Biodegradation of PAHs: analysis and stimulation of degrading bacterial populations

Biodegradación de HAPs: análisis y estimulación de poblaciones bacterianas degradadoras

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BIODEGRADACIÓN DE HAPs:
ANÁLISIS Y ESTIMULACIÓN DE POBLACIONES BACTERIANAS DEGRADADORAS

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A mis padres y hermanas

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Abbreviations

ARDRA	Amplified Ribosomal DNA Restriction Analysis
BTEX	Benzene, Toluene, Ethylbenzene, Xylenes
CERCLA	Comprehensive Environmental Response, Compensation and Liabiity Act
CSIC	Centro Superior de Investigaciones Científicas
CTC	5-cyano-2,3-tolyl-tetrazolium
DAPI	4',4'-diamidino-2-phenylindole
DGGE	Denaturing Gradient Gel Electrophoreses
DNA	Deoxyribonucleic acid
DPG	Diphosphatidylglycerol
EEA	European Environmental Agency
EPA	Environmental Protection Agency
FISH	Fluorescent *in situ* Hybridization
FISH-MAR	FISH Microautoradiography
GC-MS	Gas Chromatography- Mass spectrometry
GRL	General Reference Levels
HMN	Heptamethylnonane
HMW-PAHs	High Molecular Weight PAHs
HPLC	High Performance Liquid Chromatography
INT-formizan	Iodonitrotetrazolium formizan
IRNASE	Instituto de Recursos Naturales de Sevilla
LMW-PAH	Low Molecular Weight PAHs
MALDI-TOF	Matriz Assisted Laser Desorption/Ionization Time-of-flight
MLSA	Multilocus Sequence Analysis
MPN	Most Probable Number
NAPL	Non Aqueous Phase Liquid
NIST	National Institute of Standards and Technology
OTU	Operational Taxonomic Unit
PAH	Polycyclic Aromatic Hydrocarbon
PAH-RHD$_\alpha$	PAH ring hydroxilating dioxygenase
PC	Phosphatidylcholine
PCB	Polychlorinated Biphenyls
PCR	Polymerase Chain Reaction
PE	Phosphatydilethanolamine
PG	Phosphatidylgylglycerol
PLFA	Phospholipid fatty acids
PME	Phosphatidylmonomethylethanolamine
RAPD	Random Amplified Polymorphic DNA
RISA	Ribosomal Intergenic Spacer Analysis
RNA	Ribonucleic acid
rRNA	ribosomic RNA
RSGP	Reverse Sample Genome Probing
SIP	Stable Isotope Probing
SSCP	Single Strand Conformation Polymorphism
TGGE	Temperature Gradient Gel Electrophoreses
T-RFLP	Terminal Restriction Fragment Length Polymorphism
TPHs	Total Petroleum Hydrocarbons

List of Papers

This Thesis is based on the following publications:

I. **Gallego**, **S**., Vila, J., Tauler, M., Nieto, J.M., Breugelmans, P., Springael, D., Grifoll, M. (2012) Microbial community structure and function analysis of a pyrene-degrading marine consortium. Submitted to *FEMS Microbiology Ecology*.

II. **Gallego, S**., Vila, J., Nieto, J.M., Urdiain, M., Rosselló-Móra, R., Grifoll, M. (2010) *Breoghania corrubedonensis* gen. nov. sp. nov., a novel *alphaproteobacterium* isolated from a Galician beach (NW Spain) after the *Prestige* fuel oil spill, and emended description of the family *Cohaesibacteraceae* and the species *Cohaesibacter gelatinilyticus. Systematic and Applied Microbiology* , 33:316–321.

III. Tejeda-Agredano, M.C., **Gallego, S.**, Niqui-Arroyo J.L., Vila, J., Grifoll, M., Ortega-Calvo, J.J. (2011) Effect of interface fertilization on biodegradation of polycyclic aromatic hydrocarbons present in nonaqueous-phase liquids. *Environmental Science and Technology,* 45:1074–1081.

IV. Tejeda-Agredano, M.C., **Gallego, S**., Vila, J., Grifoll. M., Ortega-Calvo, J-J., Cantos, M. (2012) Influence of the sunflower rhizosphere on the bioaccessibility and biodegradation of PAHs in soil. Submitted to *Soil Biology & Biochemistry*.

1.1 Polycyclic Aromatic Hydrocarbons

1.1.1 Structure. Polycyclic aromatic hydrocarbons (PAHs) are a group of compounds that are formed by two or more fused benzene rings in linear, angular, or cluster arrangements. The simplest structure consists of 2 rings (naphthalene) and very complex structures can be formed by more than 6 rings and alkyl groups. By definition, they contain only C and H atoms, although N, S and O atoms may readily substitute the benzene ring to form heterocyclic aromatic compounds, commonly found with the PAHs (Wilson and Jones, 1993). Because of their toxicity, their frequent occurrence and their persistence in the environment, PAHs are considered to be priority pollutants. In 1979 the U.S. Environmental Protection Agency included the "16 EPA PAHs" in the Consent Decree Priority Pollutant list (Keith and Telliard, 1979) (Table 1). This list is frequently used by regulatory authorities to identify site contamination and to specify monitoring parameters. The 16 EPA PAHs have, in general terms, become the standard set of compounds involved in many environmental studies of PAHs.

1.1.2 Physico-chemical properties. The most important physico-chemical properties in PAHs are the aqueous solubility, vapour pressure, Henry coefficient, and octanol-water partition coefficient. These properties can be used to deduce a variety of environmental partition coefficients which apply between air, water, soil, sediment, vegetation, and other biota (Mackay and Callcott, 1998). These physico-chemical parameters change with the temperature and are usually measured at 20-25ºC. A compilation of these parameters are shown in Table 1.

