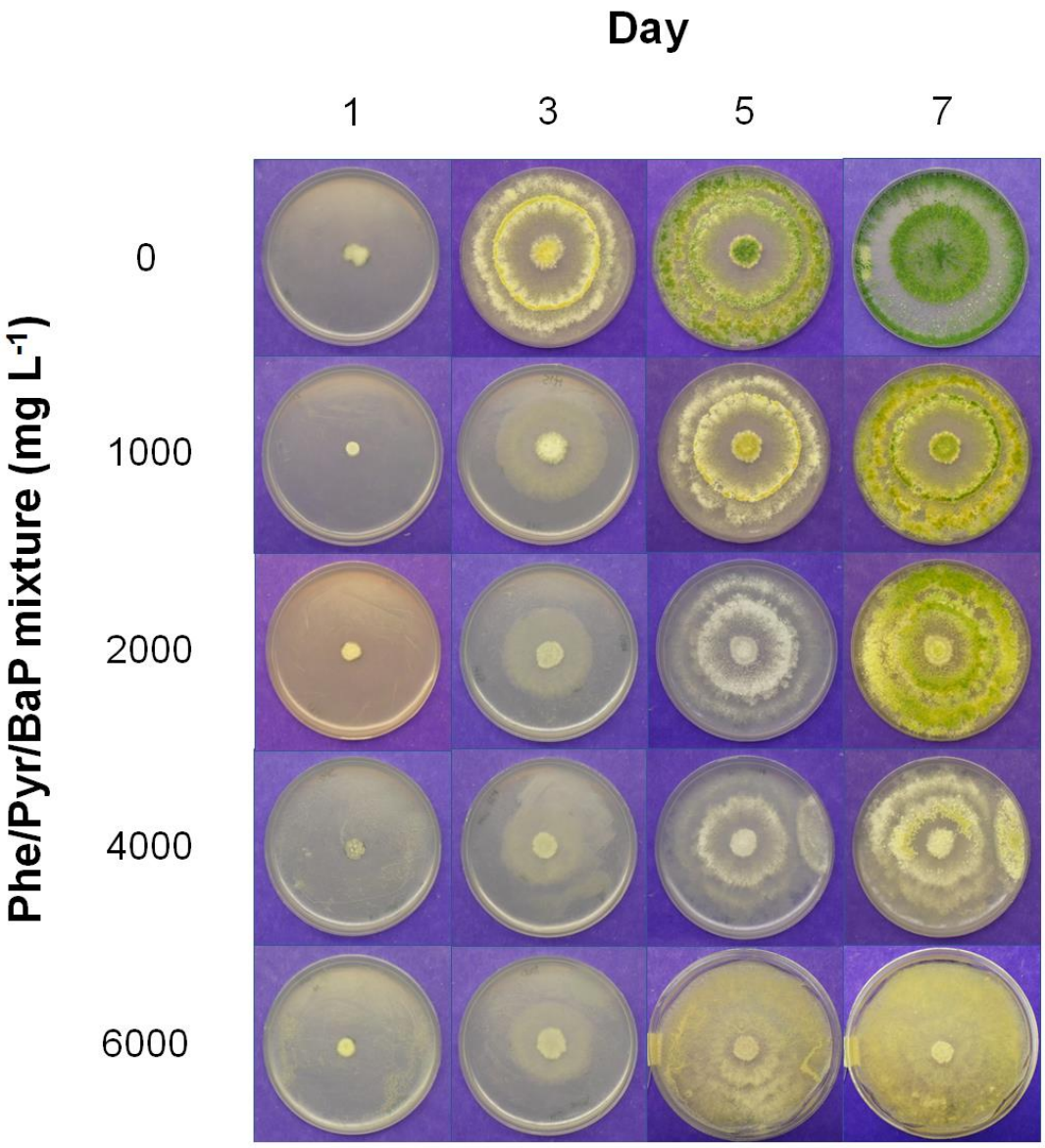


Fig. 3. Effect of different concentrations of Phe, Pyr and BaP on the radial growth and morphology of *Trichoderma asperellum* H15

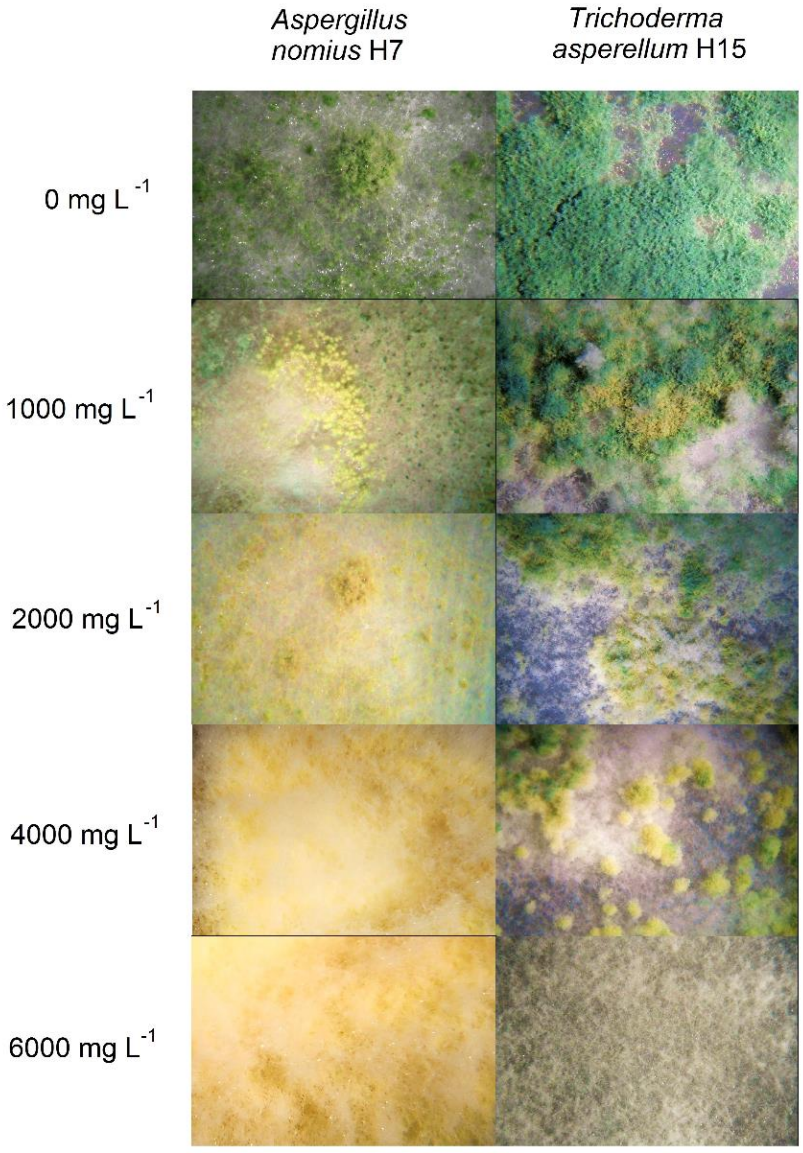


Effect of PAHs on sporulation

We found that the presence of PAHs affected at different extent the sporulation process in *Aspergillus nomius* and *Trichoderma asperellum*. Whereas sporulation in *A. nomius* H7 was observed from day 5 in control plates, the presence of 1000 and 2000 mg l^{-1} of PAHs retarded the start of sporulation until day 10, showing to be minimal at 2000 mg L^{-1} and completely absent at 4000 and 6000 mg l^{-1} (Fig. 4). In addition, a gradual change in conidia pigmentation from green

to yellow was observed from 1000 mg l^{-1} of PAHs, being evident at 2000 mg l^{-1} (Fig. 4). This effect appeared to be dose-dependent and stronger in *A. nomius* H7. On the other hand, *T. asperellum* H15 showed similar effects in sporulation as those observed in *A. nomius* H7. However there was a difference regarding the doses at which sporulation was inhibited, as strain H15 was able to sporulate even at 4000 mg l^{-1} and the conidial pigmentation changes were less marked (Fig. 4). The impact of the PAH mixture on fungal sporulation was notorious, as both strains presented a delay in the sporulation and showed changes in conidial pigmentation.

Fig. 4. Stereomicroscopic evaluation of the effect of PAHs on the morphology of fungal strains (magnification 20X).



Fungal sporulation is a complex process involving the action of many regulatory genes affecting cell specialization and intercellular communication, among others. The central regulatory pathway of conidiation have been described in *Aspergillus nidulans*, with at least three essential modulatory genes identified (*brl*A, *aba*A and *wet*A) (Adams and Yu, 1998). Previous work in our group have shown that concentrations from 200 mg L^{-1} of Phe can delay the expression of essential genes in the central regulatory pathway of *Aspergillus niger*, particularly of *wet*A (Vasquez, 2010). The *wetA* gene is required in the late phase of conidiation for the synthesis of cell wall layers (Tao and Yu, 2011) and mutants lacking this gene produces normal conidiophores, but the conidia never become pigmented and autolyse (Sewall *et al.*, 1990). Furthermore, *wet*A orthologous have been identified in several *Trichoderma* species as key genes in conidiation process (Carreras-Villasenor *et al.*, 2012). Thus it is likely that a PAH-induced delay in the expression of conidiation central genes may be responsible, or at least in part, for the observed alterations regarding sporulation delay and pigmentation changes in both strains, especially for *A. nomius*.

Conclusions

Our data suggest that fungal strains exposed to high doses of PAHs significantly vary its growth rate and sporulation characteristics due to cell membrane structural alterations and the partial inhibition of the central conidiation pathway. The results are relevant for the better understanding of fungal adaptation in PAH-polluted environments, for the selection of suitable fungal strains able to tolerate, grow and degrade high amounts of PAHs as well for developing and implementing adequate strategies for the remediation of contaminated soils.

Acknowledgments

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CHAPTER 5

CONSTRUCTION OF PAH-DEGRADING MIXED MICROBIAL CONSORTIA BY INDUCED SELECTION IN SOIL

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Construction of PAH-degrading Mixed Microbial Consortia by induced Selection in Soil

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Abstract

PAH contamination of soils has become an environmental priority. Bioremediation of PAH-contaminated soils through bioaugmentation with microbial consortia along with biostimulation treatments can be an effective strategy for the cleanup of oil spills and environmental accidents. In this work, an induced microbial selection method using PAH-polluted soils was successfully used to construct two fungal-bacterial consortia exhibiting high degradation levels of both low and high molecular weight PAHs. Six fungal and eight bacterial native strains were used to construct mixed consortia with the ability to tolerate high amounts of phenanthrene, pyrene and benzo[a]pyrene and utilize these compounds as sole carbon source. In addition, we used two GEM PAH-degrading fungal strains producing ligninolytic enzymes. After screening for microbial antagonists, the selection process was performed at microcosm and monitored using PCR-DGGE, CO_2 evolution and PAH quantitation. Resulting consortia (C1 and C2) were able to degrade up to 91,5% Phenanthrene, 64,2% Pyrene and 64,9% Benzo[a]pyrene in soil after a two weeks treatment period. The results indicate that both microbial consortia presented high potential for soil bioremediation by bioaugmentation and biostimulation, and may be effective for the treatment of sites polluted with PAHs and other high molecular weight hydrocarbons due to their elevated tolerance to aromatic compounds, their capacity to utilize them as energy source and the induced selection process.

Keywords: Polycyclic Aromatic Hydrocarbons **(**PAHs), degrading consortia, bioremediation, microbial antagonism, microbial selection, soil pollution.

Introduction

Polycyclic Aromatic Hydrocarbons are an important group of organic pollutants containing two or more fused aromatic rings, mainly produced as the result of the thermal decomposition, incomplete combustion and pyrolysis of diverse organic molecules (Mrozik and Piotrowska-Seget, 2010). Although several natural and anthropogenic sources contribute to the release of PAHs into the environment, particularly, petrochemical activities and their related wastes exert a strong negative impact on the environment and contributes to the majority of PAHs and other hydrocarbons released into soils and water bodies. PAHs are considered persistent pollutants with potential harmful effects on the flora and fauna of affected habitats, resulting in the absorption and accumulation of several toxic products and active intermediaries of their metabolism in diverse organisms. Because of their persistence and potential cytotoxic, carcinogenic, genotoxic and mutagenic effects, the removal of PAHs and other hydrocarbon compounds from contaminated soils has become an increasing environmental priority (US-EPA, 2008).

There is a variety of mechanisms by which PAHs are removed naturally from the environment, including several forms of oxidation, adsorption, volatilization, bioaccumulation and biodegradation. Although chemical, physicochemical and thermal technologies are available for remediation of impacted soils, microbial degradation is considered the main natural degradation form of PAHs in soils. More than 50 genera of bacteria and fungi contain species capable of degrading low molecular weight (LMW) or high molecular weight (HMW) PAHs under aerobic or anaerobic conditions (Cerniglia and Sutherland, 2010; Seo *et al.*, 2009). However, microorganisms in soils rarely occur as individual organisms, but instead they live in complex ecological communities with different levels of interactions which could largely influence PAH degradation efficiency. For the above, the use of a microbial consortium as inoculum for soil bioaugmentation may be advantageous to efficiently degrade mixtures of PAHs, since a single microbial species hardly possess the ability to metabolize both LMW and HMW-PAHs and also because a microbial consortium may show an improved adaptation, survival and permanence in contaminated soils. In this work, we successfully used an induced microbial selection method using PAH-spiked soils to construct two mixed microbial consortia exhibiting high degradation levels of low and high molecular weight PAHs.

Materials and Methods

Native degrading microorganisms

Five fungal (*Aspergillus flavus* H6, *Aspergillus nomius* H7, *Rhizomucor variabilis* H9, *Trichoderma asperellum* H15, *Aspergillus fumigatus* H19) and eight bacterial native strains (*Klebsiella pneumoniae* B1, *Enterobacter* sp. B3, *Bacillus cereus* B4, *Pseudomonas aeruginosa* B6, *Pseudomonas aeruginosa* B7, *Streptomyces* sp. B8, *Klebsiella* sp. B10, *Stenothrophomonas maltophilia* B14) were preselected for the construction of consortia. This selection was made based on their high PAH tolerance levels, the ability to use them as sole carbon source and the

reported ability to metabolize PAHs. (Zafra *et al.*, 2014a; Zafra *et al.*, 2014b). Fungal strains were maintained aerobically at 30°C in Potato Dextrose Agar (PDA) plates containing 0.1% Maya crude oil and bacterial strain were maintained in liquid Basal Saline Medium (g l^{-1}: NaCl, 0.3; $(NH_4)_2SO_4$, 0.6; K_2HPO_4, 0.75; KH_2PO_4; 0.25; $MgSO_4.7H_2O$, 0.15; KNO_3, 0.6; yeast extract, 0.125) using 0.1% Maya crude oil as sole carbon source.

Degrading genetically engineered microorganisms (GEMs)

In addition to native degrading microorganisms, two GEM strains of *Aspergillus niger* possessing the lignin peroxidase (LiP5 strain) and manganese peroxidase (MnP7 strain) genes from the ligninolytic organism *Phanerochaete chrysosporium* were used in the construction of consortia (Cortés-Espinosa and Absalón, 2013). LiP5 and MnP7 strains were maintained on PDA plates as described above for native fungal strains.

Evaluation of microbial antagonism

In vitro evaluation of fungi to fungi, bacteria to bacteria, fungi to bacteria and bacteria to fungi antagonism was carried out using Toyama´s medium plates (g l^{-1}: $(NH_4)_2SO_4$, 1; $MgSO_4.7H_2O$, 0.5; KH_2PO_4, 3; $CaCl_2.2H_2O$, 0.5; NaCl, 0.1; $FeSO_4$, 0.001; glucose, 10, pH 6.5) (Wunder *et al.*, 1994) with and without 2% Maya heavy crude oil. For fungi to fungi antagonism, $1x10^4$ spores from each strain were spotted on opposite sides of agar plates and incubated at 30°C until observing inhibition halos or antagonist effects between fungal colonies. Bacteria to bacteria antagonism was evaluated by streaking $1x10^4$ CFU of each strain in a grid pattern, allowing direct contact between strains. Fungi to bacteria antagonism was evaluated by centrally spotting 10^4 spores of a fungal strain (antagonist) and incubating at 30°C. After observing sporulation (indicative of secondary metabolism), $1x10^4$ CFU of each bacterial strain were radially streaked aside the fungal colony and incubated until bacterial growth was observed or inhibited by fungi. Bacteria to fungi antagonism was evaluated by spotting $1x10^4$ CFU of each bacterial strain (antagonists) in the periphery of the plates and centrally inoculating $1x10^4$ spores of a fungal strain at the same time.

Microbial selection of degrading consortia in solid state culture

The construction of two microbial consortia able to degrade PAHs in contaminated soils was performed by microbial self-selection assays in sterile soil using LMW and HMW-PAHs as selective factor. Assays were performed in microcosm solid culture systems using either sterile sugarcane bagasse (34.34% carbon, 0.18% nitrogen, 0.00343% phosphorous), corn stover (35.7% carbon, 0.465% nitrogen, 0.000031% phosphorous) or wheat straw (37.85% carbon, 0.555% nitrogen, 0.000187% phosphorous) as fungal growth support and biostimulation agent. Sterile

agroindustrial residues (0.35 g dry weight) were placed in 50 ml glass flasks moistened with Czapeck medium (g L^{-1}: sucrose, 30; sodium nitrate, 3; dipotassium phosphate, 1; magnesium sulfate, 0.5; potassium chloride, 0.5; ferrous sulfate, 0.01; pH 7.3) to reach 30% moisture content, inoculated with 2×10^6 spores g^{-1} of (1) only native fungal strains or (2) native plus GEM degrading fungal strains, and incubated for 5 d at 30°C. Pre-inoculated agroindustrial wastes were then mixed with 6.65 g of sterile soil (sandy loam with 2.4% organic matter, 1.4% total organic carbon, 0.063% nitrogen, 0.0023% phosphorous and pH of 8.41) previously spiked with 1000 mg Kg^{-1} of a mixture of Phenanthrene, Pyrene and Benzo[a]Pyrene (1:1:1 ratio) and inoculated with 2×10^6 CFU g^{-1} of each bacterial strain. Inoculated agroindustrial residue/contaminated soil mixtures were incubated at 30°C for 14 days, flushing headspaces every 48 h for 10 min with sterile and moistened air to preserve aerobic conditions and avoid carbon dioxide accumulation. Microcosm systems with non-contaminated soil were inoculated to determine the effect of PAHs on the growth of microorganisms. Abiotic controls were included to assure that the disappearance of PAHs was caused by biodegradation, as well as to compensate for adsorption losses. Assays were carried out in triplicate.

PCR-DGGE monitoring

Genomic DNA was isolated from 0.25 g IDM (soil plus agroindustrial residue) by using the PowerSoil DNA isolation Kit (MoBio, USA). The V3 region of the bacterial 16s rRNA was amplified using primers GC-341F (CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TA CGG GAG GCA GCA G) and 518R (ATT ACC GCG GCT GCT GG) (Muyzer *et al.*, 1993). Amplification conditions consisted of an initial denaturation at 95°C for 5 m, followed by 20 cycles of 95°C for 1 m, 65°C for 30 s (with a decrease of 1°C each cycle) and 72°C for 45 s, followed by 20 cycles with a constant annealing temperature of 55°C. Fungal 18s rRNA was amplified using primers CG-18sF (5´-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAT TCC AGC TCC AAT AGC GTA-3´) and 18sR (5´-TCG GCA TAG TTT ATG GTT AAG A-3´). Two-step touchdown amplification consisted of an initial denaturation at 95°C for 5 m, 10 cycles of 95°C for 40 s, 67°C for 30 s (with a decrease of 1°C per cycle), 72°C for 30 s and 25 cycles of 95°C for 30 s, 57°C for 30 s and 72°C for 30 s, with a final extension step of 72°C for 5 m. PCR products were verified on 1% agarose gels before DGGE analysis. DGGE was performed in a CBS Denaturing Gradient Gel Electrophoresis System (DGGE-1001) (C.B.S. Scientific, USA). PCR products (10 µl) were subjected to electrophoresis for 14 h at 80V through 8% polyacrylamide gels with a formamide/urea denaturant gradient of 40% to 100%, at 60°C. After electrophoresis, gels were silver stained according to Sanguinetti *et al.* (1994) and photographed.

Heterotrophic activity and PAH analysis

CO_2 production in microcosms was measured using an Agilent 6890 series Gas Chromatograph equipped with a thermal conductivity detector and a GS-CarbonPLOT column. CO_2 was reported as milligrams of CO_2 per g of initial dry matter (IDM). Residual PAHs were extracted from 1 g of IDM (soil plus agroindustrial residue) with the addition of 25 ml of a dichloromethane-acetone solution (7:3 ratio) using an Anton Paar Multiwave 3000 SOLV apparatus for 20 min, according to EPA 3546 method. The resulting extracts were evaporated, suspended in 2 ml of acetonitrile and analyzed in an HP Agilent 1100 HPLC system equipped with a C18 reverse-phase column, with an UV absorbance detector set at 245-360 nm under isocratic conditions in acetonitrile:water (90:10) and a flow rate of 1 ml/min.

Statistical Analysis

Data were analyzed by Analysis of Variance (ANOVA) followed by a multiple comparison test (LSD) with SPSS Statistics Software version 19 (IBM), considering statistically significant differences those with a p value <0.05.

Results and discussion

Microbial antagonism between PAH-degrading strains

Microbial antagonism tests showed inhibitory effects of *Pseudomonas aeruginosa* strains towards fungi, in particular *P. aeruginosa* B7 which inhibited the growth of all of the evaluated fungal strains (Table 1, Fig 1a, b) whereas strain B6 only showed an apparent inhibitory effect towards *Rhizomucor variabilis* H9. This inhibition could be explained by the reported production of several antimicrobial compounds by PAH-degrading bacteria, which may include wide spectrum antifungals as indole-3-acetic acid (Mordukhova *et al.*, 2000) and phenazine-1-carboxamide (Kumar *et al.*, 2005; Naik and Sakthivel, 2006). The remaining bacterial strains did not show any antagonistic effect towards fungal strains. An antagonistic effect of *Aspergillus flavus* H6**,** *Aspergillus nomius* H7 *and Aspergillus fumigatus* H19 towards *Streptomyces* sp. B8 was also observed (Fig 1c, 1d). On the other hand, no growth inhibitory effects were observed between bacteria or between fungi, except for a slight inhibition between *A. niger* strains and *T. asperellum* H15 (Table 2). The addition of 2% crude oil to Toyama´s medium plates did not alter the inhibitory effects observed between strains compared when no hydrocarbon was added. Results indicated that evaluated PAH-degrading strains, with the exception of *P. aeruginosa* B7 and *Streptomyces* sp. B8, were suitable to construct PAH-degrading mixed consortia with no major antagonistic effects between them.

Effect of PAHs on the microbial growth in solid culture

Common methods for consortia construction often consists in mixing microorganisms, in approximately equal ratios, able to transform the compound of interest prior application. Although straightforward, this type of selection does not have into account the possible inhibitory effects occurring between inoculated microorganisms, as well as the survival of each of the strains when applied to the environmental matrix. Simply put, our experiments basically consisted of an induced natural selection, where the microorganisms better adapted to the new pollution conditions gradually displaces and replaces those organisms not well adapted. Pollution of soils with PAHs may lead to a toxic, but at the same time enrichment effect on soil microbial populations (Gadd, 2007; Gloer, 2007). Toxic effects are effective in the selection of the most adapted strains, and enrichment occur when microorganisms are able to use the pollutant as a source of carbon and energy. As we used sterile soils and inoculated only PAH-degrading/tolerant microorganisms, this selection is expected to have an effect favoring the survival of the most adapted strains for PAH degradation.

Fig 1. Growth inhibitory effects from bacteria to fungi (a, b) and fungi to bacteria (c, d) in Toyama´s medium plates. Tested bacterial strains were *Klebsiella pneumoniae* B1, *Enterobacter* sp. B3, *Bacillus cereus* B4, *Pseudomonas aeruginosa* B6, *Pseudomonas aeruginosa* B7, *Streptomyces* sp. B8, *Klebsiella* sp. B10 and *Stenothrophomonas maltophilia* B14.

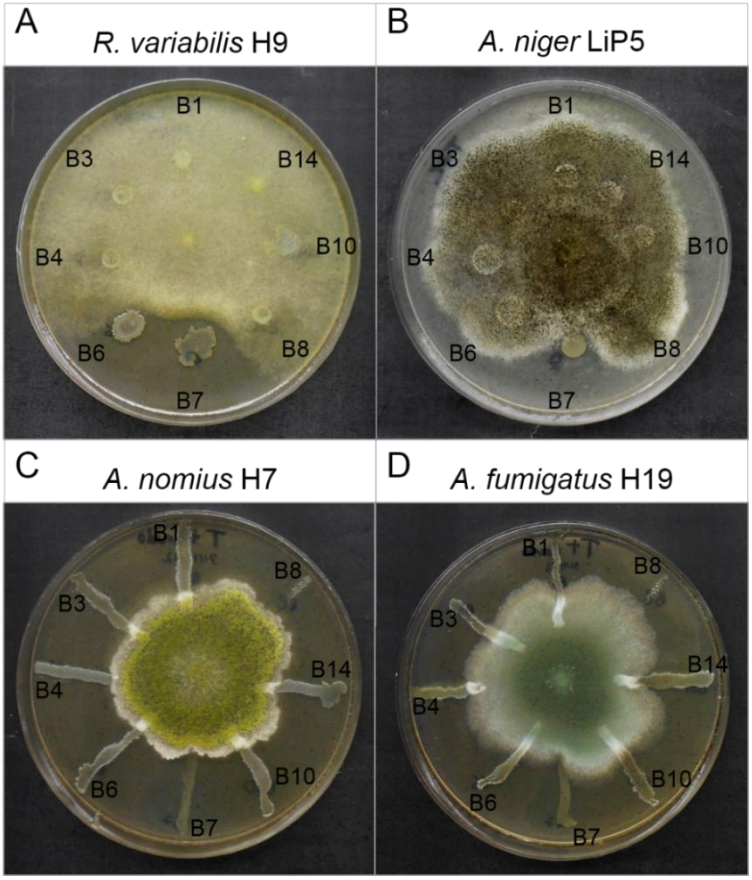


Table 1. Bacteria to fungi and fungi to bacteria inhibitory activity strains in antagonism tests. -: no inhibition; ±: partial inhibition; +: inhibition. Tested strains were *Klebsiella pneumoniae* B1, *Enterobacter* sp. B3, *Bacillus cereus* B4, *Pseudomonas aeruginosa* B6, *Pseudomonas aeruginosa* B7, *Streptomyces* sp. B8, *Klebsiella* sp. B10 and *Stenothrophomonas maltophilia* B14, *Aspergillus flavus* H6, *Aspergillus nomius* H7, *Rhizomucor variabilis* H9, *Trichoderma asperellum* H15, *Aspergillus fumigatus* H19, *Aspergillus niger* LiP5 *and Aspergillus niger* MnP7

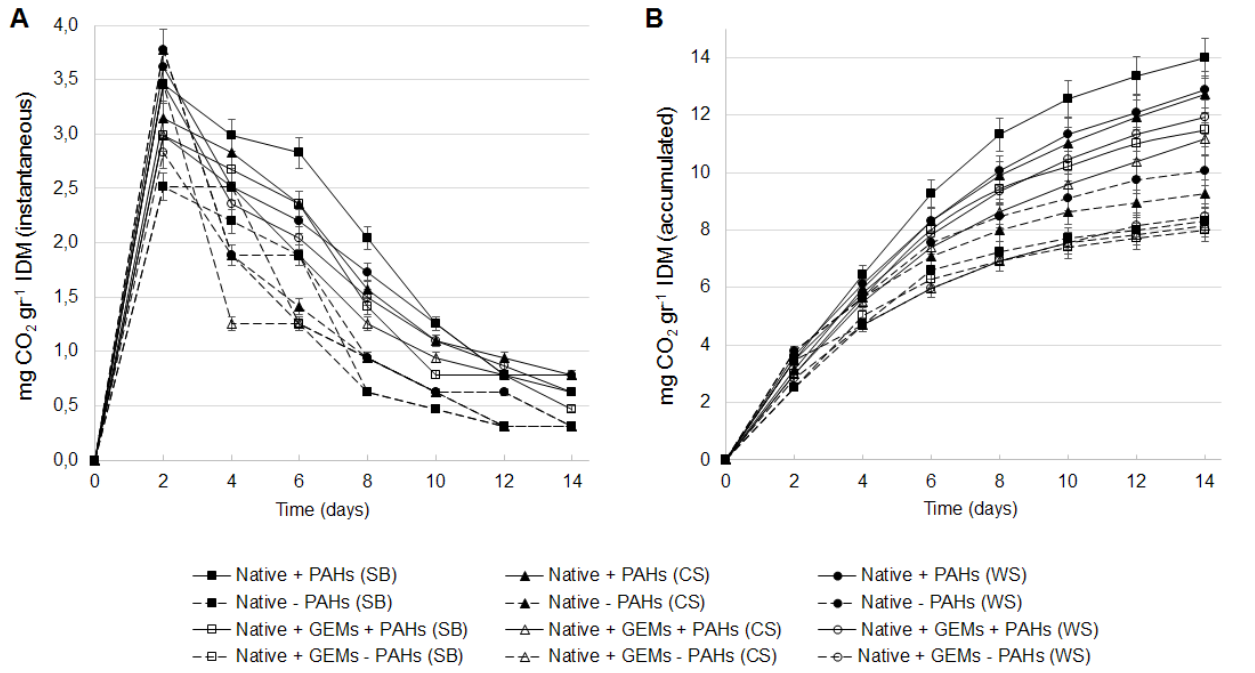
	Bacteria to fungi							*Fungi to bacteria*						
	H6	H7	H9	H15	H19	LiP5	MnP7	H6	H7	H9	H15	H19	LiP5	MnP7
B1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B4	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B6	-	-	±	-	-	-	-	-	-	-	-	-	-	-
B7	+	+	+	+	+	+	+	-	-	-	-	-	-	-
B8	-	-	-	-	-	-	-	+	+	-	-	+	-	-
B10	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B14	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 2. Bacteria to bacteria and fungi to fungi inhibitory activity in antagonism tests. -: no inhibition; ±: partial inhibition; +: inhibition. Tested strains were *Klebsiella pneumoniae* B1, *Enterobacter* sp. B3, *Bacillus cereus* B4, *Pseudomonas aeruginosa* B6, *Pseudomonas aeruginosa* B7, *Streptomyces* sp. B8, *Klebsiella* sp. B10 and *Stenothrophomonas maltophilia* B14, *Aspergillus flavus* H6, *Aspergillus nomius* H7, *Rhizomucor variabilis* H9, *Trichoderma asperellum* H15, *Aspergillus fumigatus* H19, *Aspergillus niger* LiP5 *and Aspergillus niger* MnP7

	Bacteria to bacteria									*Fungi to fungi*						
	B1	B3	B4	B6	B7	B8	B10	B14		H6	H7	H9	H15	H19	LiP5	MnP7
B1	-	-	-	-	-	-	-	-	H6	-	-	-	-	-	-	-
B3	-	-	-	-	-	-	-	-	H7	-	-	-	-	-	-	-
B4	-	-	-	-	-	-	-	-	H9	-	-	-	-	-	-	-
B6	-	-	-	-	-	-	-	-	H15	-	-	-	-	-	±	±
B7	-	-	-	-	-	-	-	-	H19	-	-	-	-	-	-	-
B8	-	-	-	-	-	-	-	-	LiP5	-	-	-	-	-	-	-
B10	-	-	-	-	-	-	-	-	MnP7	-	-	-	-	-	-	-
B14	-	-	-	-	-	-	-	-								

As observed in Fig 2, the presence of PAHs in inoculated soil microcosms led to a rapid initial increase in CO_2 levels, producing significantly higher amounts of CO_2 by day 14 than non-contaminated microcosms systems. This indicated a utilization of PAHs by the inoculated strains as carbon source, in accordance with previous reports using the same strains (Zafra *et al.*, 2014a). It is interesting to find that microcosms inoculated only with native organisms produced more CO_2 than those inoculated with native plus GEM strains. This could indicate a competition between GEM and native organisms for adaptation in soil, leading to a decrease in CO_2 levels. Systems using sugarcane bagasse as texturizing produced more CO_2 than corn stover and wheat straw-amended soils, indicating a better texturizing role and possibly providing an alternative carbon source for microbial growth. In fact sugarcane bagasse, as well as corn stover, are known to have a variety of extractable sugars and glucans that can be readily used by microorganisms, especially by fungi, during the first stages of growth (Templeton *et al.*, 2010).

Fig 2. Instantaneous (A) and accumulated (B) CO_2 production during microbial selection in a soil contaminated with 1000 mg of PAHs kg-1 soil.



Inoculum survival in PAH-contaminated soils

PCR-DGGE monitoring showed that most of the inoculated fungal strains survived the selection process and prevailed until day 14, except for *Aspergillus fumigatus* H19 which was not detected in any of the microcosms systems (Fig 3a). As H19 strain was found to have moderate tolerance levels to PAHs (Zafra *et al.*, 2014a), its disappearance was probably due to a poor adaptation to contaminated soil. *Rhizomucor variabilis* H9 did not survived in presence of GEM strains using wheat straw as texturizing and similarly, GEM strains *Aspergillus niger* LiP5 and MnP7 survived until day 14 when grown with sugarcane bagasse and corn stover, but not with wheat straw (Fig 3a), suggesting an important role of the selection of the agroindustrial residue on the survival of some strains. This was also corroborated taking into account that wheat straw-amended systems presented the lowest CO_2 production levels (Fig 2); besides, wheat straw is known to possess less extractable carbohydrates than sugarcane bagasse and corn stover (Zhang *et al.*, 2011). Attempts of fungal isolation after 14 days were consistent with DGGE results (data not shown). On the other hand, *Enterobacter* sp. B3 and *Streptomyces* sp. B8 DGGE specific bands disappeared by day 14 in all microcosms systems. Previous antagonism tests showed an inhibition of B8 strain by several fungal strains, but not an inhibition of B3 strain. As Fig 3b shows, the remaining five bacterial strains survived until day 14, independently of the presence/absence of fungal GEM strains. Taking into account the results from isolation and DGGE, four fungal (*A. flavus* H6, *A. nomius* H7, *R. variabilis* H9, *T. asperellum* H15) and five bacterial strains (*K. pneumoniae* B1, *B. cereus* B4, *P. aeruginosa* B6, *Klebsiella* sp. B10 *S. maltophilia* B14) were selected to compose a first degrading microbial consortium denominated as C1. A second consortium denominated C2 was constructed using the same strains used in C1, plus the *Aspergillus niger* LiP5 and MnP7 GEM strains.

Degradation of PAHs in soil during consortia selection

While microcosm systems inoculated only with native microorganisms exhibited high degradation levels of PAHs, the addition of GEM strains improved the degradation, particularly of HMW-PAHs, in soils biostimulated with sugarcane bagasse and corn stover (Fig 4). Biostimulation with sugarcane bagasse led to high levels of degradation of Phe, Pyr and BaP during the selection of consortium C1, achieving 87.8%, 48.2 and 56.6% degradation respectively after 14 days; however, the highest degradation rates were observed during the selection of the consortium C2 reaching degradation levels of 91.5% for Phe, 64.2% for Pyr and 64.9% for BaP (Fig 5). This tendency was also observed when corn stover was used as texturizing and alternative carbon source, where GEM-inoculated soils presented 83.1% Phe, 53.5% Pyr and 61.3% BaP degradation. Since the only difference between treatments consisted in the presence of GEM strains, these differences can be directly attributed to them. The use of GEM in bioremediation have been shown to be an effective way to improve the degradation rate of pollutants (Layton *et al.*, 2012; Urgun-Demirtas *et al.*, 2006). Previous work with LiP5 and MnP7 strains showed their ability to degrade LMW-PAHs in soil (Cortés-Espinosa and Absalón,

2013; Cortes-Espinosa *et al.*, 2011), as both lignin and manganese peroxidases from *P. chrysosporium* are ligninolytic enzymes highly effective for the initial oxidation of PAHs (Singh, 2006; Wang *et al.*, 2009). Our results corroborated the involvement of these enzymes in the degradation of both LMW and HMW-PAHs in soil. Other enzymes, as fungal laccases, peroxidases and dioxygenases are probably involved in PAH oxidation by the used consortia, as we reported previously for *Trichoderma asperellum* (Zafra *et al.*, 2014b).

Fig 3 Survival of inoculated strains in PAH-spiked soils after 14 days**.** (A) Fungal 18s rRNA and (B) bacterial 16s rRNA DGGE profiles are shown. Letters at the top of the gel indicate treatments; SB: sugarcane bagasse. CS: corn stover. WS: wheat straw. Red arrows indicate the bands disappearing after 14 days.

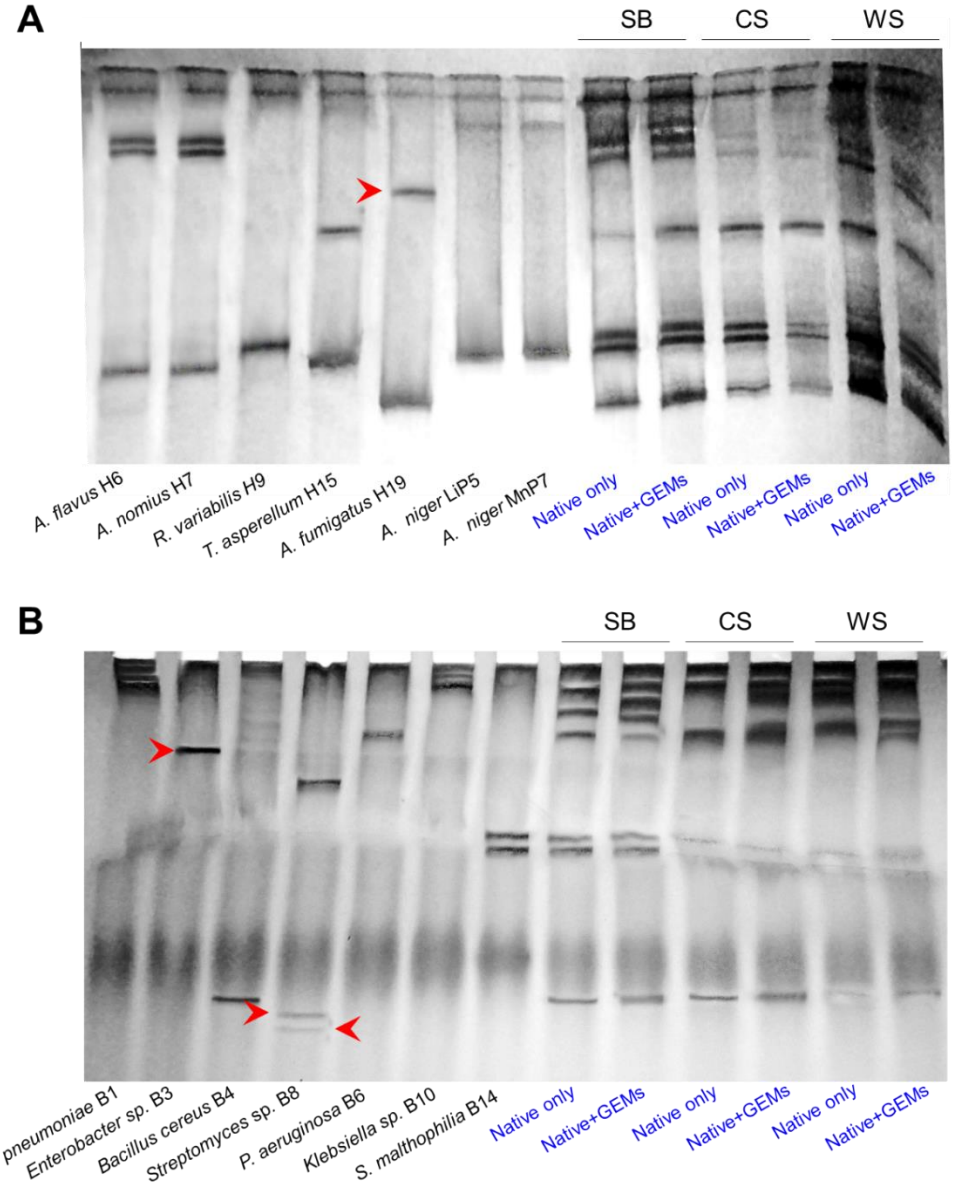
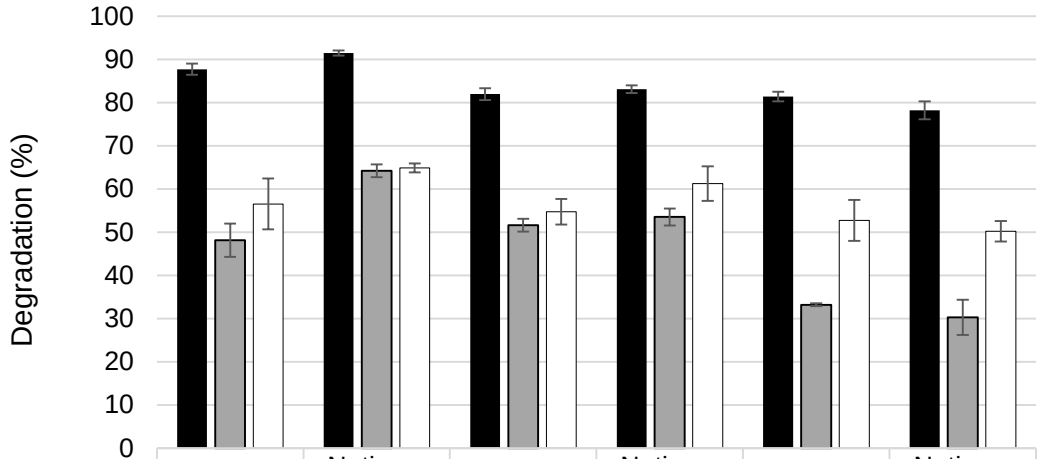


Fig 4. PAH degradation in solid culture during consortia selection in a soil contaminated with 2000 mg Kg^{-1} PAHs. SB: sugarcane bagasse; CS: corn stover; WS: wheat straw.