At room temperature PAHs are solid crystalline substances (Kästner, 2000a). The hydrophobicity of PAHs, expressed in the *n*-octanol to water partition coefficient (K_{ow}), increases as the molecular size of PAHs increases. Boiling points also increase with increasing molecular weight, while their liquid vapour pressure, Henry's Law constants and aqueous solubilities decrease with molecular size. In contrast, the melting point of PAHs appears not to be closely correlated with molecular weight. The vapour pressure of PAHs is rather low, except for PAHs with 2 aromatic rings. As a consequence, most PAHs do not tend to volatilize. Moreover, PAHs are hydrophobic compounds whose persistence within the environment is due chiefly to their low aqueous solubility. They adsorb onto soils and particulates, influencing their bioavailability and biodegradation.

1.1.3 Toxicity. PAHs may cause different toxic effects and do not have one single type of toxic action (Brummelen *et al.,* 1998). Different toxicity mechanisms may play a role, which depend on the compound, the type of exposure (acute or chronic), the organisms, and the environmental conditions. In general, four toxicity mechanisms are distinguished: nonpolar narcosis, phototoxicity, biochemical activation and disturbance of hormone regulation.

Nonpolar narcosis or baseline toxicity is an unspecific mode of toxicity, resulting from the accumulation of the toxicant in the biological membrane. The structure of biological membranes is disturbed, which may affect the regulation of essential membrane bound processes such as osmoregulation and neurotransmission. Narcosis can develop quickly during short-term exposure. This toxicity can be correlated with the octanol water partition coefficient that significantly determines the accumulation of a compound in biological membranes. Examples of PAHs which exert this toxic effect are naphthalene, fluorene and phenanthrene.

Phototoxicity is a specific toxicity mechanism which occurs in the presence of UV light. Some PAH have the ability to absorb UV light energy. UV radiation induces the formation of free radicals, which damage a variety of macromolecules, leading to a toxic effect. Photoexcited PAH molecules transfer the UV energy to an oxygen molecule, creating an oxygen radical that can disrupt cell membranes via lipid peroxidation. Anthracene, fluoranthene, pyrene, chrysene, benz(*a*)anthracene,

Table 1. Physical-chemical properties and toxicological properties of PAHs, including molecular weight (MW), LeBas molar volume (V_M), melting point (Mp), boiling point (B_p), vapour pressure (P_v), aqueous solubility (S), Henry's law constant (H), octanol-water partition coefficient (K_{ow}), mutagenicity (Mut.), and carcinogenicity to humans (Carc.). T=25ºC, unless denoted differently. (Sources: Sims and Overcash, 1983; Mueller *et al.*, 1996; van Agteren *et al.*, 1998; Pothuluri and Cerniglia 1998; IARC, 1987; van Brummelen *et al.*, 1998; Kästner, 2000a.)

PAHs		MW (g/mol)	V_M (cm^3/mol)	Mp (ºC)	Bp (ºC)	Pv (mm Hg)	S (mg/l)	H ($Pa \cdot m^3$/mol)	K_{ow}	Mut.	Carc.
Naphtalene	$C_{10}H_8$	128	148	81	218	$4.92 \cdot 10^{-2}$	31.0	43.01	3.37	-	
Acenaphtylene	$C_{12}H_8$	152		92	265	$2.9 \cdot 10^{-2}$	3.93		4.07		
Acenaphthene	$C_{12}H_{10}$	154	173	96	278	$2.0 \cdot 10^{-2}$	3.80	12.17	4.33	+	
Fluorene	$C_{13}H_{10}$	166	188	116	295	$1.3 \cdot 10^{-2}$	1.90	7.87	4.18		U
Phenanthrene	$C_{14}H_{10}$	178	199	101	339	$6.80 \cdot 10^{-4}$	1.10	3.24	4.57	-	U
Anthracene	$C_{14}H_{10}$	178	197	216	340	$1.96 \cdot 10^{-4}$	$4.5 \cdot 10^{-2}$	3.96	4.54	-	U
Fluoranthene	$C_{16}H_{10}$	202	217	111	375	$6.0 \cdot 10^{-6}$	0.26	1.04	5.22	+	U*
Pyrene	$C_{16}H_{10}$	202	214	156	360	$6.85 \cdot 10^{-7}$	0.132	0.92	5.18	±	U
Benzo(*a*)anthracene	$C_{18}H_{12}$	228	248	160	435	$5.0 \cdot 10^{-9}$	$1.1 \cdot 10^{-2}$	0.58	5.91	+	A
Chrysene	$C_{18}H_{22}$	228	251	255	448	$6.3 \cdot 10^{-7}$	2·10-3	$1.22 \cdot 10^{-2}$	5.75	+	U*
Benzo(*b*)fluoranthene	$C_{20}H_{12}$	252		167		$5.0 \cdot 10^{-7}$	$1.2 \cdot 10^{-3}$		6.57		B
Benzo(*k*)fluoranthene	$C_{20}H_{12}$	252		217	480	$5.0 \cdot 10^{-7}$	$5.5 \cdot 10^{-4}$		6.84		B
Benzo(*a*)pyrene	$C_{20}H_{12}$	252	263	175	495	$5.0 \cdot 10^{-7}$	$3.8 \cdot 10^{-3}$	$4.6 \cdot 10^{-2}$	6.04	+	A
Dibenzo(*a,h*)anthracene	$C_{22}H_{14}$	278	300	267	524	$1.0 \cdot 10^{-10}$	$6 \cdot 10^{-4}$	$1.7 \cdot 10^{-4}$	6.75	+	A
Benzo(*g,h,i,*)perylene	$C_{22}H_{12}$	276		222		$1.0 \cdot 10^{-10}$	$2.6 \cdot 10^{-4}$		7.23		U
Indeno(*1,2,3-c,d*)pyrene	$C_{22}H_{12}$	276		163		$1.0 \cdot 10^{-10}$	$6.2 \cdot 10^{-2}$		7.66		B

+: positive Ames assay; -: negative Ames assay. A: probably carcinogenic; B: possibly carcinogenic; U:unclassifiable as to carcinogenicity to humans, *classified as weak carcinogens in Pothuluri and Cerniglia, 1998.

benzo(*k*)fluoranthene, benzo(*a*)pyrene, dibenz(*a,h*)anthracene and benzo(*g,h,i*)perylene have been reported to be phototoxic. Phototoxicity can develop relatively rapidly and can be observed both in acute and chronic toxicity experiments (McDonald and Chapman, 2002).