	Native (SB)	Native + GEMs (SB)	Native (CS)	Native + GEMs (CS)	Native (WS)	Native + GEMs (WS)
Phenanthrene	87,76	91,52	82,00	83,11	81,46	78,28
Pyrene	48,18	64,23	51,63	53,53	33,19	30,27
Benzo[a]Pyrene	56,55	64,90	54,75	61,27	52,76	50,22

Although Phe was degraded at a higher rate than Pyr and BaP, these PAHs were degraded at a higher extent than previous reports using mixed microbial consortia (Jacques *et al.*, 2008; Kim and Lee, 2007; Li *et al.*, 2008; Wu *et al.*, 2013). This was not probably due only to the action of fungal enzymes, but also to important bacterial mechanisms responsible for PAH oxidation. In fact, most of the bacterial species composing C1 and C2 consortia possess reported hydrocarbon-degrading abilities: *Stenothrophomonas maltophilia* is one of the few bacterial organisms able to degrade and use both LMW and HMW-PAHs as sole carbon source (Boonchan *et al.*, 1998; Juhasz *et al.*, 2000), *Pseudomonas aeruginosa* is known to produce PAH-oxidative enzymes and secrete rhamnolipids (Zhao *et al.*, 2011), *Bacillus cereus* is able to degrade Both LMW and HMW-PAHs (Mohandass *et al.*, 2012; Tuleva *et al.*, 2005) as well as *Klebsiella pneumoniae* (Ping *et al.*, 2014).

Conclusions

In this study we constructed two PAH-degrading microbial consortia, one of them including two GEM strains, with remarkably high tolerance levels and high degradation rates of LMW and

HMW PAHs. Our results indicate that constructed microbial consortia presented high potential for soil bioremediation by bioaugmentation and biostimulation, and may be effective for the treatment of sites polluted with PAHs and other high molecular weight hydrocarbons due to their elevated tolerance to aromatic compounds, their capacity to utilize them as energy source and the induced selection process. Further studies testing the degradation capabilities of both consortia using field-contaminated soils are necessary to better comprehend its bioremediation potential.

Acknowledgments

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CHAPTER 6

SELECTION OF OPTIMAL CULTURE CONDITIONS FOR PAH DEGRADATION IN SOILS BY MIXED MICROBIAL CONSORTIA

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Selection of Optimal Culture Conditions for PAH degradation in Soil by Microbial Consortia

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Abstract

In this study, we evaluated different biotic and abiotic variables to determine the optimal conditions for the bioremediation of PAH-polluted soils by microbial consortia using a combination of bioaugmentation and biostimulation at microcosm. We used microcosms systems to evaluate the influence of variables such as relative humidity, soil/agroindustrial residue ratio (using sugarcane bagasse and corn stover) and the use of two PAH-degrading mixed microbial consortia on PAH degradation in soil during 30 days. Assays were carried out using sterile soils spiked with 2000 mg kg^{-1} of a mixture of phenanthrene, pyrene and benzo[a]pyrene. Inoculated microcosms produced the highest amounts of accumulated CO_2 under conditions consisting of 40% relative humidity and 90:10 soil/agroindustrial waste ratio, for both sugarcane bagasse and corn stover. However, optimal conditions for PAH degradation consisted of 30% relative humidity and a ratio of 95:5 soil/agroindustrial residue. The highest removal values were obtained using the consortium C2 (including two GEM strains) and biostimulation with corn stover at 30% relative humidity and a soil/agroindustrial waste ratio of 95:5, reaching removal values of 96.25% phenanthrene, 80.47% pyrene and 71.15% benzo[a]pyrene at day 30. Consortium C1 (only native strains) also reached high PAH removal values, comparable with those of consortium C2. PCR-DGGE monitoring showed that most of the microorganisms composing microbial consortia prevailed until day 30. In conclusion, we stablished the optimal conditions for PAH removal in soils by bioaugmentation with mixed fungal/bacterial consortia and biostimulation with widely available agroindustrial feedstocks.

Keywords: Polycyclic Aromatic Hydrocarbons **(**PAHs), GEMs, soil bioremediation, degrading consortia, culture conditions, degradation.

Introduction

Polycyclic Aromatic Hydrocarbons (PAHs) are a class of environmental recalcitrant pollutants that arise from petrochemical operations and the incomplete combustion of fossil organic matter (Mrozik and Piotrowska-Seget 2010). PAHs are organic compounds consisting of two or more fused aromatic rings in a linear or grouped arrangement; therefore they are recalcitrant compounds in soils, being highly hydrophobic and therefore readily absorbed in the gastrointestinal tract of mammals, having a rapid distribution in a variety of tissues with a marked tendency to fatty deposits. Because of its high toxicity and persistence, PAHs are considered persistent environmental pollutants that may have a detrimental effect on the flora and fauna of affected habitats, resulting in the absorption and accumulation of toxic chemicals in food chains and in some cases, serious health problems or genetic defects in humans (WHO-IARC 2010).

Bioremediation, based on the use of microorganisms to degrade the contaminants, is a promising technology because of its high efficiency and cost-effectiveness. For over three decades it has been shown that microorganisms such as bacteria, fungi and algae possess specific catabolic activities that can be exploited for the remediation of soils and water impacted with low (LMW) and high molecular weight (HMW) PAHs (Haritash and Kaushik 2009). As PAH degraders are common members of the native soil microbial communities, microorganisms such as bacteria, fungi and algae having specific PAH catabolic activities can be exploited for the remediation of soils impacted with low and high molecular weight PAHs (Cerniglia and Sutherland 2010; Haritash and Kaushik 2009).

One way to overcome the numerous barriers in the degradation of PAHs in soil, especially those of high molecular weight, is the use of defined consortia of fungi and bacteria (Boonchan *et al.* 2000). These barriers include the inability of bacteria to transport HMW PAHs inside the cell due to the molecular size, the PAH not being a substrate for the available enzymes or not being a suitable inductor for transport or degrading enzymes (Juhasz and Naidu 2000). While bioaugmentation is a good strategy to improve PAH degradation in soils, biostimulation (consisting in the addition of fertilizers, texturizing agents and aeration to improving the growth conditions of soil native degrading populations) is also an effective strategy that used along bioaugmentation could significantly improve PAH degradation. These two approaches can be applied through *in-situ* techniques such as land farming, composting and biopile for the degradation of PAHs and other hydrocarbons in soil. However, the carefully selection of the conditions for PAH biodegradation is a key step for an eventual application of these strategies at field-scale. We previously constructed two microbial consortia with high potential for PAH degradation in soils (manuscript in preparation), one of them containing two Genetically Engineered Microorganisms (GEMs) strains. Thus, the objective of this work was to evaluate different biotic and abiotic variables to determine the optimal conditions for the bioremediation of PAH-polluted soils by combining bioaugmentation and biostimulation at microcosm.

Materials and Methods

Degrading microbial consortia

Two PAH-degrading microbial consortia were used in this study. Consortium C1 was composed by four fungal (*Aspergillus flavus* H6, *Aspergillus nomius* H7, *Rhizomucor variabilis* H9, *Trichoderma asperellum* H15) and five bacterial native strains (*Klebsiella pneumoniae* B1, *Bacillus cereus* B4, *Pseudomonas aeruginosa* B6, *Klebsiella* sp. B10, *Stenothrophomonas maltophilia* B14) (Zafra *et al.* 2014), while consortium C2 was composed by the same organisms composing consortium C1, with the addition of two GEM strains of *Aspergillus niger* expressing the lignin peroxidase (LiP5 strain) and manganese peroxidase (MnP7 strain) genes from *Phanerochaete chrysosporium* (Cortés-Espinosa and Absalón 2013). Microorganisms were maintained aerobically at 30°C in Potato Dextrose Agar (PDA) plates containing 0.1% Maya crude oil (fungi) or liquid Basal Saline Medium (g l^{-1}: NaCl, 0.3; $(NH_4)_2SO_4$, 0.6; K_2HPO_4, 0.75; KH_2PO_4; 0.25; $MgSO_4.7H_2O$, 0.15; KNO_3, 0.6; yeast extract, 0.125) using 0.1% Maya crude oil as sole carbon source (bacteria).

Ideal time of addition of fungi to soil

Microcosm assays were performed to stablish the end of fungal trophophase and the beginning of idiophase, moment when the contaminated soil and bacterial inoculum ideally could be added to the previously grown fungi. For this, 0.35 gr of the two sterile sugarcane bagasse (34.34% carbon, 0.18% nitrogen, 0.00343% phosphorous) or corn stover (35.7% carbon, 0.465% nitrogen, 0.000031% phosphorous) were inoculated with 2×10^6 spores g^{-1} of each fungal strain and incubated for 10 d with Czapeck medium (g L^{-1}: sucrose, 30; sodium nitrate, 3; dipotassium phosphate, 1; magnesium sulfate, 0.5; potassium chloride, 0.5; ferrous sulfate, 0.01; pH 7.3). Idiophase was indirectly located by measuring each 24 h the released CO2 by gas chromatography (described hereinafter).

Solid State Fermentation at microcosm

Once defined the ideal time of addition of soil, pre-inoculated agroindustrial wastes were then mixed with 6.65 g of sterile soil (sandy loam with 2.4% organic matter, 1.4% total organic carbon, 0.063% nitrogen, 0.0023% phosphorous and pH of 8.41) previously spiked with 2000 mg Kg^{-1} of a mixture of phenanthrene, pyrene and benzo[a]pyrene (1:1:1 ratio) and inoculated with 2×10^6 CFU g^{-1} of each bacterial strain. Inoculated agroindustrial residue/contaminated soil mixtures were incubated at 30°C for 30 days, flushing headspaces every 48 h for 10 min with sterile and moistened air to preserve aerobic conditions and avoid carbon dioxide accumulation. Systems with non-contaminated soil were inoculated to determine the effect of PAHs on the growth of microorganisms. Abiotic controls were included to assure that the disappearance of

PAHs was caused by biodegradation, as well as to compensate for adsorption losses. Assays were carried out in triplicate.

Heterotrophic activity and PAH measurements

CO_2 evolution in microcosm was measured every 48 h using an Agilent 6890 series Gas Chromatograph equipped with a thermal conductivity detector and a GS-CarbonPLOT column. CO_2 was reported as milligrams of CO_2 per g of initial dry matter (IDM). Residual PAHs were extracted from 1 g of IDM (soil plus agroindustrial residue) with the addition of 25 ml of a dichloromethane-acetone solution (7:3 ratio) using an Anton Paar Multiwave 3000 SOLV apparatus for 20 min, according to EPA 3546 method. The resulting extracts were evaporated, suspended in 2 ml of acetonitrile and analyzed in an HP Agilent 1100 HPLC system equipped with a C18 reverse-phase column, with an UV absorbance detector set at 245-360 nm under isocratic conditions in acetonitrile:water (90:10) and a flow rate of 1 ml/min.

PCR-DGGE monitoring

Genomic DNA was isolated from 0.25 g IDM (soil plus agroindustrial residue) by using the PowerSoil DNA isolation Kit (MoBio, USA). The RNA polymerase β subunit (*rpoB*) bacterial gene was amplified using primers *rpoB*1698f (5'- CGC CCC CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC C AAC ATC GGT TTG ATC AAC-3') and *rpoB*2041r (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3') (Dahllof *et al.* 2000). Amplification conditions consisted of an initial denaturation at 95°C for 5 m, followed by 25 cycles of 95°C for 30 s, 51°C for 1 m, 72°C for 45 s and a final extension at 72°C for 10 m. Fungal 18s rRNA was amplified using primers CG-18sF (5´-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAT TCC AGC TCC AAT AGC GTA-3´) and 18sR (5´-TCG GCA TAG TTT ATG GTT AAG A-3´). Two-step touchdown amplification consisted of an initial denaturation at 95°C for 5 m, 10 cycles of 95°C for 40 s, 67°C for 30 s (with a decrease of 1°C per cycle), 72°C for 30 s and 25 cycles of 95°C for 30 s, 57°C for 30 s and 72°C for 30 s, with a final extension step of 72°C for 5 m. PCR products were verified on 1% agarose gels before DGGE analysis. DGGE was performed in a CBS Denaturing Gradient Gel Electrophoresis System (DGGE-1001) (C.B.S. Scientific, USA). PCR products (10 µl) were subjected to electrophoresis for 14 h at 80V through 8% polyacrylamide gels with a formamide/urea denaturant gradient of 40% to 100%, at 60°C. After electrophoresis, gels were silver stained according to Sanguinetti *et al.* (1994) and photographed.

Statistical Analysis

Data were analyzed by Analysis of Variance (ANOVA) followed by a multiple comparison test (LSD) with SPSS Statistics Software version 19 (IBM), considering statistically significant differences those with a *p* value <0.05.

Results and discussion

Ideal time of addition of fungi to soil

Fungal idiophase is a state in which organisms does not grow, but remain metabolically active. Locating the beginning of idiophase is relevant in order to stablish the moment where the production of secondary metabolites begin, including some important oxidoreductases such as laccases and peroxidases (D'Souza-Ticlo *et al.* 2009). Evaluated fungal strains growing on sugarcane bagasse and corn stover exhibited typical growth curves and a similar behavior, where the end of trophophase and the beginning of idiophase were clearly identifiable (Fig 1). Based on growth curves, we concluded that ideal time of addition of fungi to soil was located approximately at day 5, as idiophase started at day 6.

Influence of abiotic factors on heterotrophic activity of microbial consortia in soil

We used microcosm systems to assess the influence of variables such as the agroindustrial residues used for biostimulation, the relative humidity of the systems, soil/agroindustrial residue ratio and the consortium to be used for the degradation of PAHs. In general, most of the instantaneous CO_2 production by both consortia took place during days 2-4, regardless of the growth conditions used. This was presumably due to the addition of the bacterial inoculum along with fresh culture medium to systems in which the fungal inoculum was already growing using agroindustrial residues as support. An increased production of instantaneous CO_2 was also observed in systems with higher humidity percentages (30 and 40%) for both consortia, independent of the soil/agroindustrial residue ratio. On the other hand, an abrupt decrease in CO_2 production was observed from day 8 to 12, which, however, did not decrease to baseline levels until the last days of the degradation assays.

We used two different agroindustrial residues as texturizing agents, as well as to provide support for fungal growth and an alternative carbon source. The influence of sugarcane bagasse on CO_2 production by microbial consortia is shown on figures 2 and 3. For the consortium C1, accumulated amounts of CO_2 produced in systems with 40% humidity almost doubled the amounts produced on systems with only 20% humidity (36.89 mg CO_2 g^{-1} vs 19.55 mg CO_2 gr^{-1} IDM). The presence of PAHs in soils reduced the production of CO_2 until day 2, but from day 4 CO_2 levels remained higher than those observed in soils without PAHs. Significant differences in accumulated CO_2 production were observed when comparing spiked vs. non-spiked soils at

soil/bagasse ratios of 100:0 (p=0.015), 95:5 (p=0.03) and 90:10 (p=0.014) under 40% humidity. Similarly, a positive effect of the biostimulation with sugarcane bagasse on CO_2 production was also observed either in presence or absence of PAHs at a relative humidity of 20%. The highest production of instantaneous and accumulated CO_2 was obtained in PAH-spiked soils, a ratio of 90:10 soil/bagasse and 40% humidity (36.89 mg CO_2 g^{-1} IDM), followed by microcosms with PAH-spiked soils, a 95:5 soil/bagasse ratio and 40% humidity (35.41 mg g^{-1} CO_2 IDM). The results obtained with the consortium C2 were similar to those of consortium C1, even though the final amounts of accumulated CO_2 were higher for the consortium C2. An increased production of instantaneous CO_2 in systems with a higher humidity content was also seen (fig 3), as the accumulated amounts of CO_2 produced on systems with 40% humidity were also higher than those produced in systems with only 20% humidity.

Figure 1. Metabolic activity of fungal strains in solid culture prior to addition to soil.

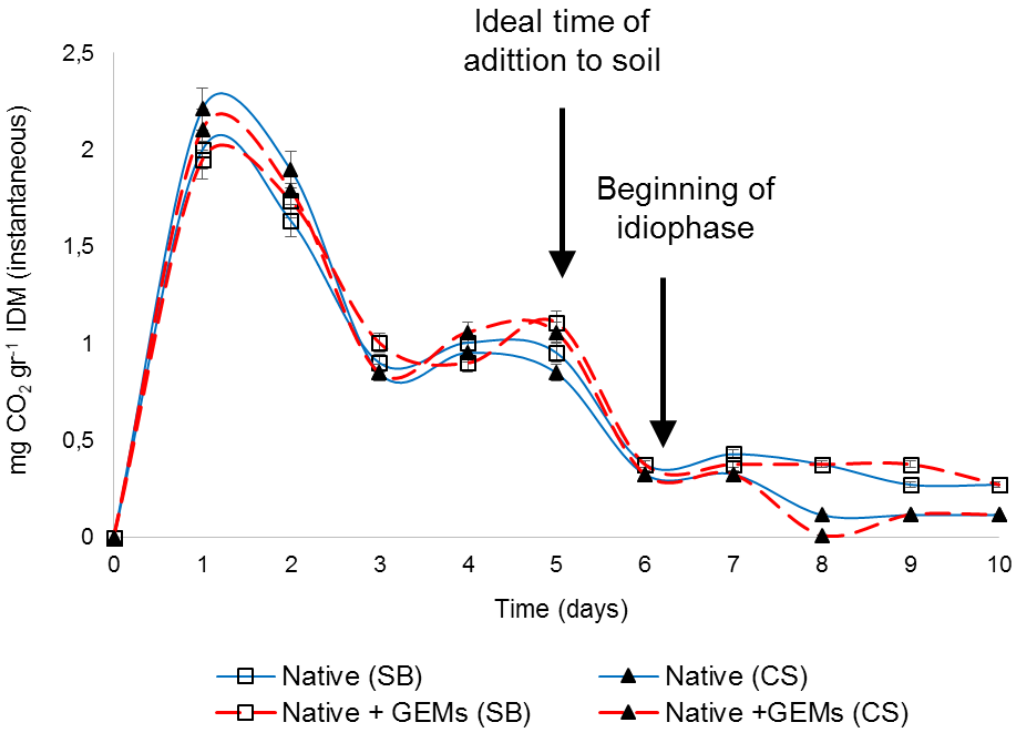


Fig 2. Instantaneous and accumulated CO_2 production by the degrading consortium C1 in a soil contaminated with 2000 mg of PAHs kg^{-1} soil. Soils were amended either with sugarcane bagasse or corn stover.

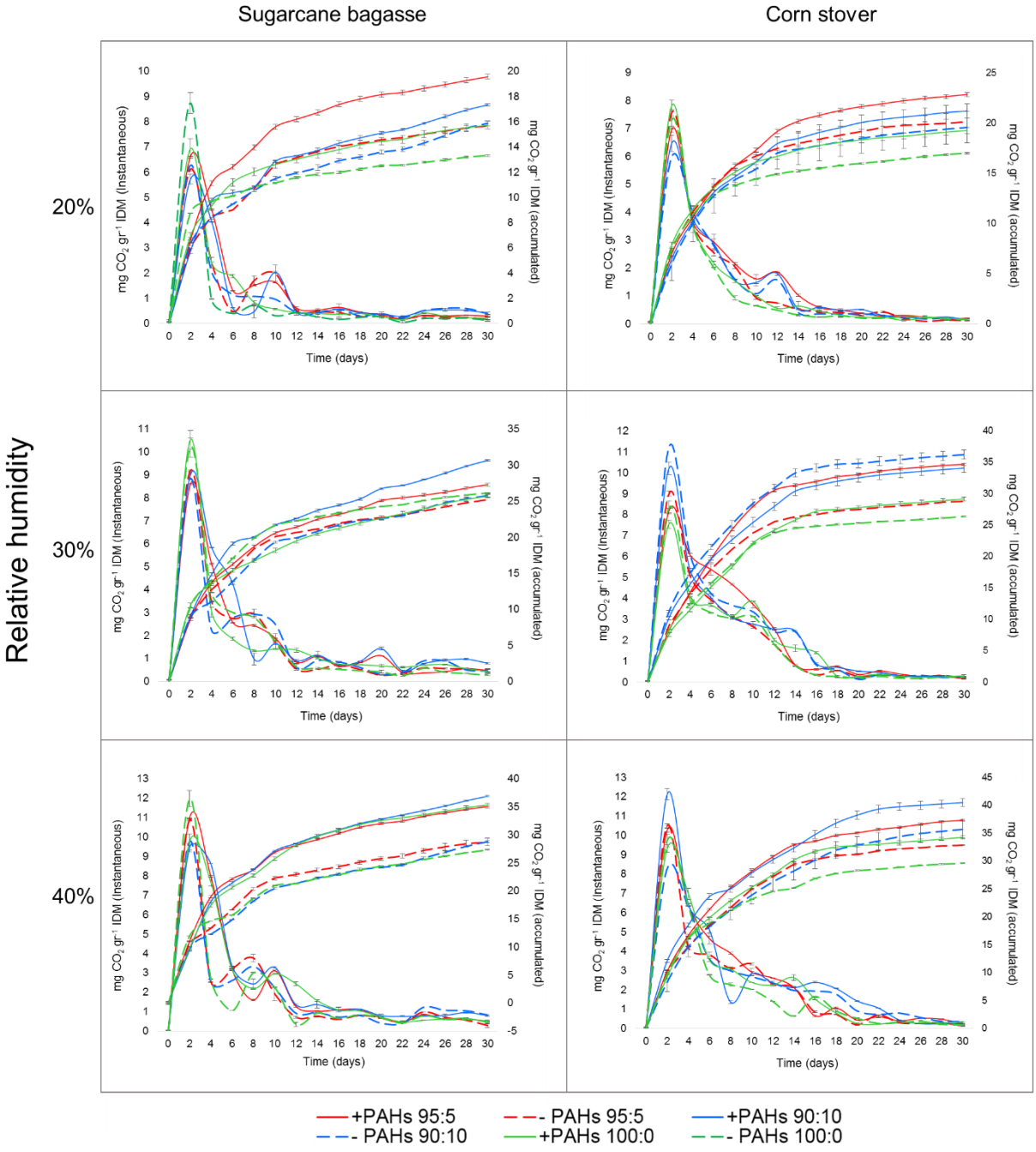
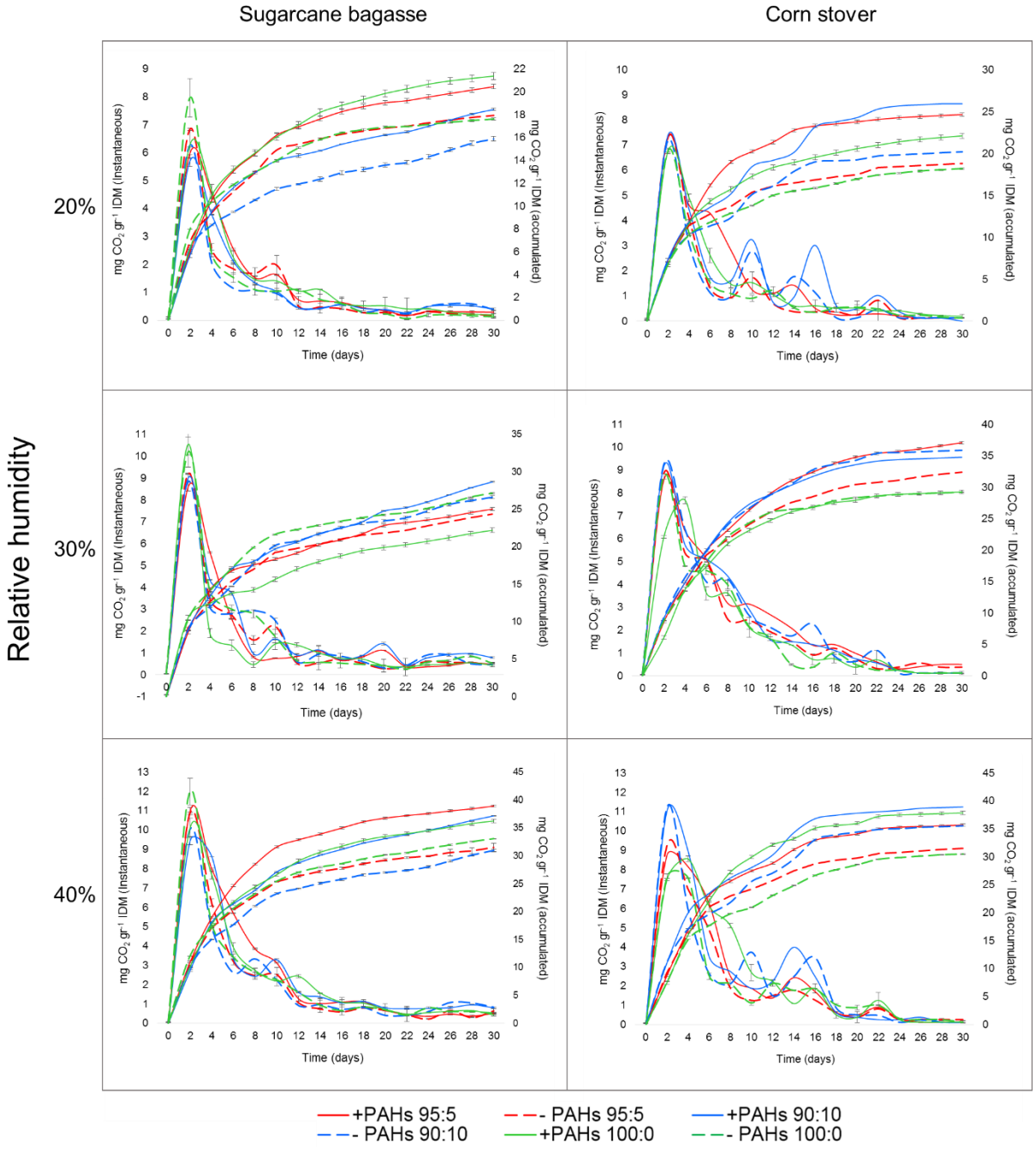


Fig 3. Instantaneous and accumulated CO_2 production by the degrading consortium C2 in a soil contaminated with 2000 mg of PAHs kg^{-1} soil. Soils were amended either with sugarcane bagasse or corn stover.



On the other hand, the use of corn stover also promoted an increase in instantaneous CO_2 production by both consortia on microcosms having the higher percentage of humidity (40%), regardless of the soil/corn stover ratio. However, when using this texturizing the differences between non-biostimulated (100:0 soil/corn stover ratio) and biostimulated soils were more clearly seen; without biostimulation with corn stover, the amounts of CO_2 were consistently lower than those observed in biostimulated soils (fig 2). The best conditions for the growth of consortia, revealed by means of the CO_2 production, consisted in a relative humidity of 40% and a 90:10 soil/corn stover ratio in PAH-spiked soils (40.48 mg CO_2 g-1 IDM), even though systems with 95:5 soil/corn stover ratio also produced high amounts of accumulated CO_2 (37.3 mg CO_2 g-1 IDM). It should be noted however, that under low humidity values (20%) the consortium C2 seems to grow better in presence of corn stover than consortium C1 (fig 3).

Survival of degrading consortia in soils

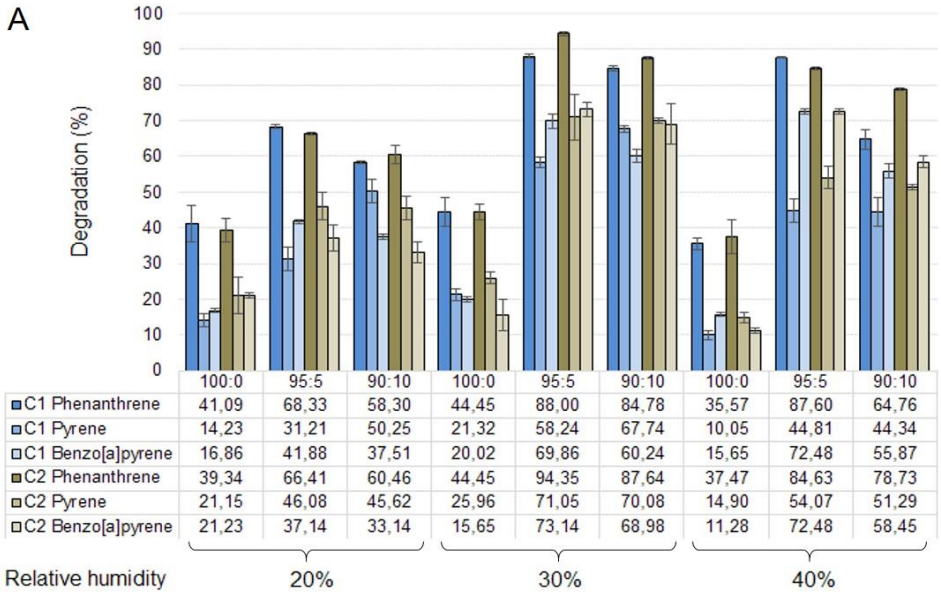
Most of bacterial and fungal strains composing both microbial consortia were detectable until day 30 in PAH-spiked and non-spiked soils. The exception was *R. variabilis* H9, which was only detectable until day 20 (Fig 5) either as a part of C1 or C2 consortia. *R variabilis* was the only member of consortia not belonging to Ascomycota, and even having high tolerance levels it could be displaced as observed in soil microcosms. Interestingly, GEMs strains LiP5 and MnP7 were no longer detectable after day 20 in any of the two agroindustrial residues used. Previous results in our lab also showed a limited survival of these GEM strains, particularly when wheat straw was used as the biostimulation agent (Chapter 5; manuscript in preparation). The poor survival of GEM strains in presence of other organisms could provide an unexpected advantage, as current normative restrict the release of GEMs or field applications. The above could represent a high degrading efficiency with limited risks, since it is desirable to use GEMs with poor survival, able to degrade pollutants but at the same time conditioned to the presence of specific conditions to survive. On the other hand, all of the bacterial strains survived until day 30, independently of the presence or absence of fungal GEM strains. The results confirmed the findings of our previous report describing high tolerance levels and an improved adaptive ability of the strains composing both microbial consortia (Zafra *et al.* 2014).

PAH degradation under different growth conditions

As observed in figure 4, bioaugmentation/biostimulation treatments of PAH-spiked soils with both consortia produced degradation levels exceeding 90% for phenanthrene and 70% for pyrene and benzo[a]pyrene. The best results were obtained with the consortium C2, particularly when the 95:5 soil/corn stover ratio was used with a relative humidity of 30%, degrading up to 96.25% phenanthrene, 80.47% pyrene and 71.15% benzo[a]pyrene by day 30. PAH degradation with sugarcane bagasse also showed to be high under the same conditions, reaching degradation levels of 94.35% for phenanthrene, 71.05% for pyrene and 70.08 for benzo[a]pyrene with the

consortium C2. Consortium C1, composed only by native strains, also reached high PAH removal values, comparable with those of consortium C2. In general, pyrene was the most difficult PAH to degrade from soils, while phenanthrene was more easily degraded. Notably, the culture conditions under which the highest levels of CO_2 were produced not necessarily corresponded to those where the highest degradation of PAHs were achieved. This was clearly the case for microcosms with soil/agroindustrial waste ratio of 90:10 and humidity of 30/40%.

Figure 4. PAH degradation in solid culture by microbial consortia in a soil spiked with 2000 mg of PAHs kg^{-1} soil. Soils were amended with A) sugarcane bagasse and B) corn stover as texturizing. Soil/agroindustrial waste ratio and relative humidity are indicated.



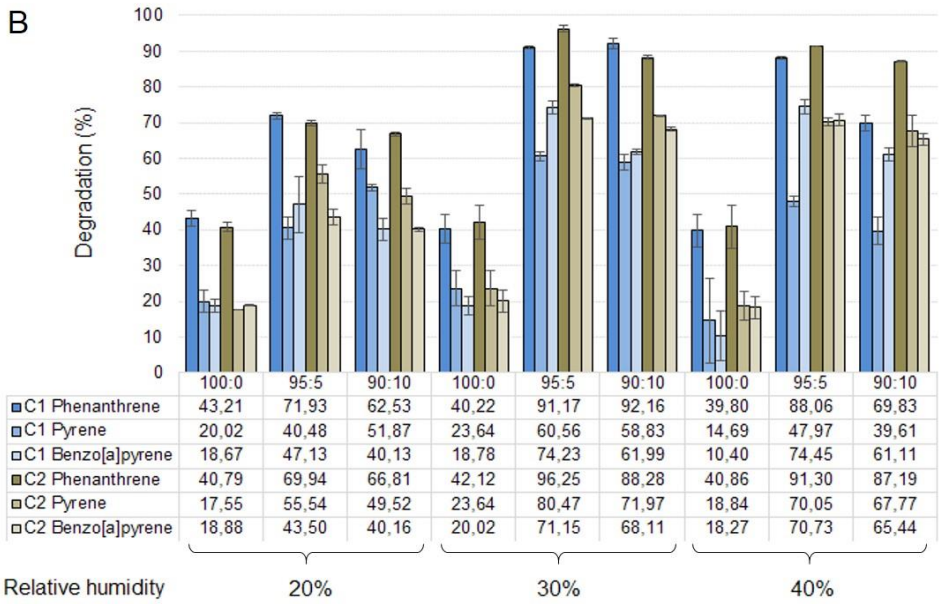
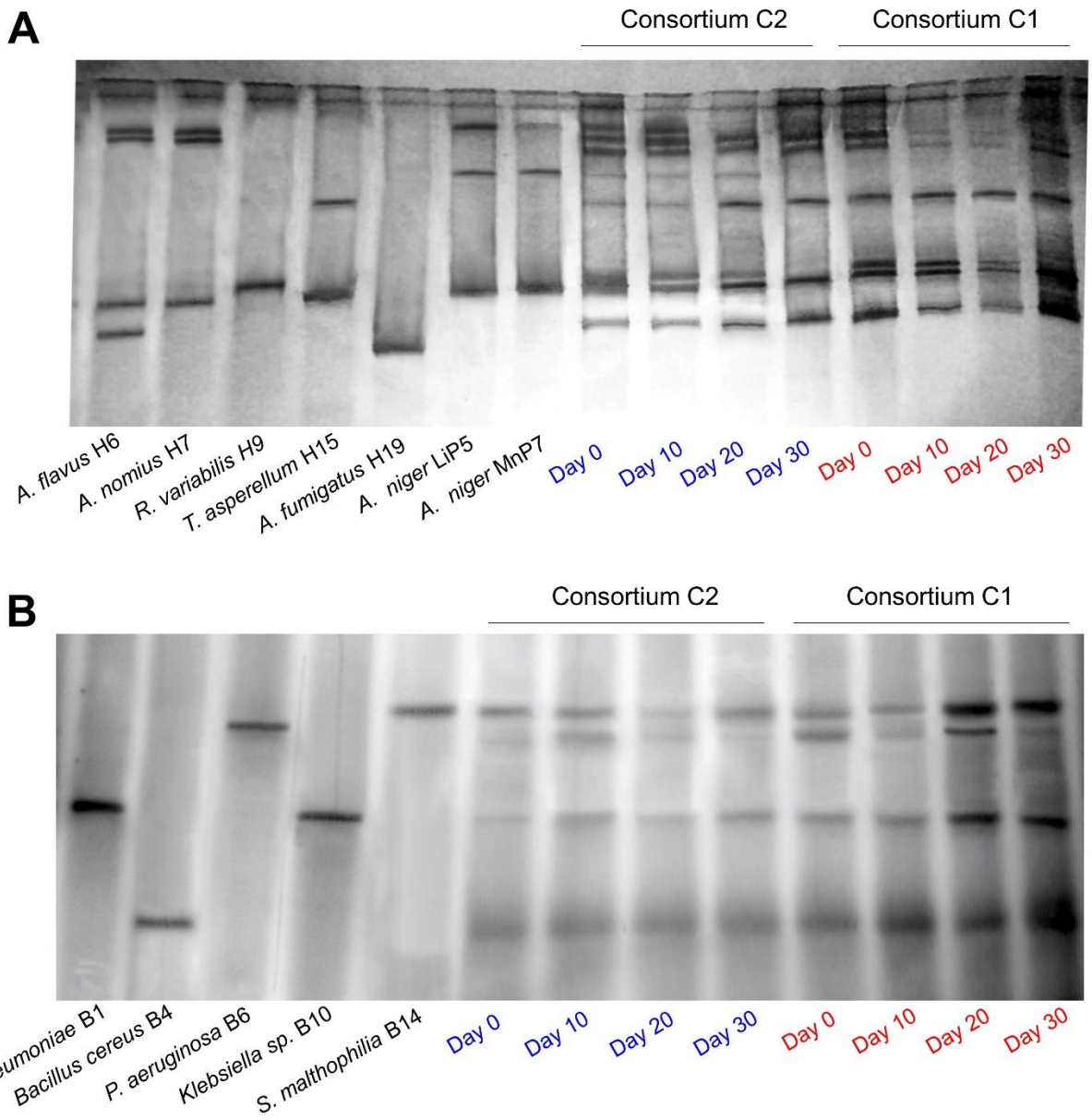


Fig 3 Survival of degrading consortia in PAH-spiked soils biostimulated with corn stover after 30 days**.** (A) Fungal 18s rRNA DGGE profiles; (B) bacterial *rpo*B DGGE profiles.



Numerous reports have demonstrated the positive effects of the addition of agroindustrial residues on the bioremediation of hydrocarbons (Zhang *et al.* 2008; Barathi and Vasudevan 2003). The amendment of soil with lignocellulosic residues may result in and increased degradation of PAHs through a number of interactions including the increase of aeration, reduction of soil bulk density and compaction, favoring the microbial diversity and overall hydrocarbonoclastic activity (Shahsavari *et al.* 2013). In this work, the biostimulation with corn stover induced a higher degradation of PAHs than sugarcane bagasse. This can be explained, at least in part, by the fact that corn stover have more extractive sugars and free sucrose than sugarcane bagasse, along with its higher texturizing ability (Templeton *et al.* 2010). The use of corn stover for the biostimulation of soils is advantageous, because it is a widely and currently available feedstock.

Conclusions

In this work we stablished the optimal conditions for PAH degradation in soils by combining bioaugmentation with fungal-bacterial consortia and biostimulation with sugarcane bagasse and corn stover. Native and GEM organisms showed to be exceptionally efficient PAH degraders in soil under the selected treatment conditions, which could make possible to scale up this bioremediation process. Further studies using contaminated field soils at higher scale, along with deeper molecular techniques are necessary to better address the mechanisms for PAH degradation by both consortia at field-scale conditions.