Biochemical activation is the best-known toxicity mechanism. It implies the subsequent adduct formation when certain PAHs are converted during enzymatic transformation, into highly reactive compounds which may form covalent bonds with macromolecules such as proteins and DNA, the adducts. DNA adducts may provoke mutations which may result in carcinogenesis and teratogenesis. Biochemical activation is a highly specific (chronic) toxicity mechanism, limited to a specific group of PAHs. Not all organisms are equally equipped with the enzyme system needed to carry out biochemical activation.

Human metabolism of PAHs is mediated by cytochrome P_{450} (CYP)1A1 in the initial oxidation step and by glutathione transferase (GST) M1 in the conjugation step. Both of these enzymes are polymorphic and able to modulate the levels of DNA adducts. Successive oxidation steps predominantly produce reactive diol-epoxides that can then be converted to carbonium ions as the reactive electrophiles that can then covalently bind to DNA. The monitoring of DNA adducts has been used in risk assessment for human exposure to PAHs.

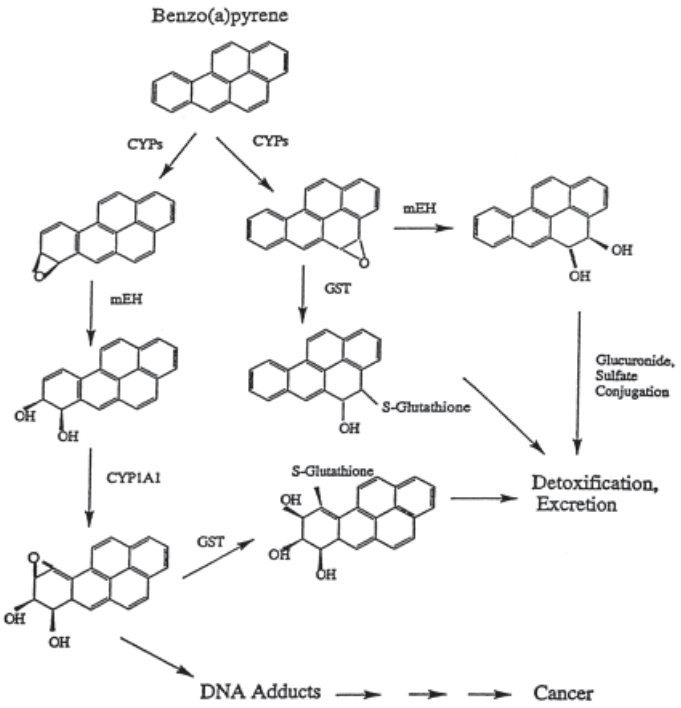

Figure 1. Pathways of benzo(*a*)pyrene activation and detoxification. CYPs: cytochrome P_{450}; mEH: mitochondrial epoxide hydrolase: GST: glutathione S-transferase (Adapted from Eaton *et al*., 1998).

For example, the oxidation of benzo(*a*)pyrene produces 7,8-epoxide, which then is transformed to a *trans*-dihydrodiol by an epoxide hydrolase. This 7,8-*trans*-dihydrodiol can be later epoxidated in other positions, some of the resulting diol-epoxides having a high mutagenic and carcinogenic potential. The diol-epoxides can be combined with glutathione by a glutathione transferase, which results in their detoxification. The

epoxides that are not combined with glutathione are converted to the corresponding phenols and diols. However, these metabolites are not polar enough to be excreted and are also combined with glucuronic or sulphuric acids for subsequent excretion (Shaw and Connell, 1994; Mastrangelo *et al*., 1996; Hemminki *et al*., 1997) (Figure 1). In fact, experiments realized with benzo(*a*)pyrene in animals have demonstrated the apparition of malignant tumors after administration or inhalation of this PAH (Collins *et al*., 1991; Pickering, 1999).

There are some evidences for the existence of the disturbance of hormone regulation by PAHs. Disturbance is thought to occur either by direct interaction of PAH metabolites with hormone receptors or, indirectly, by interference with hormone metabolism. This type of toxicity requires chronic exposure. Benzo(*a*)pyrene, for example, reduces testosterone production in rats (Chung *et al*., 2011) while several key components of the thyroid hormones homeostasis are disturbed by the action of PAHs (Mastorakos *et al*., 2007).

All these toxic effects can only be exerted if organisms are able to take up the PAHs, therefore, besides toxicity, the PAH concentration, bioavailability, and uptake mechanisms are crucial parameters. Organisms that can metabolize PAHs extensively have relatively low residue concentrations and will be less susceptible to narcosis and phototoxicity. On the other hand, organisms that have a high rate of PAH metabolism are more likely to be the victim of adduct formation or disturbance of the hormone regulation.

1.1.4 Occurrence. Historically, PAHs have been produced by three different processes: diagenetic origin (such as petrogenesis of organic material and coal formation), pyrolytic origin (incomplete combustion of organic material), and biogenesis. Generally, the PAHs are found as part of non-aqueous phase liquids (NAPLs) such as crude oil and derivatives (gas-oil, fuel oil...) and mineral coal products (coal tar, creosote...). These materials are very complex mixtures with the constituent compounds exhibiting a wide range of chemical, physical and toxicological properties.