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CHAPTER 7

COMPARATIVE METAGENOMIC ANALYSIS OF PAH BIOREMEDIATION IN SOIL BY A MIXED MICROBIAL CONSORTIUM

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Comparative metagenomic analysis of PAH degradation in soil by a mixed microbial consortium

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Abstract

Metagenomics can be used to monitor microbial communities, providing access to the taxonomic and functional gene composition of soil microbial communities and thus giving information on potentially novel biocatalysts or enzymes and phylogenetic and functional relationships as well. In this study, we used a taxonomic and functional metagenomic approach to analyze some of the effects (e.g. displacement, permanence, disappearance) produced between native microbiota and a previously constructed PAH-degrading microbial consortium during the bioremediation of a soil polluted with PAHs, and provided insights regarding the metabolic pathways and specific enzymes involved in the bioremediation process. Bioaugmentation with the fungal-bacterial consortium and biostimulation with corn stover produced appreciable changes in the microbial diversity of polluted soils, shifting native microbial communities in favor of degrading specific populations. Functional metagenomics showed changes in gene abundance suggesting a bias towards aromatic hydrocarbon and intermediary degradation pathways, greatly favoring PAH mineralization. In contrast, pathways favoring the formation of toxic intermediates such as cytochrome P450-mediated reactions were found to be significantly reduced in bioaugmentated soils. PAH biodegradation using the microbial consortium was faster and reached higher degradation values when compared with other mixed microbial consortia in soil, as a result of an increased co-metabolic degradation. The main differences between inoculated and non-inoculated soils were observed in aromatic ring-hydroxylating dioxygenases, laccase, protocatechuate, salicylate and benzoate-degrading enzyme genes. Based on our results, we propose several concurrent metabolic pathways taking place in soils during PAH degradation.

Keywords: Polycyclic Aromatic Hydrocarbons **(**PAHs), Metagenomics, Soil pollution, bioremediation, aromatic-ring-hydroxylating dioxygenases, cometabolism

Introduction

Contamination of soils with hydrocarbons has become a worldwide environmental issue, because of the potential toxic effects on animals, humans, plants and microorganisms. Continuous contamination with crude oil and its derivatives favor the deposition and accumulation of xenobiotics and toxic compounds in soils. Polycyclic Aromatic Hydrocarbons (PAHs) are considered priority environmental pollutants because of their high toxicity and persistence. PAHs are molecules with physical and chemical characteristics that greatly contribute to their persistence in soil, possessing toxic, mutagenic and teratogenic properties (Haritash and Kaushik, 2009). During the last century there has been an increase in the amount of PAHs released into the environment from anthropogenic sources and atmospheric deposition from natural sources (Juhasz and Naidu, 2000). Microbial degradation is considered the main natural degradation form of hydrocarbons in soils (Haritash and Kaushik, 2009; Juhasz and Naidu, 2000). Bioremediation, based on the use of microorganisms to degrade the contaminants, is a promising technology because of its high efficiency and cost-effectiveness. For over three decades it has been shown that microorganisms such as bacteria, fungi and algae possess specific catabolic activities that can be exploited for the remediation of soil and water impacted with low and high molecular weight PAHs (Cerniglia and Sutherland, 2010; Seo *et al.*, 2009).

Current knowledge about the microbial, functional and metabolic diversity and the impact produced by introduced microbial populations on native communities during the degradation of PAHs in soil is still limited. Metagenomics can be used to monitor microbial communities, providing access to the taxonomic and functional gene composition of soil microbial communities and thus giving information on potentially novel biocatalysts or enzymes and phylogenetic and functional relationships between them (Thomas *et al.*, 2012). Recent studies using Next-Generation Sequencing technologies during hydrocarbon bioremediation processes have shown the usefulness of these technologies to identify, monitor and estimate proportions of crude oil (Coulon *et al.*, 2012; dos Santos *et al.*, 2011) and diesel degrading populations (Yergeau *et al.*, 2012a; Yergeau *et al.*, 2012b) present in soils and bioreactors. Thus, metagenomic sequencing of soil microbial communities involved in PAH degradation can provide insights regarding the microbial populations, functional and metabolic profiles and specific enzymes involved during the bioremediation of soils.

In this study, we used a taxonomic and functional metagenomic approach to analyze the metabolic profiles and main enzymes involved during the bioremediation of a soil polluted with low and high molecular weight PAHs, as well as some of the effects (e.g. displacement, permanence, disappearance) produced between soil native microbiota and a PAH-degrading microbial consortium.

Materials and Methods

Soil samples

Uncontaminated soil samples obtained from the Xalostoc region in Tlaxcala, Mexico (19°24'08"N 98°02'54"W, 18°C annual average temperature) were used in this study. Soil was sandy loam with 2.4% organic matter, 1.4% total organic carbon, 0.063% nitrogen, 0.0023% phosphorous and pH of 8.41. Homogeneous samples were obtained at 30 cm depth in a simple random sampling, according to procedures described by US-EPA (1996). Soil samples were dried, homogenized and sieved with a 2 mm test sieve. Unsterile soil was then spiked with 2500 mg Kg^{-1} of a mixture of Phenanthrene (Phe), Pyrene (Pyr) and Benzo[a]pyrene (BaP) (1:1:1 ratio)

PAH-degrading consortium

A microbial PAH-degrading consortium (C1), composed by four fungal (*Aspergillus flavus* H6, *Aspergillus nomius* H7, *Rhizomucor variabilis* H9, *Trichoderma asperellum* H15) and five bacterial native strains (*Klebsiella pneumoniae* B1, *Bacillus cereus* B4, *Pseudomonas aeruginosa* B6, *Klebsiella* sp. B10, *Stenotrophomonas maltophilia* B14) was used for the bioaugmentation of PAH-contaminated soils. (Zafra *et al.*, 2014b). Individual strains composing the consortium were maintained aerobically at 30°C in Potato Dextrose Agar (PDA) plates containing 0.1% Maya crude oil (fungi) or liquid Basal Saline Medium (g l^{-1}: NaCl, 0.3; $(NH_4)_2SO_4$, 0.6; K_2HPO_4, 0.75; KH_2PO_4; 0.25; $MgSO_4.7H_2O$, 0.15; KNO_3, 0.6; yeast extract, 0.125) using 0.1% Maya crude oil as sole carbon source (bacteria).

Microcosm treatability tests

Treatability assays were performed in microcosm solid culture systems using sterile corn stover (35.7% carbon, 0.465% nitrogen, 0.000031% phosphorous) as fungal growth support, texturizing and biostimulation agent. Corn stover (0.35 g dry weight) was placed in 100 ml glass flasks moistened with Czapeck medium (g L^{-1}: sucrose, 30; sodium nitrate, 3; dipotassium phosphate, 1; magnesium sulfate, 0.5; potassium chloride, 0.5; ferrous sulfate, 0.01; pH 7.3) to reach 30% moisture content, inoculated with 2×10^6 spores g^{-1} of each fungal strain and incubated for 5 d at 30°C. Pre-inoculated corn stover was then mixed with 6.65 g of PAH-contaminated soil and inoculated with 2×10^6 CFU g^{-1} of each bacterial strain. Inoculated corn stover/contaminated soil mixtures were incubated at 30°C for 30 days, flushing headspaces every 48 h for 10 min with sterile and moistened air to preserve aerobic conditions and avoid carbon dioxide accumulation. Non-inoculated systems (only biostimulated with corn stover) were used to compare the effect of the bioaugmentation with C1 consortium on PAH degradation, as well on taxonomic composition and functional profiles. Microcosms were sampled at days 0 (post-inoculation), 16 and 30 for PAH quantitation and DNA extraction. Assays were carried out in triplicate.

Microcosm heterotrophic activity and PAH measurements

Heterotrophic activity in microcosms was measured each 48 h by means of CO_2 production, using an Agilent 6890 series Gas Chromatograph equipped with a thermal conductivity detector and a GS-CarbonPLOT column. CO_2 was reported as milligrams of CO_2 per g of initial dry matter (IDM). Residual PAHs from soils were extracted from 1 g of IDM (soil plus corn stover) with the addition of 25 ml of a dichloromethane-acetone solution (7:3 ratio) using an Anton Paar Multiwave 3000 SOLV apparatus for 20 min, according to EPA 3546 method. The resulting extracts were evaporated, suspended in 2 ml of acetonitrile and analyzed in an HP Agilent 1100 HPLC system equipped with a C18 reverse-phase column, with an UV absorbance detector set at 245-360 nm under isocratic conditions in acetonitrile:water (90:10) and a flow rate of 1 ml/min.

Metagenomic sequencing and bioinformatic analysis

Metagenomic DNA was isolated from 2 g IDM (soil plus corn stover) using the PowerSoil DNA isolation Kit (MoBio Laboratories Inc., USA) following the manufacturer's instructions. Extracted DNA preparations were quantified and quality checked using a Nanodrop 1000 Spectrophotometer (Thermo Scientific). Paired-end whole genome shotgun sequencing was performed using an Illumina MiSeq 2x250 platform. Resulting metagenomic unassembled reads were quality checked with FastQC v0.10.1 software (Andrews, 2012), compared and annotated using the MG-RAST server (Meyer *et al.*, 2008). Quality check included sequence quality filtering, length filtering and dereplication steps. Taxonomic analysis were made using the M5nr protein database (Wilke *et al.*, 2012) with a maximum e-value of $1e^{-5}$ and a minimum identity cutoff and alignment length of 60% and 15 bp respectively. SEED subsystems annotation and KEGG orthology was used for functional hierarchical classification using a maximum e-value of $1e^{-5}$, a minimum identity cutoff of 60% and an alignment length cutoff of 15 bp. Metagenomic data were also analyzed with standalone BLASTX v2.2.29 (Altschul *et al.*, 1997) using a custom database containing sequences from ring hydroxylating dioxygenases, monooxygenases, ligninolytic peroxidases and laccases, and subsequently annotated with MEGAN5 (Huson *et al.*, 2011). Results from taxonomic and functional analysis were visualized within the MG-RAST server (Meyer *et al.*, 2008), MEGAN5 and STAMP version v2.0.3 (Parks and Beiko, 2010) programs.

Statistical Analysis

Data from CO_2 and PAH measurements were analyzed by Analysis of Variance (ANOVA) followed by a multiple comparison test (LSD) with SPSS Statistics Software version 19 (IBM), considering statistically significant differences those with a p value <0.05. Statistical analysis of metagenomic profiles was performed with the STAMP v2.0.3 software.

Results and discussion

Heterotrophic activity in soil microcosms

Soil microcosms inoculated with consortium C1 presented a rapid initial increase in CO_2 production by day 2, with a slightly lower production by biostimulation-only microcosms. Bioaugmentated/biostimulated microcosms produced more CO_2 than biostimulated-only soils during the first two weeks, but the tendency reverted from day 16 and higher instantaneous CO_2 production was observed for biostimulated-only microcosms, even though accumulated CO_2 levels by day 30 were almost identical (Fig. 1). In general, CO_2 production through the biodegradation process in soil was higher when compared with reported values of similar mixed consortia in microcosms, where maximum accumulated production only reached 1 mg CO_2 gr^{-1} IDM after 60 d growth (Jacques *et al.*, 2008). As expected, inoculation of soils promoted an increase in respiratory levels, especially taking into account that the spiking of soils with high amounts of PAHs could greatly affect the quantity and diversity of native soil populations, which could explain the differences in CO_2 production observed during the first two weeks. Regardless of soil inoculation, biostimulation treatment with corn stover promoted an increase in CO_2 levels, as uncontaminated but non-biostimulated microcosms produced lower but not significantly different CO_2 levels than both of the biostimulated systems.

PAH biodegradation in soil

Despite producing CO_2 amounts similar to biostimulated-only microcosms, PAH degradation was significantly higher in soils treated with consortium C1. As observed in Figure 2, Phe was degraded at a higher rate during the first 16 days than Pyr and BaP by consortium C1 (58.12%, 36.64% and 27.16%, respectively) but interestingly Pyr, and more notoriously BaP, were degraded at a higher rate after day 16 until day 30 reaching 59.66% and 58.98% degradation. These results are in accordance with our previous report using a similar native degrading consortium, where HMW-PAH degradation in sterile soils was relatively low at initial stages, but at later stages increased importantly (Zafra *et al.*, 2014b). In fact, an increased co-metabolic degradation of HMW PAHs such as Pyr and BaP in a mixture with LMW PAHs can be achieved in response to the induction of LMW PAH degradation pathways (Juhasz and Naidu, 2000; Yuan *et al.*, 2003). PAH biodegradation with consortium C1 was also faster and showed higher degradation values when compared with other mixed microbial consortia in soil (Kim *et al.* 2007; Li *et al.* 2009).

Figure 1. Microbial activity (CO_2 production) of inoculated and non-inoculated soil microcosms

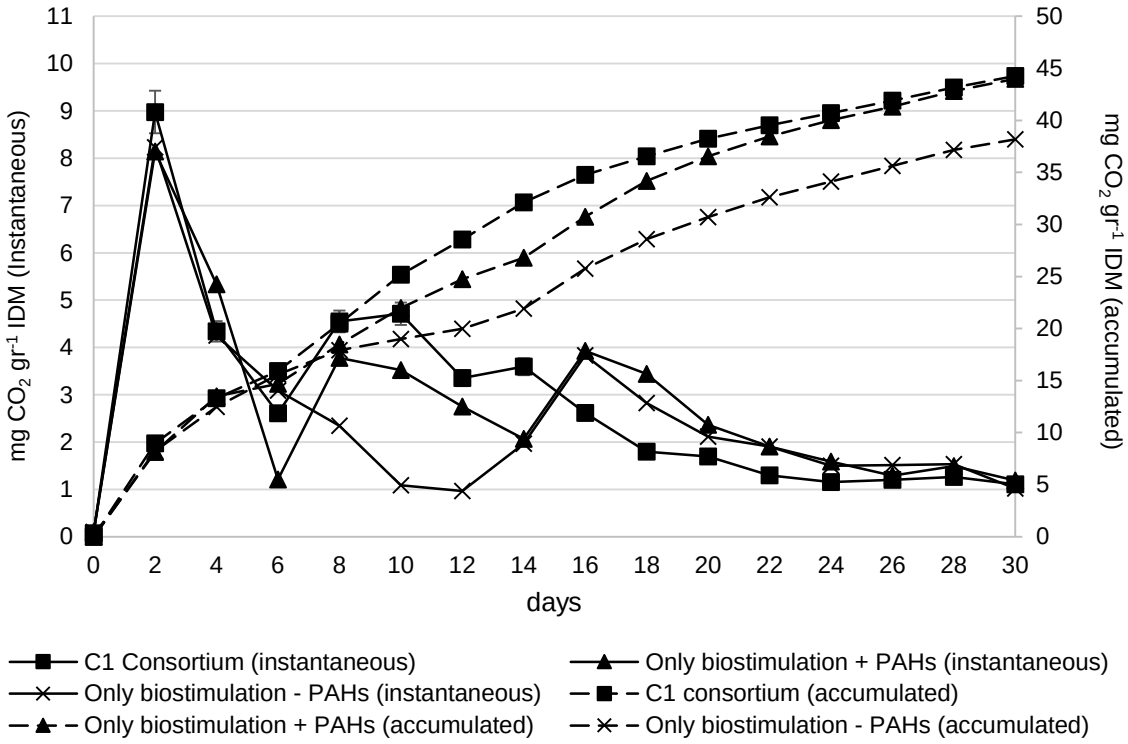
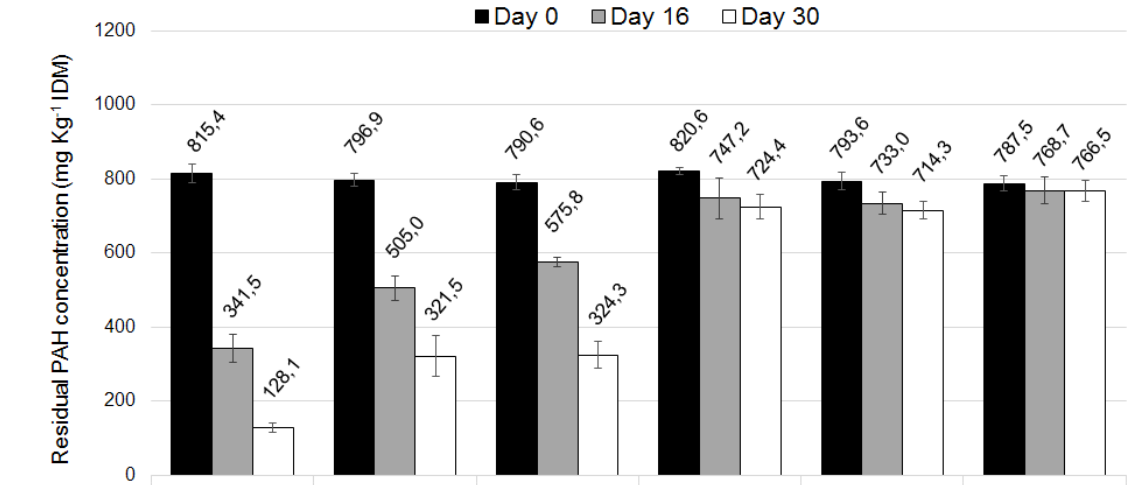


Figure 2. PAH degradation by C1-inoculated and biostimulated-only soil microcosms with non-sterile soil spiked with 2500 mg Kg^{-1} Phe, Pyr and BaP.



Even though microbial growth rates are not necessarily related to the degree of pollutant degradation (Montgomery *et al.*, 2010), a higher growth rate along with an effective PAH biodegradation is suggestive of their utilization as a carbon source by microbial populations. Moreover, a decrease in the microbial growth rate could be indicative of microorganisms reaching stationary phase and secondary metabolism, producing hydrocarbon-degrading enzymes that could be positively influencing the oxidation of PAHs (Conesa *et al.*, 2002; Gochev and Krastanov, 2007). Control microcosms where only corn stover was added presented significantly lower degradation rates of Phe (11.73%), Pyr (10.00%) and BaP (2.67) by day 30 in comparison to inoculated soils. In fact, most of the degradation was produced during the first stage of the process, in a growth-dependent PAH degradation triggered by the presence of culture media and corn stover at day 0.

Taxonomic analysis of soil bioremediation

Taxonomic analysis of metagenomic data from soils showed an overall predominance of bacterial and a lesser portion of eukaryotic and archaeal organisms, regardless of their treatment or sampling day (Fig. 3A). Archaeal communities remained stable through the bioremediation process, showing only slight differences in community composition with the exception of control microcosms at day 30 (Fig. 3B). In contrast, soil inoculation with the microbial consortium resulted in appreciable shifts in bacterial populations, as treatment with consortium C1 produced a marked increase over time of Firmicutes, Actinobacteria, and Bacteroidetes taxa in detriment of Proteobacteria (Fig. 3C). However, the proportion of Proteobacteria was visibly higher in inoculated microcosms than in biostimulation-only microcosms, and the opposite was observed for Firmicutes as control microcosms contained a higher proportion of this taxa than inoculated systems. As the microbial consortium contained members of Firmicutes (*Bacillus cereus* H4) and Proteobacteria (*K. pneumoniae* H1, *P. aeruginosa* H6, *Klebsiella* sp. H10 and *S. maltophilia* B14), this displacement could indicate a better adaptation of Proteobacteria and a positive effect over other communities, particularly in Bacteroidetes/Chlorobi group, whose proportion in control microcosms was lower.

Figure 3 (next page). Taxonomic composition of different phyla based on the metagenomic libraries from microcosms. (A) domain level; (B), Archaeal phyla level; (C) Bacterial phyla level; (D) Proteobacteria orders; (E) Fungal orders; (F) Ascomycota orders; (G) and (H), Bacterial and fungal taxa included in the degrading consortium.

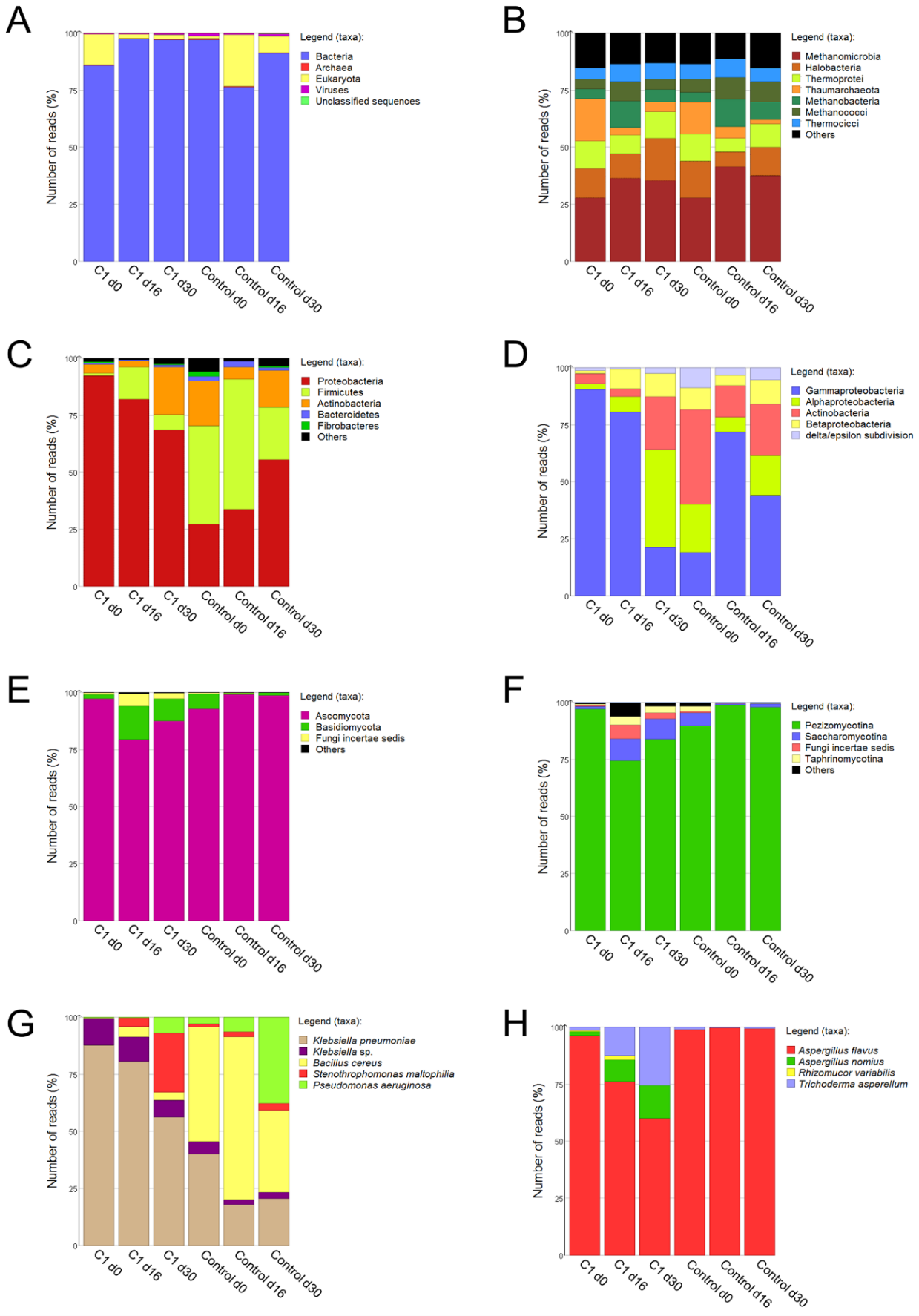
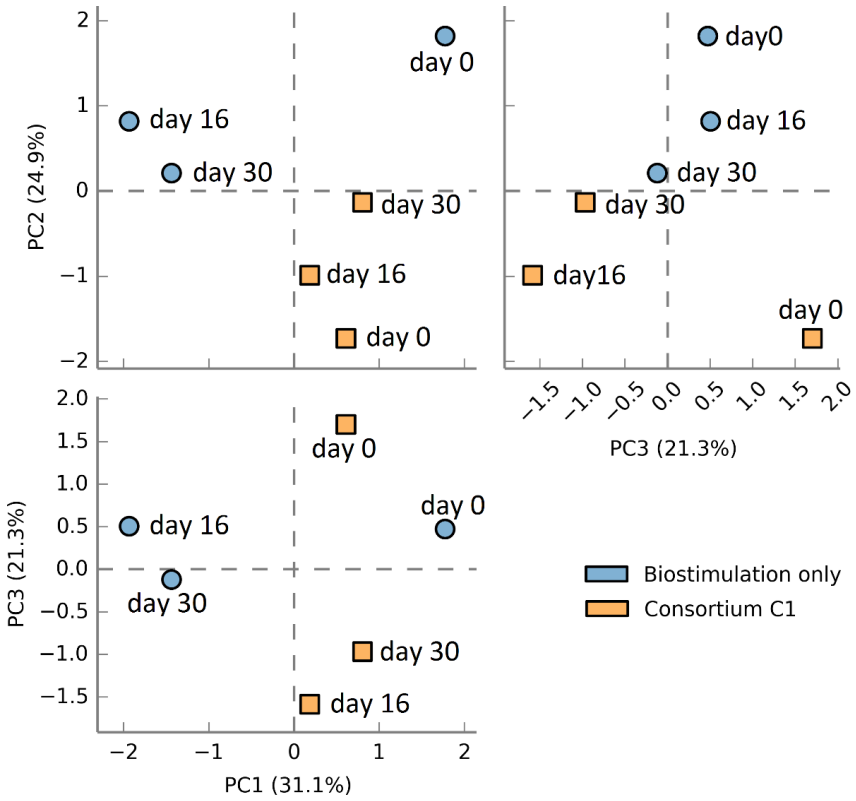

A
Number of reads (%)
Legend (taxa):
Bacteria
Archaea
Eukaryota
Viruses
Unclassified sequences
C1 d0
C1 d16
C1 d30
Control d0
Control d16
Control d30
B
Legend (taxa):
Methanomicrobia
Halobacteria
Thermoprotei
Thaumarchaeota
Methanobacteria
Methanococci
Thermococci
Others
C
Legend (taxa):
Proteobacteria
Firmicutes
Actinobacteria
Bacteroidetes
Fibrobacteres
Others
D
Legend (taxa):
Gammaproteobacteria
Alphaproteobacteria
Actinobacteria
Betaproteobacteria
delta/epsilon subdivision
E
Legend (taxa):
Ascomycota
Basidiomycota
Fungi incertae sedis
Others
F
Legend (taxa):
Pezizomycotina
Saccharomycotina
Fungi incertae sedis
Taphrinomycotina
Others
G
Legend (taxa):
Klebsiella pneumoniae
Klebsiella sp.
Bacillus cereus
Stenothrophomonas maltophilia
Pseudomonas aeruginosa
H
Legend (taxa):
Aspergillus flavus
Aspergillus nomius
Rhizomucor variabilis
Trichoderma asperellum

Figure 4. Principal Component Analysis of taxonomic profiles at species level using the ANOVA and Tukey-Kramer tests.



Within Proteobacteria, Alpha and Gammaproteobacteria were the most abundant taxa (Fig. 3D), both containing known species of PAH degraders (Bautista *et al.*, 2009; Boonchan *et al.*, 2000; Chen *et al.*, 2008). Similar results have been reported previously in microcosms containing heavy crude oil-contaminated soils, where members of Gammaproteobacteria were initially dominant in the contaminated microcosms but after 90 days of incubation were superseded by Firmicutes and Alphaproteobacteria (Bordenave *et al.*, 2007). Fungal populations remained relatively stable over time, being Ascomycota the most abundant taxa with a minor proportion of sequences belonging to Basidiomycota and incertae sedis (Fig. 3E). Within Ascomycota phylum, Pezizomycotina was the most prominent group (Fig. 3F). This is not unexpected as Pezizomycotina groups all fungal strains included in the degrading consortium, except for *R. variabilis* H9.

Population dynamics of bacterial and fungal species conforming the degrading consortium showed important shifts across time, exhibiting marked differences compared to control microcosms. *Stenotrophomonas maltophilia* showed to be the most prominent bacterial organism in inoculated microcosms with a constant increase, concomitant with PAH degradation, while

Klebsiella populations showed a tendency to disappear over time (Fig. 3G). A similar situation was observed with fungal species, where *Aspergillus flavus* predominated in soils inoculated with consortium C1 and *Trichoderma asperellum* and *Aspergillus nomius* increased over time (Fig. 3H). *Rhizomucor variabilis* populations disappeared after day 16 suggesting a poor adaptation to soil and presumably a displacement by other microorganisms. These findings are in accordance with previous results at our laboratory using PCR-DGGE to monitor the survival of consortium C1in PAH-contaminated sterile soils, where *R. variabilis* H9 was no longer detectable after day 20 (manuscript in preparation). With the exception of *Rhizomucor variabilis*, the microbial species conforming the degrading consortium remained relatively stable across time in control microcosms, with a higher proportion of *Bacillus cereus, Klebsiella pneumoniae and Aspergillus flavus.* In addition, PCA grouping analysis showed clear differences between soil metagenomes as a consequence of soil inoculation (Fig. 4).

Functional and metabolic analyses of PAH bioremediation

SEED functional subsystems annotation showed a predominance of genes belonging to metabolism pathways in soils, especially for carbohydrate, aminoacid and energy metabolism (Fig. 5A). Even though aromatic degradation pathways did not show appreciable differences in soils at SEED level 1, a deeper look focusing on degradation pathways for aromatic compounds revealed a dominance of peripheral pathways for aromatic degradation genes in both inoculated and non-inoculated soils, while metabolism of central aromatic intermediaries were also found at relatively high levels (Fig. 5B). Interestingly, gene abundance of aromatic anaerobic degradation pathways was higher, although non-significantly different, in non-inoculated microcosms. As all treatments used corn stover as a texturizing, this difference could be directly attributed to the presence of members of the degrading consortium, which could potentially led to a higher proportion of hydrocarbon aerobic-degradation genes in inoculated soils.

Figure 5 (next page). SEED subsystems functional heatmap of metagenomes from inoculated and non-inoculated microcosms during the bioremediation treatment. A) Differences at level 1 subsystems; B) Differences in subsystems related to metabolism of aromatic compounds

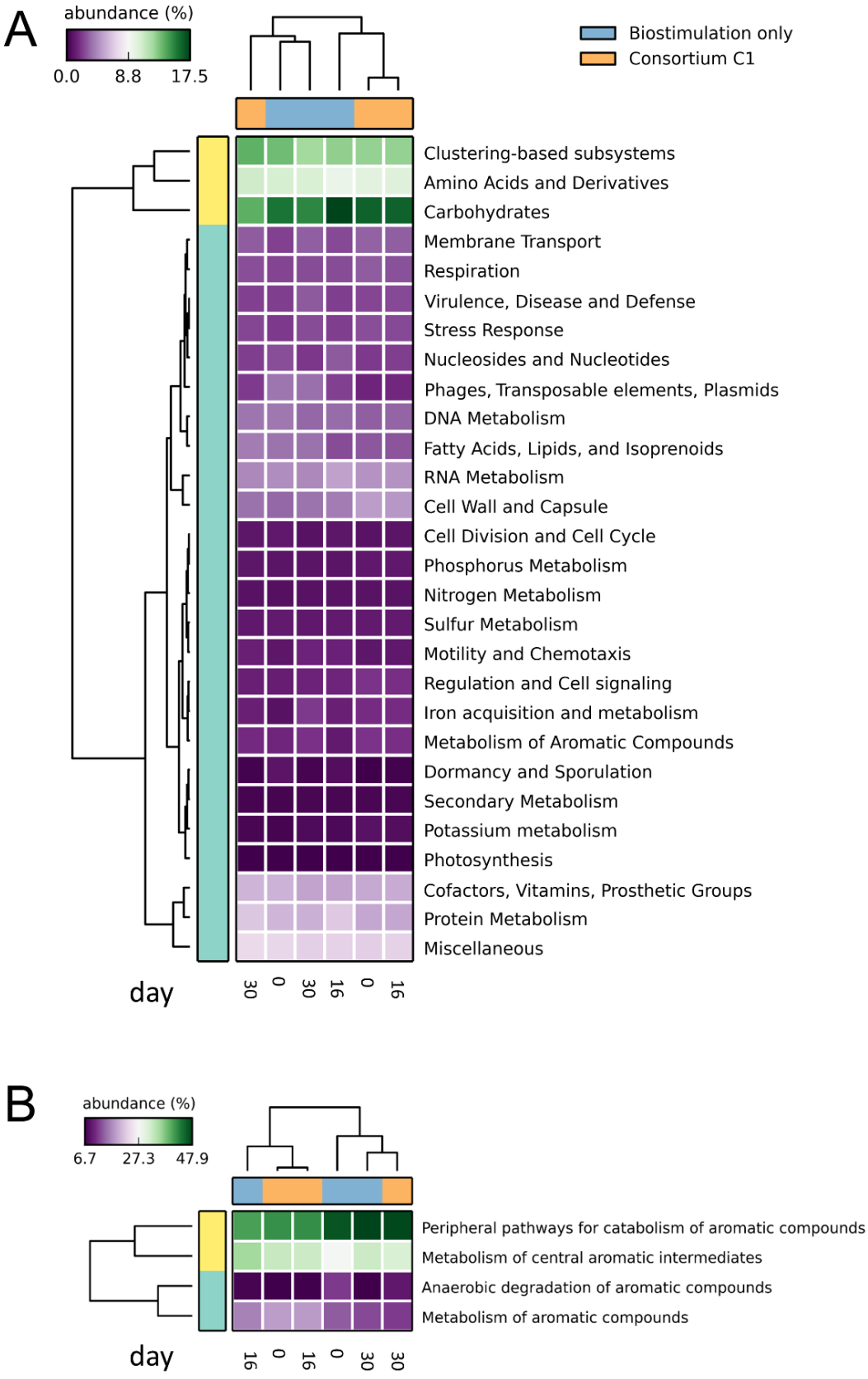
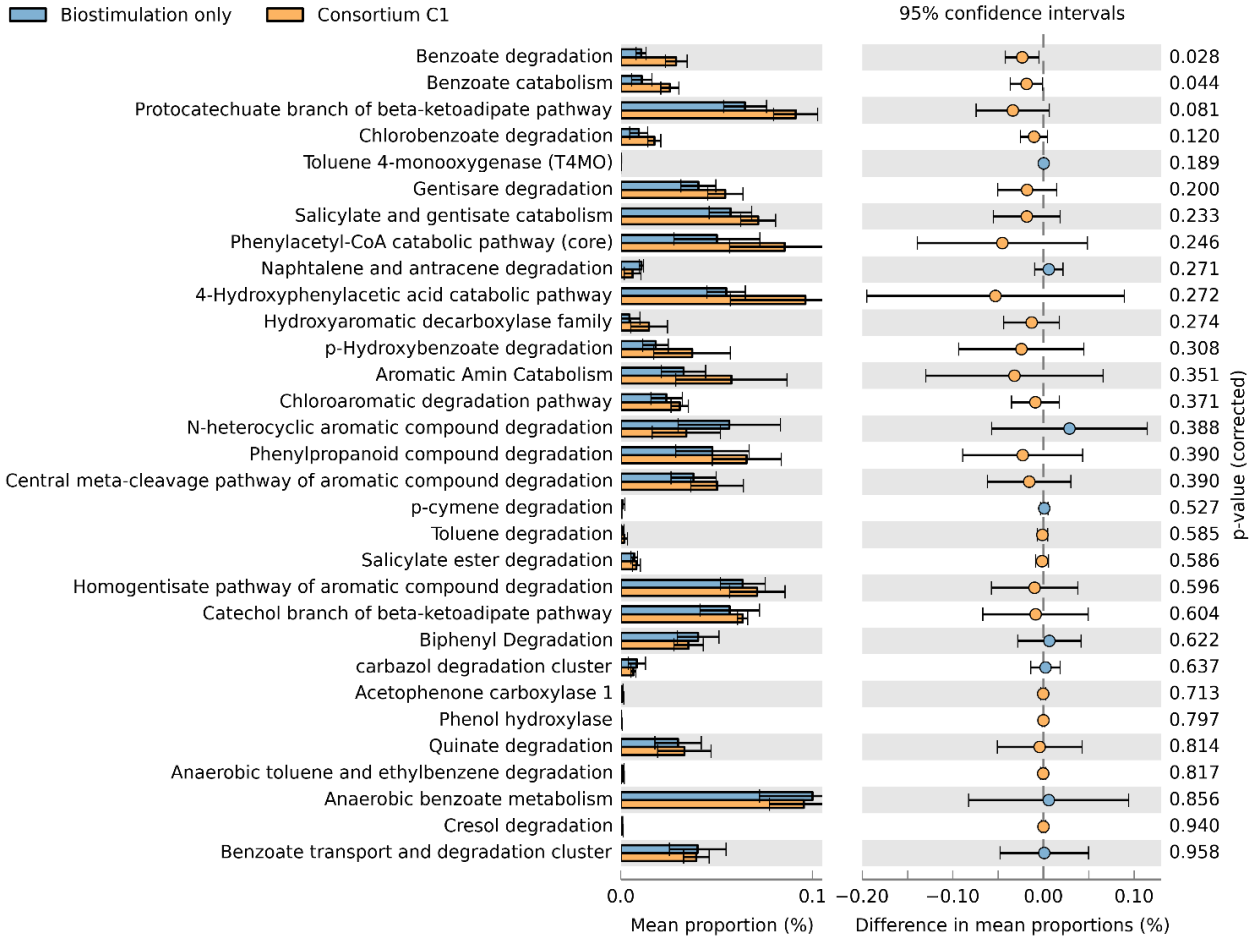
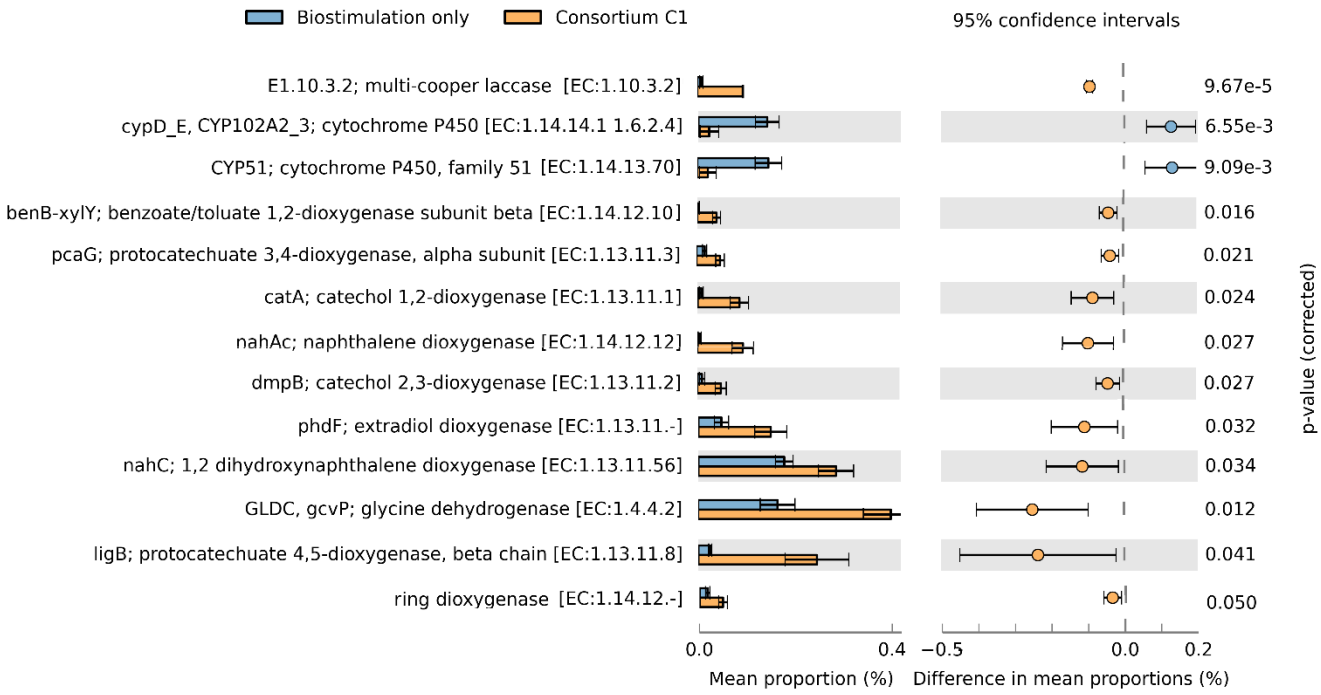

A
abundance (%)
0.0
8.8
17.5
Biostimulation only
Consortium C1
Clustering-based subsystems
Amino Acids and Derivatives
Carbohydrates
Membrane Transport
Respiration
Virulence, Disease and Defense
Stress Response
Nucleosides and Nucleotides
Phages, Transposable elements, Plasmids
DNA Metabolism
Fatty Acids, Lipids, and Isoprenoids
RNA Metabolism
Cell Wall and Capsule
Cell Division and Cell Cycle
Phosphorus Metabolism
Nitrogen Metabolism
Sulfur Metabolism
Motility and Chemotaxis
Regulation and Cell signaling
Iron acquisition and metabolism
Metabolism of Aromatic Compounds
Dormancy and Sporulation
Secondary Metabolism
Potassium metabolism
Photosynthesis
Cofactors, Vitamins, Prosthetic Groups
Protein Metabolism
Miscellaneous
day
30 0 30 16 0 16
B
abundance (%)
6.7
27.3
47.9
Peripheral pathways for catabolism of aromatic compounds
Metabolism of central aromatic intermediates
Anaerobic degradation of aromatic compounds
Metabolism of aromatic compounds
day
16 0 16 0 30 30

Fig 6. SEED extended error bar plot of genes from soil metagenomes belonging to the metabolism of aromatic compounds subsystem. Pairwise comparisons were made with a two-sided Welch´s t-test.