Crude oil or petroleum derives from ancient fossilized organic materials, such as zooplankton and algae which have remained during million of years at high pressure and temperature conditions (100-150ºC) (Blumer, 1976). Crude oil is a thick dark liquid containing many hydrocarbon compounds and small amounts of sulfur, nitrogen, oxygen and metallic constituents. The main components of crude oil can be purify and subdivided in different fractions: saturated (alkanes or paraffins, branched or isoalkanes, cycloalkanes or naphthenes) aromatic (characterized by the presence of at least one benzene ring, alkyl-benzenes, alkyl-PAHs), resin and asphaltenic fractions (Rosini, 1960; Speigh, 1991; Sugiura *et al*., 1997; Mills *et al*., 1999).

Mineral coal is a fossil fuel occurring in the subsurface that is composed primarily of carbon along with variable quantities of other elements such as hydrogen, sulfur, oxygen and nitrogen. There are different types of coal such as peat, lignite, anthracite and graphite. The pyrolysis (900-1200ºC) and subsequent distillation (200-400ºC) of the coal tar product, produces an oily mixture called creosote widely used in wood preservation. Coal tar creosote is composed of approximately 85% polycyclic aromatic hydrocarbons, 10% phenolic compounds, and 5% N-, S- and O- heterocycles (Mueller *et al*. 1989). Pyrogenic hydrocarbons such as creosote characteristically contain more unsubstituted parent PAHs than their alkylated homologues, whereas the petrogenic hydrocarbons such as petroleum and its derivatives are more abundant in alkylated PAHs due to the lower temperature formation (Blumer, 1976; Page *et al*., 1996). There are numerous sites polluted with creosote in Europe and United States (Mueller *et al*., 1989), however, due to the toxic effects (mutagenic and carcinogenic effects) of its components, nowadays the production and use of creosote in Europe and United States is forbidden (Commission Directive 2001/90/EC).

1.2. Environmental PAH pollution

1.2.1 Sources. Generally, the processes producing environmental PAH pollution are subdivided into natural and anthropogenic sources. Natural sources of PAHs include volcanic eruptions, forest and bush fires, biogenic reactions in plants and bacteria (Borneff *et al.*, 1968; Suess, 1976), and thermal geologic reactions associated with fossil-fuel and mineral production.

Anthropogenic sources are generally considered the most significant sources of PAH production and introduction into the environment. Anthropogenic sources include the combustion of fossil-fuels, the production and use of coal tar and its distillation product creosote, accidental spills during transport, production and storage, industrial processes, waste incineration and urban sewage and industrial sewage effluents (Cerniglia, 1992).

The anthropogenic inputs of PAHs into the environment often divided into point sources and low-level input sources. In many industrially developed countries the point source contamination is the most common, and is often the focus of environmental concern and regulatory compilations. Point sources include spills and mismanagement (waste treatment, sludge disposal, leaking tanks) during industrial operations like gasification/liquefaction of fossil-fuels, coke production, catalytic cracking, carbon black production and use, asphalt production and use, coal-tar production and use, refining/distillation of crude oil and oil-derivated products, wood treatment processes, wood-preservative (creosote/anthracene-oil) production, landfill waste dump sites, burning/incineration, and fuel/oil storage, transportation, processing, use and disposal. Generally, all of these activities result in small contaminated areas with relatively high PAH contaminants often associated with other types of contaminants like petroleum hydrocarbons, xenobiotic chemicals, and heavy metals (Mueller *et al*., 1996).

Low-level inputs generally result from atmospheric deposition of incomplete combustion of fossil-fuels, typically from industrial activities and automobile exhaust. However, land application of sewage sludge and large-scale spillage and disposal of oily wastes may also contribute. Atmospheric PAH depositions are usually much dispersed sources, but cover significant amounts of land surfaces and sediments. PAH concentrations from these sources are typically quite low in soil and sediments, although on a global scale atmospheric deposition is the main pathway for PAHs to reach the terrestrial, aquatic and marine environment through precipitation and percolation (Grifoll *et al*., 1990; Fernández *et al*. 1992; Casellas *et al*. 1995).

1.2.2 Environmental fate. The environmental fate of PAHs is important since they are the largest class of chemical carcinogens known today. The possible fates of PAHs in the environment include volatilization, photoooxidation, chemical oxidation, bioaccumulation, adsorption to soil particles, leaching and microbial degradation (Cerniglia, 1992; Kästner, 2000b).

In soils, volatilization accounts for loss of naphthalene and methylnaphthalenes. For non volatile compounds the volatilization is negligible (Park *et al*., 1990a; 1990b). Abiotic reactions generally account for two- and three-ring PAH compounds and it is not statistically significant for the reduction of PAHs containing more than three aromatic rings (Bossert and Bartha, 1986; Wild and Jones, 1993). Moreover, these compounds have the negative impact of aging, which decrease their bioavailability by sequestration in the soil matrix.

In aquatic systems, naphthalenes and alkylderivatives are relatively soluble. However, PAHs of more than three rings are high hydrophobic and they tend to link to organic material and settle in the sediments. Some PAHs can be photooxidized, volatilized and biodegraded, but the PAHs that remain in the bottom of aquatic systems are generally only biodegraded by microbial activity and predominantly in the upper

surfaces than in the lower layers of the sediments (Gardner *et al*., 1979; Cerniglia and Heitkamp, 1989).

All these processes result in the redistribution of the pollutants into the environment. However microbial degradation, catalyzed by bacteria, algae, fungi and yeast is the main process for removal of pollutants though mineralization, partial degradation or transformation.

1.2.3 Legal frame. Polluted sites are of great concern due to their potential toxic effects to public health. As a consequence, developed countries have adopted regulatory programs addressed to specific problems and have set up standard guidelines, regulations, and protocols in order to clean up these polluted areas. Initially, the efforts were focussed in water and air quality improvement. The interest about soil quality increased after several industries caused serious public health damages by chemical waste disposal under residential areas such as Love Canal in United States (1976) and Lekkerkerk in Netherlands (1978). In 1980, with the federal law CERCLA (Comprehensive Environmental Response, Compensation and Liability Act), commonly known as Superfund, the United States of America started to regulate and to fund the cleanup of several hazadous waste sites. CERCLA set up a trust funded by taxes on the chemical and petroleum industries and provided federal authority to respond to releases or threatened releases of hazardous substances that may endanger public health or the environment. This program, mediated by the US EPA, authorizes the short-term removal and long-term remediation of hazardous substances, includes wide information about polluted sites, and states the prohibitions and requirements in relation with polluted areas in United States.

In 1996, the European Union established the 96/61/EC Council Directive which contains general aspects regarding the pollution prevention and control in air, water and soils. The application of this directive required the cooperation of the state members in the identification and localization of polluted sites, especially those used for industrial, military activities and waste dumpsites. The European Environmental Agency (EEA) estimated in the year 2000 that there were approximately 300.000 contaminated areas and 1.500.000 potentially contaminated areas in the European Union (Prokop *et al*., 2000).

In Spain, the first action was taken in the period of 1990-1995 by the Government, which developed a National Plan for the Remediation of Contaminated Soils (BOE 172 of 20-7-1995) with the objective of identifying polluted sites, create a database, develop a risk assessment methodology, calculate the cost of the actions, propose a program of action, evaluate the remediation costs and consider a legal framework. As a result, in 1998 the Law 10/1998 of 21st April on Waste was approved including a definition for contaminated areas, the need for a list of potentially polluting activities and a subsequent inventory of polluted sites, the responsibilities associated, and the standards for allowable levels of contamination for soils depending on their future uses.

Later , the Royal Decree 9/2005 (BOE-15, 2005) was approved which established the general reference levels (GRL) for specific pollutants such as the tolerable levels of total petroleum hydrocarbons (TPHs), polychlorinated biphenyls (PCBs), HAPs or BTEX (volatile monoaromatic compounds present in crude oil and derivatives such as gasolines) among others.

1.3. Biodegradation and bioremediation of PAHs

There is considerable interest in the use of biological systems to remediate PAH contaminated environments. Biological remediation procedures have been developed for some hazardous organic pollutants, and have shown to be successful under field conditions, both in terms of pollutant elimination efficiency and economic feasibility. Costs associated with thermal and other physicochemical techniques are much

expensive than biological treatments and most of the times the environment as a suitable living system is destroyed (Wilson and Jones, 1993). Bioremediation treatments are based on the ubiquitous ability of microorganisms to degrade pollutants, as long as adequate environmental conditions are maintained. Indeed, many bacterial, fungal and algal strains have been shown to degrade a wide variety of PAHs containing from two to five aromatic rings (Mueller *et al*., 1996). However, bioremediation of polluted environments with PAHs does not always produce the desired results. The PAHs are usually rapidly degraded until a certain residual concentration is reached after which degradation is hardly observed. This phenomenon, known as the hockey-stick shaped kinetics (Alexander, 1999), may be caused by limited concentration of nutrients, sorption of the PAHs to organic matter causing a lower availability, diffusion of the compound into micropores which are inaccessible for the biodegradation, and the absence of efficient PAH degrading bacteria.

1.3.1 Factors affecting PAH biodegradation There are several factors which may affect the outcome of bioremediation experiences, the most important being the following: environmental conditions, such as toxicity, pH, temperature and humidity (in case of soils), presence of suitable microorganisms able to degrade the pollutants, and the molecular structure of the contaminant, their concentration and bioavailability.

a) Environmental conditions.

Important environmental factors which may affect PAH biodegradation are the presence of toxicants, the oxygen content, pH, temperature, humidity and nutrient content.

Presence of toxicants. In some cases hydrocarbon contamination may be associated with high levels of other pollutants. High concentrations of toxic contaminants such as heavy metals may slow down metabolic processes and prevent the growth of biomass. The degree and mechanisms of toxicity depends on the concentration, type of metals and the microorganisms exposed. Moreover, bioremediation experiences involving the introduction of non native species (bioaugmentation) may be also affected by natural inhibitors present in some polluted waters and soils (National Research Council, 1993; Alexander, 1999; Scragg, 2005).

Oxygen. PAH degradation is carried out by heterotrophic microorganisms that require a final electron acceptor. Generally, aerobic degradation of hydrocarbons (oxygen as final acceptor) is considerably faster than anaerobic processes. This means that a supply of oxygen will be needed to maintain aerobic conditions if rapid degradation is required. Nearly all bioremediations in practical use are aerobic. However, an increasing body of research on the degradation of PAHs shows that many of them can be degraded in the absence of oxygen, often using nitrate, sulphate or ferrous iron as an alternate electron acceptor. In addition, problems encountered with aerobic processes such as the production of “overwhelming” levels of biomass, which the consequent problem of clogging and insufficient supply of oxygen, may be solved with anaerobic processes using nitrate, Fe (III), sulphate and carbon dioxide as electron acceptors (Lovley *et al*., 1989; Holliger and Zehnder, 1996; Heider and Fuchs, 1997; Mcnally *et al*., 1998; Widdel and Rabus, 2001; Jones *et al*., 2008a).