The inoculation of soils promoted a significant increase in the abundance of genes involved in benzoate degradation and metabolism, protocatechuate degradation and chloroaromatic compounds degradation, as well non-significant increments in the abundance of gentisate degradation, salicylate and gentisate catabolism genes (Fig. 6). A similar set of enzyme-encoding genes has been found to have an important role in the degradation of aromatic compounds, producing key metabolic intermediates including salicylate, 3-chlorobenzoate and hydroquinone that act as strong inducers of PAH degradative enzymes (Uchiyama and Miyazaki, 2013). A well-studied molecule is salicylate, which induces naphthalene dioxygenase in bacteria and also stimulates the degradation of HMW-PAHs by bacteria (Chen and Aitken, 1999; Shamsuzzaman and Barnsley, 1974). The observed differences in gene abundance indicate an overall increase in aerobic pathways involved in the degradation of aromatic compounds into products that can enter the tricarboxylic acid cycle, greatly favoring the mineralization of PAHs.

Fig 7. Extended error bar plot of genes from soil metagenomes where two-sided Welch´s t-test produced a *p*-value ≤0.05. All genes shown are restricted to aromatic and intermediate degradation subsystems

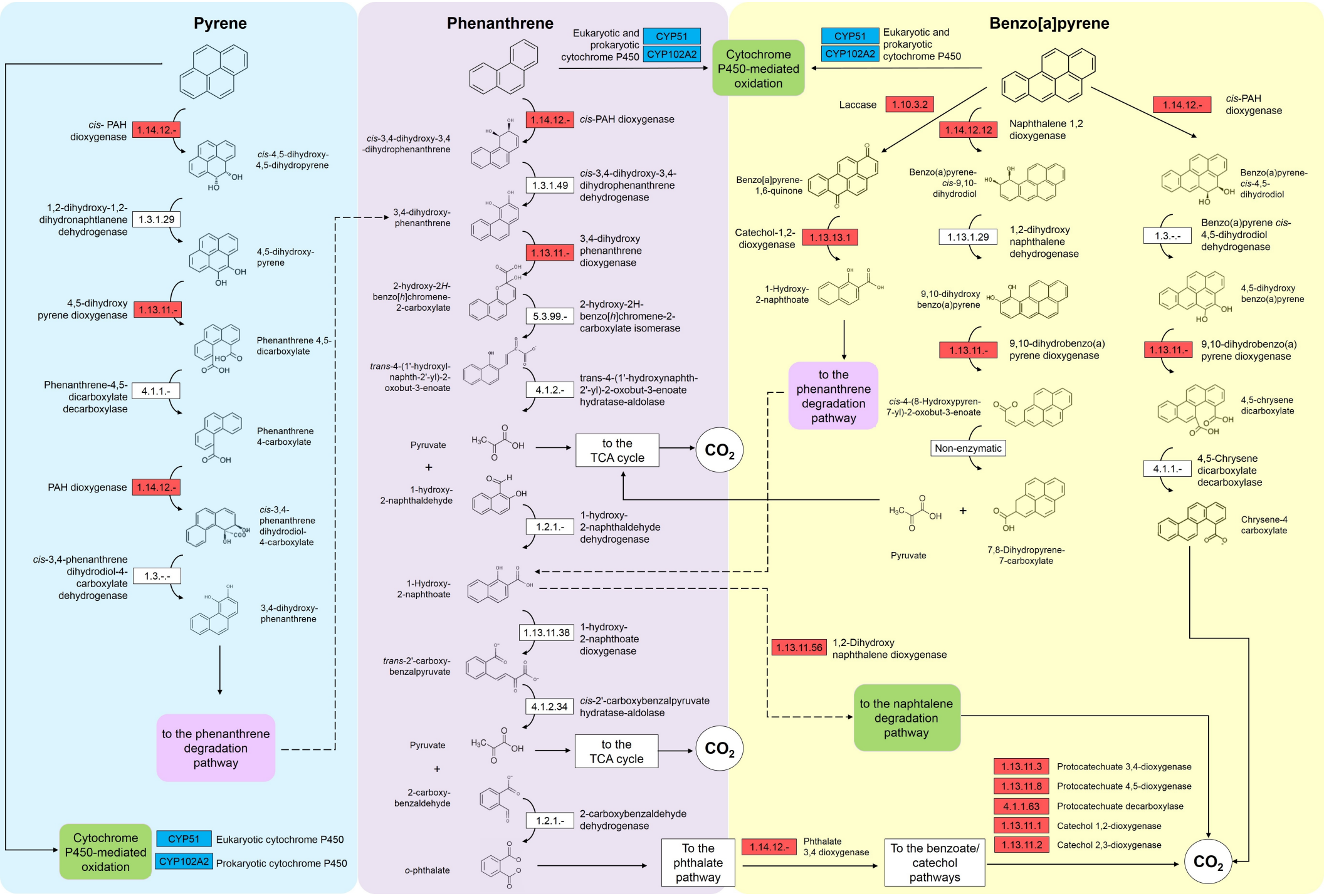


This was also corroborated when aerobic specific ring hydroxylating cleavage enzymes such as naphthalene 1,2-dioxygenase, extradiol dioxygenase, ring hydroxylating dioxygenase, benzoate/toluate-1,2 dioxygenase, catechol 1,2-dioxygenase, catechol 2,3-dioxygenase, protocatechuate 3,4-dioxygenase, protocatechuate 4,5-dioxygenase and 1,2-dihydroxynaphthalene dioxygenase were found at significantly higher levels in inoculated microcosms when compared to non-inoculated controls (Fig. 7). Such enzymes have also been found at elevated levels in metagenomic analyses of the bioremediation of soils and sediments contaminated with diesel (Yergeau *et al.*, 2012b), and crude oil (Kimes *et al.*, 2013; Mason *et al.*, 2014). Even though these enzymes have been mainly described in bacteria, several non-ligninolytic fungal species are also known to produce *meta* and *ortho*-cleavage dioxygenases potentially involved in PAH degradation (Fetzner, 2012; Hadibarata *et al.*, 2007). In addition, laccase gene abundance was also found to be significantly increased in inoculated soils, indicating the involvement of eukaryotic organisms in both the initial oxidation and intermediate steps of PAH degradation. A particular case is *Trichoderma asperellum* H15, which has been found to produce both ring-cleavage dioxygenases and laccase in presence of PAHs (Zafra *et al.*,

2014c). Our results showed a higher abundance of laccase genes concomitant with the survival and permanence of *T. asperellum* H15 in inoculated soils (Fig. 3H). Remarkably, the abundance of bacterial and eukaryotic genes involved in PAH metabolism via cytochrome P450 monooxygenases were found to be significantly reduced when the microbial consortium was inoculated to soils (Fig. 7). This indicate a bias towards PAH mineralization pathways, potentially decreasing the formation of dead-end metabolites, toxic intermediates (e.g. *trans*-dihydrodiols) and conjugates (e.g. *o*-glucuronides, *o*-glucosides and methoxy molecules) responsible for the formation of DNA adducts and subsequent carcinogenesis in mammals (Warshawsky, 1999).

Taking into account the results from the functional analysis, we found several concomitant degradation pathways taking place in inoculated soils for the degradation of Phe, Pyr and BaP (Fig. 8). Initial oxidation steps are driven by laccases and ring hydroxylating dioxygenases, while subsequent steps involve the action of dioxygenases and decarboxylases, promoting the further degradation of PAHs via central metabolic pathways. It is interesting to find significantly higher proportions of several broad specificity enzyme-encoding genes in inoculated soils, such as naphthalene 1,2-dioxygenase (EC 1.14.12.12), possessing a wide substrate specificity which eventually permits the *cis*-hydroxilation of several other aromatic compounds including HMW-PAHs (Selifonov *et al.,* 1996). Our results showed that the same enzyme, along with a similar bacterial ring cleavage dioxygenase referred to here as *cis*-PAH dioxygenase (EC 1.14.12.-) have an important role in the initial and intermediate steps of Phe, Pyr and BaP degradation in inoculated soils. Assuming that both inoculated and native soil microorganisms are able to use broad specificity enzymes and common pathways to metabolize multiple PAHs, the inducers for the degradation of a single PAH could eventually co-stimulate the degradation of a wide range of PAHs, as has been reported (Chen and Aitken, 1999).

Figure 8 (next page). Proposed metabolic pathways for the degradation of phenanthrene, pyrene and benzo[a]pyrene by the microbial consortium C1 in PAH-polluted soils. Red squares denotes the enzyme-encoding genes found to be significantly more abundant in inoculated soils. Blue squares denotes the enzyme-encoding genes found to be significantly less abundant in inoculated soils.


Pyrene
cis- PAH dioxygenase
1.14.12.-
cis-4,5-dihydroxy-4,5-dihydropyrene
1,2-dihydroxy-1,2-dihydronaphtlanene dehydrogenase
1.3.1.29
4,5-dihydroxy-pyrene
4,5-dihydroxy pyrene dioxygenase
1.13.11.-
Phenanthrene 4,5-dicarboxylate
Phenanthrene-4,5-dicarboxylate decarboxylase
4.1.1.-
Phenanthrene 4-carboxylate
PAH dioxygenase
1.14.12.-
cis-3,4-phenanthrene dihydrodiol-4-carboxylate
cis-3,4-phenanthrene dihydrodiol-4-carboxylate dehydrogenase
1.3.-.-
3,4-dihydroxy-phenanthrene
to the phenanthrene degradation pathway
Cytochrome P450-mediated oxidation
CYP51
Eukaryotic cytochrome P450
CYP102A2
Prokaryotic cytochrome P450
Phenanthrene
Eukaryotic and prokaryotic cytochrome P450
CYP51
CYP102A2
Cytochrome P450-mediated oxidation
1.14.12.-
cis-PAH dioxygenase
cis-3,4-dihydroxy-3,4-dihydrophenanthrene
1.3.1.49
cis-3,4-dihydroxy-3,4-dihydrophenanthrene dehydrogenase
3,4-dihydroxy-phenanthrene
1.13.11.-
3,4-dihydroxy phenanthrene dioxygenase
2-hydroxy-2H-benzo[h]chromene-2-carboxylate
5.3.99.-
2-hydroxy-2H-benzo[h]chromene-2-carboxylate isomerase
trans-4-(1'-hydroxyl-naphth-2'-yl)-2-oxobut-3-enoate
4.1.2.-
trans-4-(1'-hydroxynaphth-2'-yl)-2-oxobut-3-enoate hydratase-aldolase
Pyruvate
+
to the TCA cycle
CO2
1-hydroxy-2-naphthaldehyde
1.2.1.-
1-hydroxy-2-naphthaldehyde dehydrogenase
1-Hydroxy-2-naphthoate
1.13.11.38
1-hydroxy-2-naphthoate dioxygenase
trans-2'-carboxy-benzalpyruvate
4.1.2.34
cis-2'-carboxybenzalpyruvate hydratase-aldolase
Pyruvate
+
to the TCA cycle
CO2
2-carboxy-benzaldehyde
1.2.1.-
2-carboxybenzaldehyde dehydrogenase
o-phthalate
To the phthalate pathway
Benzo[a]pyrene
CYP51
CYP102A2
Eukaryotic and prokaryotic cytochrome P450
Laccase
1.10.3.2
1.14.12.-
cis-PAH dioxygenase
1.14.12.12
Naphthalene 1,2 dioxygenase
Benzo[a]pyrene-1,6-quinone
Benzo(a)pyrene-cis-9,10-dihydrodiol
Benzo(a)pyrene-cis-4,5-dihydrodiol
Catechol-1,2-dioxygenase
1.13.13.1
1.13.1.29
1,2-dihydroxy naphthalene dehydrogenase
1.3.-.-
Benzo(a)pyrene cis-4,5-dihydrodiol dehydrogenase
1-Hydroxy-2-naphthoate
9,10-dihydroxy benzo(a)pyrene
4,5-dihydroxy benzo(a)pyrene
1.13.11.-
9,10-dihydrobenzo(a) pyrene dioxygenase
1.13.11.-
9,10-dihydrobenzo(a) pyrene dioxygenase
to the phenanthrene degradation pathway
cis-4-(8-Hydroxypyren-7-yl)-2-oxobut-3-enoate
4,5-chrysene dicarboxylate
Non-enzymatic
4.1.1.-
4,5-Chrysene dicarboxylate decarboxylase
Pyruvate
+
7,8-Dihydropyrene-7-carboxylate
Chrysene-4 carboxylate
1.13.11.56
1,2-Dihydroxy naphthalene dioxygenase
to the naphtalene degradation pathway
1.13.11.3
Protocatechuate 3,4-dioxygenase
1.13.11.8
Protocatechuate 4,5-dioxygenase
4.1.1.63
Protocatechuate decarboxylase
1.13.11.1
Catechol 1,2-dioxygenase
1.13.11.2
Catechol 2,3-dioxygenase
1.14.12.-
Phthalate 3,4 dioxygenase
To the benzoate/ catechol pathways
CO2

Conclusions

Our results demonstrate the efficiency of a mixed microbial consortium to degrade high amounts of PAHs in soils, as a result of an increased co-metabolic degradation. Metagenomic analysis showed that inoculation of PAH-degrading consortium produced appreciable changes in the microbial diversity of PAH-contaminated soils, shifting native microbial communities in favor of degrading specific populations. Functional metagenomics showed changes in gene abundance indicating a bias towards aromatic hydrocarbons and intermediary degradation pathways, greatly favoring PAH mineralization.

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CHAPTER 8

DEGRADATION OF AROMATIC HYDROCARBONS BY *TRICHODERMA* SPECIES: AN OVERVIEW

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MINIREVIEW

Degradation of aromatic hydrocarbons by *Trichoderma* species: an overview

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Abstract

Fungi belonging to *Trichoderma* genus are ascomycetes found in soils worldwide. *Trichoderma* species have been studied in relation with diverse characteristics and biotechnological applications and are known successful colonizers of their habitats. Members of the genus have been well described as effective biocontrol organisms, through the production of secondary metabolites with potential applications as new antibiotics. Even though members of *Trichoderma* are used for the commercial production of lytic enzymes, as biological control agent and in the food industry, their use in the bioremediation of xenobiotic compounds is limited. *Trichoderma* stands out as a genus with a great range of substrate utilization, a high production of antimicrobial compounds and its ability for environmental opportunism. In this review, we focus on the recent advances in the research of *Trichoderma species* as potent and efficient aromatic hydrocarbon-degrading organisms, aiming to provide insight into its potential role in the bioremediation of soils contaminated with aromatic hydrocarbons.

Keywords: *Trichoderma, T. asperellum*, Polycyclic Aromatic Hydrocarbons (PAHs), bioremediation, xenobiotic metabolism, degradation

Introduction

Trichoderma is genus of filamentous fungi belonging to the Ascomycota division consisting of more than 100 species (Druzhinina *et al.*, 2006). They are commonly found in soils worldwide, occurring on root surfaces of plants, decaying bark and other organic materials. *Trichoderma* members have been studied in relation with diverse characteristics and biotechnological applications and are known successful colonizers of their habitats, possessing a potent enzymatic machinery (e.g. cellulases, chitinases, glucanases, proteases) for the decomposition and utilization of substrates present in soils, in special for the degradation of lignocellulosic materials (Jaklitsch, 2009). Members of the genus have also been described as effective biocontrol organisms, through the production of secondary metabolites with potential applications as new antibiotics (polyketides, pyrones, terpenes, amino acid derivatives and metabolites polypeptides) (Sivasithamparam, 1998). Furthermore, *Trichoderma* members are one of the biological control agents most commonly used against plant pathogens, and despite their mechanisms of action have not been clearly established, it is believed that the main mechanisms of control of the disease are those that act primarily on pathogens such as mycoparasitism, antibiosis, and competition for resources and space (Druzhinina *et al.*, 2011). However, it is also known that *Trichoderma* spp. interacts with plants, producing changes in their metabolism and thereby improves growth and resistance to biotic and abiotic stress.

Trichoderma species are commonly used for the commercial production of lytic enzymes, as biological control agent and in the food industry but its use in bioremediation is limited. Recent studies have shown the ability of *Trichoderma* to biotransform environmental pollutants, including hydrocarbons (Atagana, 2009; Su *et al.*, 2011). In this review we provided an overview and summarized the recent findings of the involvement of *Trichoderma* species on aromatic hydrocarbon degradation and assimilation, in order to evaluate its potential as a biological agent applied to polluted soils.

Trichoderma assimilates diverse hydrocarbon compounds

Even though *Trichoderma* is widely used for the commercial production of lytic enzymes and as a biological control agent, its use in pollutant bioremediation is scarce. Recent studies have shown the important ability of *Trichoderma* to biotransform heavy metals (Atagana, 2009; Su *et al.*, 2011) and hydrocarbons (Matsubara *et al.*, 2006). In fact, it is known that several species of the genus possess the ability to degrade and metabolize aromatic hydrocarbons, even in presence of heavy metals (Atagana, 2009; Verdin *et al.*, 2004). Numerous studies have evaluated the ability of some species of *Trichoderma* to metabolize unsaturated and aromatic hydrocarbons, crude oil, BTEX and resins. The species reported so far as hydrocarbon degraders include, but are not restricted to, *T. asperellum, T. koningii, T. pseudokoningii, T. longibrachiatum, T. hamatum, T. polysporum, T. harzianum, T. koningii, T. viride and T. virens* (Table 1).

Table 1. Reported *Trichoderma* species able to degrade hydrocarbons

Organism	Hydrocarbons	References
Trichoderma sp.	Crude oil BTEX Naphthalene Phenanthrene Chrysene Benzo[a]anthracene Benzo[a]fluoranthene Benzo[a]pyrene	(Argumedo-Delira *et al.*, 2012; Atagana, 2009; Hadibarata *et al.*, 2007; Hughes *et al.*, 2007)
Trichoderma asperellum	Phenanthrene Pyrene Benzo[a]pyrene	(Zafra *et al.*, 2014c)
Trichoderma harzianum	Crude oil Anthracene Pyrene	(Chaineau *et al.*, 1999; Ermisch and Rehm, 1989; Romero *et al.*, 2002)
Trichoderma hamatum	Crude oil	(Archegova *et al.*, 2012)
Trichoderma koningii	Crude oil Saturated hydrocarbons Pyrene	(Chaineau *et al.*, 1999; Hughes *et al.*, 2007; Ravelet *et al.*, 2000)
Trichoderma longibrachiatum	Phenanthrene Benzo[a]anthracene	(Cobas *et al.*, 2013; Rosales *et al.*, 2012)
Trichoderma pseudokoningii	Pyrene	(Chaineau *et al.*, 1999; Ravelet *et al.*, 2000)
Trichoderma polysporum	Crude oil	(Chaineau *et al.*, 1999)
Trichoderma virens	Crude oil, resins	(Hamzah *et al.*, 2012; Husaini, 2014; Singh, 2006)
Trichoderma viride	Pyrene Benzo[a]pyrene	(Ravelet *et al.*, 2000; Verdin *et al.*, 2004)

One common characteristic of these studies is the high tolerance exhibited by *Trichoderma* strains in presence of hydrocarbons and the sustained growth even in presence of other pollutants. However, degradation efficiencies greatly vary depending on individual strains and species, revealing that the chemical structure greatly influences the degradation capabilities by different species of *Trichoderma*. Based on the available reports, it is evident that unsaturated hydrocarbons degradation it is expected to be higher in comparison with those for the aromatic hydrocarbons and resins.