Temperature, pH, salinity. The temperature affects microbial growth. At lower temperatures the rate of degradation is slower. For this reason, enrichment cultures are usually incubated at 25º to 37º degrees. However, many species multiply at lower temperatures. For bioaugmentation experiences enrichments should be established and organisms isolated at temperatures similar to those where the organisms are found or will be applied. The majority of bioremediation studies have been realized at

mesophilic conditions (20-30ºC) (Menn *et al*., 2000), however, there are several examples of successful bioremediation experiences at extreme temperatures such as in arctic (Whyte *et al*., 1999) or desert locations (Sorkoh *et al*., 1993).

The oceans and many inland waters are at basic pH, while soils have usually acidic pH. In general, the optimum pH for biodegradation processes is neutral (pH 7.4-7.8) (Dibble and Bartha, 1979). Verstraete *et al*., (1976) achieved greater biodegradation results in polluted soil by increasing the pH from 4.5 to 7.4; however, there are microbial degrading populations adapted to acidic pH (Stapleton, 1998).

Oceans and estuaries have high levels of salts (occasionally, some waters and soils too). It is possible that the salinity in estuaries and oceans may be detrimental to some species involved in biodegradation, but there are several examples of isolated degrading organisms from a salt-rich habitat that do not degrade a chemical in fresh water (Atlas and Busdosh, 1976).

Nitrogen and phosphorus (nutrient content). In natural uncontaminated environments mineral nutrients including nitrogen, phosphate, and potassium (N, P, and K) are very rarely limited. However, when an environment is contaminated with organic compounds such as PAHs, the C:N ratio can increase dramatically and can become the rate-limiting factor in bioremediation. These nutrients can be supplied to the microorganisms by supplementing the contaminated environment (soil or water) with a mineral fertilizer (biostimulation) (Breedveld and Sparrevik, 2001; Atagana, *et al*. 2003). Numerous studies have reported the use of inorganic and organic fertilizers with a positive effect on pollutant biodegradation (Swannell and MacDonagh, 1996; Venosa *et al*., 1996; Vasudavan and Rajaram, 2001). However it is important to apply the correct balance and concentration of nutrients since several studies have shown inhibiting effects on microbial biodegradation when those are applied (Morgan and Watkinson, 1992, Zhou and Crawford, 1995; Margesin and Schinner, 1997). The US EPA recommends molar ratios of C:N between 100:10 and 1000:10 (US EPA, 1995) and the molar ratio of C:N:P 100:10:1 is frequently considered optimal for contaminated soils. However, the most used ratios described in the literature vary from 100:10:1 to 300:10:1 (Bossert and Bartha 1984; Alexander, 1999; Atagana *et al*., 2003; Leys *et al*., 2005).

Supplemental Carbon Sources (cometabolism). Cometabolism can be defined as the breakdown of compounds which are not used as carbon or energy source (Scragg, 2005). It is generally accepted that microorganisms do not grow on high-molecular-weight (HMW) PAHs as sole carbon sources, therefore the organisms capable of degrading these compounds must obtain energy from other sources and cometabolize these recalcitrant PAHs. There is some evidence that degradation of HMW PAHs can be enhanced by the addition of non-specific carbon sources (Keck *et al*., 1989), for example organic matter such as humic acids has been observed to stimulate the degradation of pyrene (Holman *et al*., 2002).

b) Factors associated with the pollutant

Molecular structure. More bacteria are known to degrade low molecular weight PAHs than high molecular weight PAHs. Generally, the biodegradability of PAHs decrease with increasing the molecular weight and number of rings which results in a concomitant increase in hydrophobicity and electrochemical stability (Cerniglia, 1992; Kanaly and Harayama 2000). The recalcitrance in PAHs also increases with the number of alkyl substitutes and the distribution of their rings. PAHs that are conformed in angular arrangements are more stable than those with linear configurations. Therefore, PAHs containing two or three rings are biodegraded at reasonable rates and PAHs with four or more rings are highly persistent (Wilson and Jones, 1993; Wild and Jones 1995; Howsan and Jones, 1998).

Concentration. Pollutant concentration is important basically for two reasons: toxicity and induction. PAHs are lipophilic and tend to accumulate in the membrane lipid bilayer. These pollutants possess solvent properties, toxic effects that may disrupt membrane structure of the microorganisms. As a result of accumulated hydrocarbon molecules, the membrane loses its integrity, and increases its permeability. The toxic effects may include disruption of membrane structure resulting in leakage of important cellular constituents, destruction of membrane-based energy generation processes such as dissipation of electrical potential and pH gradients, or inhibition of membrane proteins (Sikkema *et al*., 1995).The higher the concentration of the contaminant, the more organisms will be unable to defend itself from toxic effects. On the other hand, some contaminants are only degraded when contaminant levels are above certain concentrations. This is because the enzymes involved in a particular process may be formed and active only when the substrate, one of its metabolites, or possibly a closely related molecules are present (inducible enzymes) at a certain concentration (Chen and Aitken, 1999). The concentration of the substrate in the aqueous phase may be too low to result in enzyme formation if the compound of concern is present in oil, organic solvent or other non-aqueous liquid, with the result of very low partitioning rate between the water and the non-aqueous liquid.

Bioavailability. Bioavailability can be defined as the accessibility of a compound for its biologic assimilation and possible toxicity (Alexander, 2000). The bioavailability of a chemical is determined by the rate of mass transfer relative to the intrinsic activity of the microbial cells. It is controlled by a number of physical-chemical processes such as sorption/desorption, diffusion and dissolution (Cuypers *et al*., 2002).

Earlier in this Introduction, it has been mentioned that the bioremediation of PAHs in soils and sediments generally shows a biphasic loss of PAHs: a short period of rapid PAH degradation, followed by a longer period of slow degradation (Beck *et al*., 1995; Cornelissen *et al*., 1997; 1998; Williamson *et al*., 1998). The initial phase of bioremediation is primarily limited by microbial degradation kinetics (uptake and metabolism), whereas in the second phase, the rate of PAH removal is limited by slow PAH desorption (the rate of transfer to the cell) (Bonten, 2001).This results in an incomplete removal of PAHs, that is to say, a residual PAH concentration remains in the matrix after remediation. In many cases, this is mainly related to PAH bioavailability.