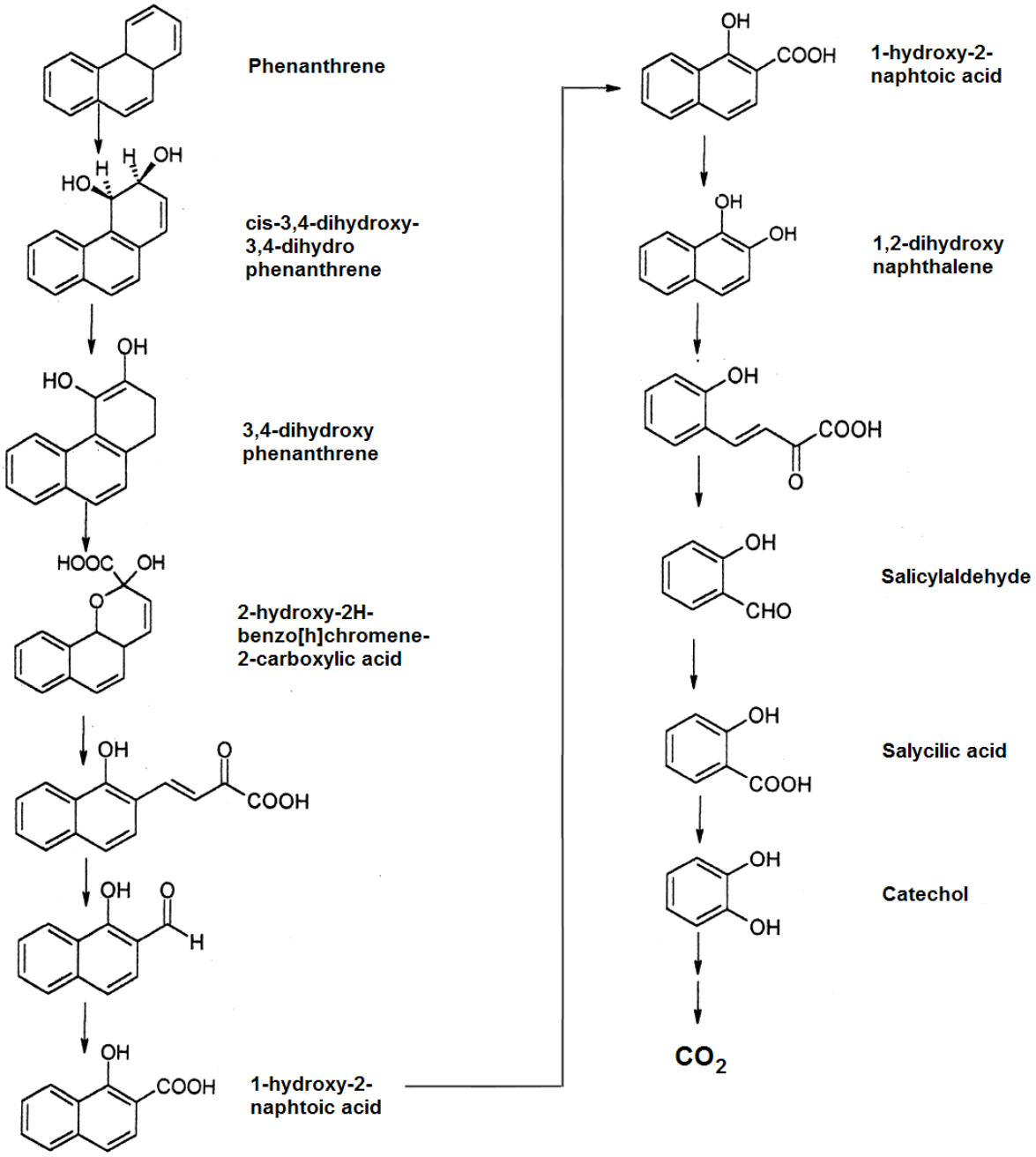
Degradation of aromatic compounds

Polycyclic Aromatic Hydrocarbons (PAHs) are one of the most important and persistent organic pollutants in soils, as they physicochemical properties confers them high recalcitrancy and resistance to microbial attacks. The degradation ability of aromatic ring-containing molecules are widely distributed in nature, and in fact, many species of aromatic-degrading bacteria and fungi have been isolated and characterized (Cerniglia and Sutherland, 2010; Juhasz and Naidu, 2000; Kanaly and Harayama, 2000). Several species within *Trichoderma* genus have been associated with the ability to degrade and metabolize PAHs such as naphthalene, phenanthrene, chrysene, pyrene and benzo[a]pyrene, (Atagana, 2009; Verdin *et al.*, 2004). The species reported as PAH degraders include *T. hamatum*, *T. harzianum*, *T. koningii*, *T. viride and T. virens* (Argumedo-Delira *et al.*, 2012; Cerniglia and Sutherland, 2010). In general, the rate of PAH degradation depends largely on the molecular weight of the molecules and the environmental compartment where they are present. Low molecular weight PAHs as naphthalene, phenanthrene or anthracene are more frequently reported as degradable by *Trichoderma* members. However, evidence point to a more relevant role of *Trichoderma* in PAH degradation in soils in presence of complex mixtures. Chaineau *et al.* (1999) and Hughes *et al.* (2007) reported a high range of PAH assimilation by *Trichoderma* strains, even in mixtures. The same was observed when *Trichoderma* sp. and *T. asperellum* were tested for its tolerance and degradation capabilities of PAHs in mixtures (Argumedo-Delira *et al.*, 2012; Zafra *et al.*, 2014b; Zafra *et al.*, 2014c).

Pathways leading to PAH degradation by *Trichoderma*

The initial steps of PAH metabolisms in many filamentous fungi involves the action of cytochrome P450 monooxygenases and epoxide hydrolases. The products may include *trans*-dihydrodiols, phenols, quinones and dihydrodiol epoxides, which eventually may be conjugated to form glucuronides, glucosides, xylosides, and sulfates, metabolites that can be more toxic than the parent molecule. Interestingly, several *Trichoderma* species possess alternate enzyme systems commonly seen in other organisms, such as multicooper laccases, peroxidases and ring-cleavage dioxygenases. These enzymes offers advantages for substrate utilization and improve their own survival in soils, but more importantly, they can be exploited for xenobiotic degradation.

Figure 1. Proposed pathway for the degradation of phenanthrene by *Trichoderma* spp. (modified from Hadibarata *et al.*, 2007)



As mentioned above, *Trichoderma* species have an advantageous enzymatic versatility. *Trichoderma* dioxygenases might be involved in the initial oxidation of the aromatic rings, forming unstable *cis*-dihidrodiols subsequently be degraded via the catechol pathway (Hadibarata *et al.* 2007). In addition, extracellular laccase production has been described for *T. atroviride, T. harzianum* and *T. asperellum* (Hölker *et al.*, 2002; Zafra *et al.*, 2014c). Laccase-mediated degradation of a range of several low and high molecular weight PAHs (e.g. phenanthrene, anthracene, benzo[a]pyrene) have been described using redox mediators such as ABTS (Xu *et al.*, 2001), representing an alternate way to initiate PAH oxidation.

As observed in our previous study with *T. asperellum*, the presence of PAHs led to a significant increase in the activity of catechol-1,2- and -2,3-dioxygenases during the initial stages of degradation in liquid cultures. In contrast, laccase activity was nearly undetectable during the initial days of degradation but increased notoriously from day 4, with a higher production in response to the presence of PAHs. In addition, increased peroxidase activity was also detected from day 6 to day 10, indicating a role of these enzymes in PAH degradation (Zafra *et al.*, 2014c). Hadibarata *et al.* (2007) reported a pathway for phenanthrene degradation, which eventually could be a common way for the mineralization of higher weight PAHs by *Trichoderma* (Fig 1). This way involves the action of catechol 1,2- and 2,3-dioxygenases in the initial steps of oxidation, with 1-hydroxy-2-naphthoic acid, salicylic acid, and catechol as major intermediaries.

T. asperellum: a highly versatile organism for soil bioremediation

Within the *Trichoderma* genus, *T. asperellum* stands out as a species with a great range of substrate utilization, a high production of antimicrobial compounds (Chutrakul *et al.*, 2008) and its ability for environmental opportunism (Ding *et al.*, 2012). *Trichoderma asperellum* has been used as a biological control agent against a wide range of disease organisms of plants including *Colletotrichum gloeosporioides, Phytophthora megakarya*, other pathogenic fungi and nematode (de los Santos-Villalobos *et al.*, 2013; Sharon *et al.*, 2007; Slusarski and Pietr, 2009; Tondje *et al.*, 2007).

The use of *T. asperellum* as a bioremediation agent of soils polluted with PAHs may present additional advantages to the use of other members of *Trichoderma*, such as its high growth rate, wide range of substrate utilization, growth-promoting effects in plants, and its versatility in the production of hydrolytic and oxidizing enzymes, including laccase (Cazares-Garcia *et al.* 2013). Since no Lignin (LiP) or Manganese (MnP) peroxidase activity has been described for *Trichoderma* species, it has been suggested that similar to bacteria, the ability of *T. asperellum* to remove PAHs lies in the release of enzymes with different substrate range of cytochrome P450 oxidases (e.g. 1,2- and 2,3-extradiol dioxygenases) (Hadibarata *et al.*, 2007). This has been corroborated in studies of degradation of phenanthrene, pyrene and benzo[a]pyrene in liquid culture and soil, where different peroxidase activities were detected and showed to be involved in

PAH degradation (Zafra *et al.*, 2014c). On the other hand, *T. asperellum* has showed substantially higher tolerance levels to low and high molecular weight PAHs than other degrading filamentous fungi (Zafra *et al.*, 2014b), being able to use them as sole carbon source. Besides, their growth rate does not appear to be affected at high levels such as 6000 mg Kg^{-1} PAHs, even though its sporulation ability is compromised at such elevated concentrations (Zafra *et al.*, 2014a).

Another important aspect of *T. asperellum* lies on their ability to metabolize plant root exudates, and in return, to enhance plant root growth. Plant growth promotion by *Trichoderma* asperellum is thought to occur via a combination of control of plant microbial pathogens (directly by mycoparasitism, antibiosis and competition for nutrients and indirectly via systemic effects on the plant) and improved plant nutrition (Harman *et al.*, 2004) via systemic effects and *T. asperellum*-mediated nutrient solubilization. Previous studies have demonstrated the potential of *Trichoderma ressei* to promote plant growth in soils polluted with diesel (Mishra and Nautiyal, 2009). It is likely that *T. asperellum* could promote similar effects in PAH-polluted soils, which summed to its adaptive ability, long term survival in soil, the ability of use PAHs as sole carbon source and its versatile enzymatic machinery makes of *T. asperellum* a promising organism for PAH remediation in soils.

Concluding remarks

This review provided an overview and explored the potential of *Trichoderma* species to be used as potential bioremediation agents in soils impacted with aromatic hydrocarbons. Despite the valuable information indicating that several *Trichoderma* species could be excellent bioremediation agents at field scale, further studies are required to identify the physiological, biochemical and molecular mechanisms of *Trichoderma* species to tolerate, accumulate, detoxify, transform and mineralize hydrocarbons.

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SUMMARIZING DISCUSSION AND CONCLUDING REMARKS

Despite being environmentally friendly and cost-effective methods, bioremediation technologies often possess drawbacks when soils polluted with complex mixtures of PAHs are treated. Due to the high hydrophobicity and recalcitrance of PAHs, as well as the low tolerance level and degrading ability of the microorganisms present in the soil, bioremediation treatments require longer times compared to chemical and physical methods and, in some cases, may generate toxic intermediate products. Therefore, special care must be taken to effectively apply a bioremediation treatment in soil. The selection of highly competent microorganisms that can easily adapt to polluted soils and possess the ability to mineralize LMW and HMW PAHs - even in the presence of high concentrations - is a key aspect of the success of the bioremediation. In addition, the careful optimization of the remediation conditions and the fate of the microbial populations during the treatment are essential factors to better understand the whole degradation process and subsequently improve it.

The objective of this thesis was to study, in a culture-dependent and -independent fashion, the process of using previously defined fungal-bacterial consortia to enhance PAH degradation in soil. In general, four separate limitations of current bioremediation studies were addressed throughout this thesis: 1) some of the problems associated with a poor adaptation of introduced microorganisms were overcome using strict isolation/selection methods that permitted the obtaining of highly tolerant organisms with improved adaptive/degradative abilities in soil, 2) the limiting conventional methods for the construction of degrading consortia were avoided with the use of an induced natural selection method using highly tolerant organisms, 3) the lack of evaluation of important variables influencing PAH degradation in soil was addressed with the thorough evaluation of biotic/abiotic factors in microcosm, and 4) the limited knowledge about the ecological and functional effects of the introduction of PAH-degrading consortia in polluted soils was overcome using NGS and taxonomic/functional metagenomic analysis. The conclusions from each chapter are summarized in this section.

As mentioned throughout this thesis, one of the main limitations for a full-scale bioaugmentation process lies in the lack of knowledge on how the different introduced microorganisms could interact and promote changes in local microbiota. In principle, bioremediation processes involving the stimulation of native organisms along with an augmentation with introduced consortia is a reasonable and effective strategy to promote the degradation of persistent pollutants. In practice, bioremediation of PAH-contaminated soils is difficult and is more likely

to be effective at field scale by the stimulation of microorganisms already present in the contaminated site, because of the difficulties with the adaptation and survival of the introduced species (Singer *et al.*, 2005). Inoculated microorganisms will interact with native species and produce an impact on the microbial community, including native PAH-degraders (Cunliffe and Kertesz, 2006).

The results presented here showed that highly tolerant organisms, having the ability to use PAHs as sole carbon source, have a better chance to adapt well to a highly polluted environment. This is clearly the case for *Stenothrophomonas maltophilia* B14 and *Trichoderma asperellum* H15, two of the most tolerant and promising strains used in the degrading consortia. As the adaptation and survival of the inoculated organisms is closely related to their ability to utilize local resources, as well as the competition with the native microbial populations, our findings point to the high tolerance level as a good indicative of microbial adaptation and survival in PAH-polluted soils, even if microbial tolerance to PAHs does not appear to be directly related to their degradative capabilities (Montgomery *et al.*, 2010). In spite of the important negative effects that degrading organisms, even being tolerant, could suffer in presence of high amounts of PAHs, most of the strains used for soil bioaugmentation were able to survive and prevail until the end of treatments. Microbial consortia composed of fungal-bacterial strains adapted well and more importantly, degraded to a high extent considerable amounts of LMW and HMW-PAHs in polluted soils.

The finding of bacterial and fungal species not previously described for the degradation of PAHs with high levels of tolerance, points to extensively polluted soils as a major reservoir of microorganisms with potential for PAH degradation. In this work *Trichoderma asperellum* H15 was one of those species, showing exceptionally high tolerance levels and degradative abilities. The discovery of this species as an excellent candidate for PAH degradation in soils, and the description of some of the enzyme systems involved in the process, provide the basis for using this organism alone on a full-scale process especially when applied along with lignocellulosic residues, as *T . asperellum* produces a wide variety of potent lignocellulolytic enzyme cocktails(Marx *et al.*, 2013). Furthermore, the use of this organism in soil could bring additional advantages, such as its ability to promote the growth of some plants (Qi and Zhao, 2013), its role as a biological control agent against plant pathogens and as inductor of resistance to disease (Hermosa *et al.*, 2012). The use of *T. asperellum* in co-culture with other organisms showed to be feasible as no significant antagonistic effects were observed.

As traditional methods for consortia construction often consist of simply mixing degrading microorganisms in approximately equal quantities prior to application, there is a significant limitation to knowing the probable antagonist effects between them once applied in soil, their potential synergistic effects and predicting their behavior in soil. In this study this issue was addressed with the use of a self-selection method directly in soil, which allowed the recreation of the natural interactions produced between the different organisms. In practice, this approach produced positive results, as the resulting consortia showed improved adaptive/degradative

abilities with most of the inoculated fungal strains surviving and prevailing until the end of the remediation process of contaminated soils. Since the microorganisms better adapted to a new pollution condition gradually displace and replace those organisms not well adapted (Gadd, 2007), the use of this method for consortia construction showed to be one of the key factors in the successful bioaugmentation of polluted soils with fungal-bacterial consortia.

Two degrading consortia were constructed using the above method and tested for PAH degradation in soils. For this, both biotic (each consortium) and abiotic factors (relative humidity, the use of several agroindustrial residues, soil/residues ratio) influencing PAH degradation in soil were evaluated for one month. Notably, two GEM strains were included in one of the consortia. Even though GEM strains contributed to overall PAH degradation, their survival in soil was reduced in comparison with other organisms composing consortium C2. Since current regulations in Mexico restrict the release of GEMs and they can only be used in confined systems (SEMARNAT, 2005), the results regarding their survival are valuable since it is desirable to use GEMs with poor survival, able to degrade pollutants but at the same time conditioned to survive under specific conditions. In this study GEM strains had a role during the initial stages of bioremediation, but rapidly disappeared being displaced by other populations. The above could mean high efficiency and limited risks, as long as they are used as a part of a consortium.

Finally, the metagenomic analysis of the bioremediation process revealed in greater detail how the introduced degrading organisms interact with native soil populations and the potential effects on the metabolic pathways responsible for PAH degradation. Important effects were observed regarding displacement and stimulation of different populations, as seen with Firmicutes and Alfa/Gammaproteobacteria. Notably, members of the consortium C1 not only survived, but also increased their proportion constantly over time. Furthermore, functional analysis revealed several PAH degradation pathways taking place in polluted soils, but remarkably, degradation pathways also showed to be complimentary. Fungal members of the consortium able to produce multicopper laccases, such as *Aspergillus flavus* (Arias *et al.*, 2014) and *Trichoderma asperellum* (Cazares-Garcia *et al.*, 2013; Marx *et al.*, 2013) could initiate the oxidation of PAHs by secreting laccases and ring-cleaving dioxygenases, while bacteria can also initiate the oxidation by extradiol dioxygenases and, in some cases, laccases as reported for *Stenothrophomonas maltophilia* (Galai *et al.*, 2011). Evidence points to a cometabolic and synergic interaction for PAH degradation, where HMW-PAHs as benzo[a]pyrene can enter the degradation pathways of LMW-PAHs such as naphthalene and phenanthrene. Taking into account the long term survival exhibited by the organisms composing the degrading consortium, the results suggested a synergic system favoring mineralization pathways and avoiding those that favor the formation of toxic intermediates. Although soil bioaugmentation showed to be responsible for most PAH degradation, biostimulation by itself also contributed, to a lesser extent, to PAH degradation.

In conclusion, the results of this thesis demonstrate the potential and feasibility of using fungal-bacterial consortia in the bioremediation of soils polluted with PAHs by combining

bioaugmentation and biostimulation, establishing the optimal conditions for PAH biodegradation in soils and providing evidence of the ecological and functional effects of the bioremediation process. Furthermore, this thesis described for the first time extreme microbial tolerance levels to PAHs, the involvement of a novel PAH-degrading microorganism and provided evidence of the role and impact of GEM strains in the bioremediation process.

The main contributions of the work described in this thesis are:

- The isolation and selection of highly tolerant and competent PAH-degrading organisms from contaminated soils, describing for the first time the extreme organotolerance levels of previously known and new fungal and bacterial degrading organisms;
- The description for the first time of the involvement of *Trichoderma asperellum* in PAH degradation and the elucidation of the enzymatic mechanisms responsible;
- The influence of PAHs on important physiological aspects, such as growth, pigmentation and sporulation in two PAH-degrading fungal strains;
- The improved construction by self-selection of two fungal-bacterial degrading consortia having remarkably high adaptive and degradative capabilities in PAH-contaminated soils;
- The study and impact of using fungal GEM degrading strains on a soil bioremediation system;
- The influence of several abiotic factors on the degradation of PAHs by microbial consortia and the selection of optimal conditions for soil bioremediation by combining bioaugmentation and biostimulation; and
- The metagenomic analysis of the bioremediation of PAH-contaminated soils by a microbial consortium, increasing the knowledge about the effects on taxonomy structure and functional/metabolic aspects of PAH degradation.

FUTURE DIRECTIONS

This study demonstrated the feasibility and effectiveness of microbial consortia to degrade high amounts of PAHs in soils. However, some questions arise from this work and could be proposed as areas of further investigation:

1. The scale up of the bioaugmentation and biostimulation processes evaluated in this work, either using degrading consortia or individual organisms (such as *T. asperellum* H15), would allow to test its effectiveness under field conditions.

2. Being a highly potent and versatile organism, *T. asperellum* H15 should be further studied for its ability to degrade total petroleum hydrocarbons in soils, as well to promote beneficial effects (e.g. plant growth induction) in remediated soils.

3. In addition to metagenomics, a metatranscriptomic approach of the PAH degradation process by microbial consortia would provide valuable insights into the expression profiles and regulation of the key genes found to be involved in PAH metabolism in soil.

4. Further analysis of the metagenomic datasets is recommended, addressing not only the abundance and diversity of genes involved in aromatic degradation, but also the microbial metabolism of aliphatic, resin and asphaltene compounds for the bioremediation of soils polluted with heavy crude oil. Genes involved in the metabolism of other persistent organic pollutants that were not discussed in this thesis (e.g. polychlorinated biphenyls) could be studied as well.

5. Further analysis of the metagenomic datasets may also allow the description of novel unidentified microorganisms in soils and novel enzymes.

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APPENDICES

APPENDIX 1

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