Several mechanisms have been suggested to explain this two-stage desorption behaviour. In soils, one of the major phenomena which affect negatively to bioavailability is the ageing or weathering. As the compound is still intact and unchanged, but has become hidden and inaccessible, such compounds are often termed sequestered instead of aged or weathered. This effect can be produced by different mechanisms (Alexander, 1999) such as: i) the compound is not sorbed, but remains in micropores at some distance from microorganisms able to degrade them; this distance (micrometers) is important for the cells which are in a porous or nonfluid environment and are not able to degrade them; ii) compounds are retained in nanopores which are far smaller than micropores and have dimensions too small for even the tiniest bacterium to penetrate; iii) compounds interact with humic acids or other environmental constituents to form molecular species that, although contain the same molecule or its metabolites are in fact new molecular species that can influence negatively the microbial degradation processes (Weissenfels *et al*., 1992; Ortega-Calvo and Saiz-Jiménez, 1998)

Microbial populations have developed numerous strategies to overcome mass transfer limitations for metabolizable substrates. One of these mechanisms is the production of biosurfactants that increase the surface area of the hydrophobic pollutants (emulsification), desorbs them from surfaces, and increase their apparent solubility (Volkering *et al*., 1998). Biosurfactants are produced by a wide variety of diverse microorganisms and have very different chemical and surface properties

(Kanga *et al*., 1997; Page *et al*., 1999; Mulligan *et al*., 2001; Ron and Rosenberg, 2001; 2002; Ábalos *et al*., 2004).

Another strategy to increase pollutant bioavaliability is the modification of cell surface properties. Microorganisms interact with the environments via their cell surfaces, and they can alter their surface properties in order to make them more hydrophobic, especially when grown in the presence of hydrophobic phases (Hanson *et al*., 1994; Stelmack *et al*., 1999; Norman *et al*., 2005). Increased hydrophobicity can be attributed to cell bound surface-active compounds such as lipopolysaccharides, lipoteichoic acids, lipoglycans, glycolipds, fatty acids, neutral lipids or phospholipids among others (Neu, 1996; Wick *et al*., 2002a; Obuekwe *et al*., 2009; Zhao *et al*., 2005).

Alterations in cell surface properties are also key determinants in biofilm formation, colonization and spread. Biofilms have been shown to be advantageous to microbial survival for many reasons including protection from toxic effects, metabolite exchange, nutrient accession and lateral gene exchange (Stoodley *et al*., 2002). Biofilm formation on PAHs crystals may favor the diffusive mass transfer of PAHs from crystals to the bacterial cells and will impact on pollutant bioavailability, indicating that biofilm formation may be a specific response to optimize substrate bioavailability (Wick *et al*., 2002b; Johnsen and Karlson, 2004).

Finally, in biodegradation situations it is necessary for the microorganism to be in close proximity with the pollutants. For this reason, numerous microorganisms have developed chemotactic mechanisms that allow movement towards higher concentrations of pollutants or its metabolites (Ortega-Calvo *et al*., 2003).

c) Presence of suitable degrading microorganisms.

Generally, the indigenous microbial populations in polluted environments have the capacity for PAH degradation. For this reason, it is important to analyze the community structure and its changes during bioremediation processes in order to be able to enhance the microbial processes, optimizing the environmental conditions. Prior to the degradation of many organic compounds such as PAHs, a period is noted in which no destruction of the chemical is evident. This time interval is called acclimation period, adaptation or lag period. The duration of the acclimation period varies from hours to months and depends on the chemicals, environments and concentration of the compound. Acclimation of a microbial community to one substrate frequently results in the simultaneous acclimation to some chemicals structurally related. Several factors affect the length of acclimation period such as temperature, pH, aireation, concentration of N and P. Environments previously exposed to the pollutant may reduce the acclimation period and enhance the degradation rates. However, environments which never have been affected by a pollutant, may have an insignificant or nonexistent microbial degrading population. In this situation, the inoculation with species able to destroy the chemical and also to tolerate the specific environmental conditions may be highly beneficial (Vogel, 1996; Aldrett *et al*., 1997; Hozumi *et al*., 2000). Several studies have shown that bioaugmentation with authoctonous microorganism or the addition of selected organisms to contaminated sites has speed up the biodegradation reducing the acclimation period (Forsyth *et al*., 1995; Grosser *et al*., 1991). However, introduced bacteria decline rapidly after introduction and their growth is poor. This decline in introduced bacteria is probably due to their failure to compete with the indigenous population (Venosa *et al*., 1991; 1992) and also to lack of adaptation to changing environmental conditions (Tagger *et al*., 1983; Venosa *et al*., 1996). If there is an indigenous flora capable of carrying out the reaction the addition of inocula are not needed.

1.3.2 PAH-degrading microbial populations.

a) Metabolism of PAHs

Bacteria and fungi metabolize a wide variety of organic contaminants, being microbial degradation the main process to remove PAHs from the environment (Prince, 1993; Sutherland *et al*., 1995). The biodegradation of a particular organic substrate may be carried out by microorganisms that are growing at the expense of that substrate, i.e. using it as a source of carbon and energy, and producing carbon dioxide, water or other inorganic compounds (mineralization). In other cases microorganisms produce only a partial degradation of the substrate converting it to metabolites that are considered dead-end products (partial degradation). A third possibility is found when a microorganism grows at the expense of another organic substrate but metabolizes the substrate of interest (cometabolism) (Alexander, 1999). Studies in our group demonstrate that a single bacterial strain can grow in PAHs mixtures carrying out all those processes simultaneously (Grifoll *et al*., 1995; López *et al*., 2005). On the other hand, the biodegradation of complex hydrocarbons may require the concerted effort of several microorganisms. The elucidation of the microbial degradation pathways is necessary to determine the extent of degradation, whether the metabolites that are formed are toxic or biologically inactive, and their environmental persistence.

PAHs are stable reduced compounds and therefore degradation generally proceeds by oxidation under either aerobic or anaerobic conditions. There are numerous studies which report a wide diversity of microorganisms able to degrade PAHs (Cerniglia, 1992; 1993; 1997; Kanaly and Harayama 2000; Prince, 2005; Kanaly and Harayama 2010). Anaerobic degradation of PAH is very slow compared to aerobic degradation and, therefore, plays no significant role in bioremediation strategies. However, nowadays there is a growing interest in the microbial populations involved in anaerobic biodegradation processes (Lovley *et al*., 1989; Langenhoff *et al*., 1996; Heider *et al*., 1999, Widdel and Rabus, 2001; Jones *et al*., 2008a; Meckenstock and Mouttaki, 2011).

The bacterial aerobic degradation of PAHs is generally initiated by the action of multicomponent dioxygenases that catalyze the incorporation of both atoms of oxygen and two electrons from NADH to form *cis*-dihydrodiols. These multi-component dioxygenases usually consist of a reductase, a ferredoxin, and an iron-sulphur protein. The subsequent dehydrogenation by a NAD^+-dependent dehydrogenase yields dihydroxylated intermediates, which are further degraded through ring-cleavage pathways with the eventual formation of citric acid cycle intermediates. PAHs can also be metabolized by monooxygenases in bacteria to form *trans*-dihydrodiols, although this activity is generally lower than the dioxygenase activity in the same organism (Heitkamp *et al*., 1988a; 1988b) (Figure 2).

The aerobic degradation of low-molecular-weight (LMW) PAHs such as naphthalene, anthracene, and phenanthrene, has been extensively studied revealing that numerous Gram-negative species such as *Pseudomonas*, *Sphingomonas*, *Burkholderia* and *Comamonas* are able to grow using these hydrocarbons (Cerniglia, 1992; Kästner, 2000a). Abundant research on the purification and characterization of the enzymes and genes involved is also available (Menn *et al*., 1993; Sanseverino *et al*., 1993). However, more recent studies, reported the ability of Gram-positive nocardioform organisms (*Mycobacterium*, *Nocardia*, and *Rhodococcus* species) to degrade PAHs containing more than three rings (Kanaly and Harayama, 2000; Kanaly and Harayama, 2010).

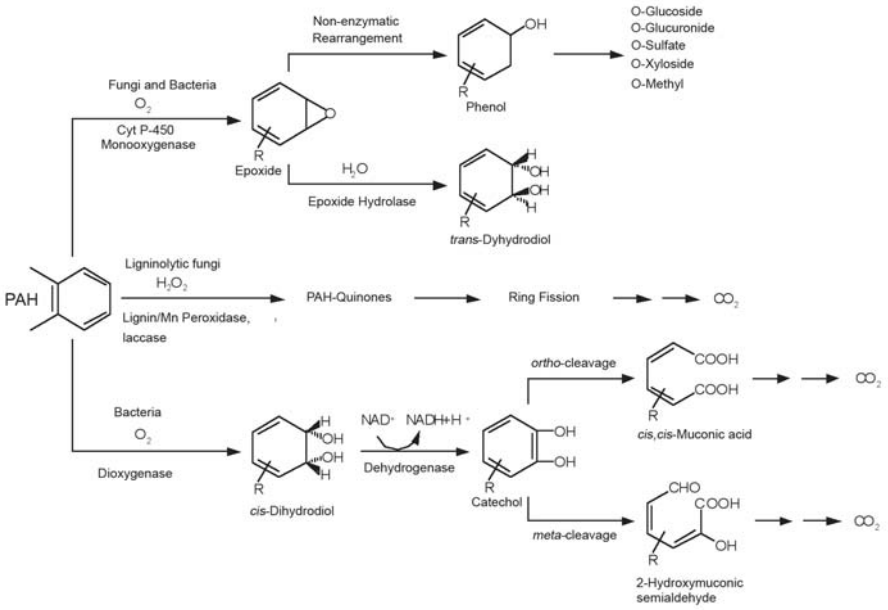

Figure 2. Pathways for the microbial catabolism of PAHs (Adapted from Kästner, 2000b)

The first strain able to mineralize pyrene described was *Mycobacterium* sp. PYR-1 (then renamed as *Mycobacterium vanbaalenii* PYR-1). The isolate was able to mineralize pyrene, although low concentrations of organic nutrients were needed (Heitkamp *et al*., 1988a). During growth this strain accumulated different metabolites, such as *cis*- and *trans*- 4,5 pyrene dihydrodiols and pyrenol, as initial microbial ring-oxidation products. Other products detected were 4-phenanthroic acid (the most abundant), 4-hydroxyperinaphthenone, cinnamic and phtalic acid as ring fission products. The detection of the *trans*-dihydrodiol indicated that *Mycobacterium vanbaalenii* was able to attack PAHs via dioxygenation and monoxygenation. This product had been previously detected during metabolic studies realized with the fungi *Cunninghamella elegans* (Cerniglia *et al.*, 1985) and was the result of a cytochrome P_{450} monooxygenation. Later studies using $^{18}O_2$ confirmed the production of the *trans* isomer from pyrene by strain *Mycobacterium vanbaalenii* PYR-1, and demonstrated that it was the result of the action of cytochrome P_{450}-dependent monooxygenases (Heitkamp *et al*., 1988b). *Mycobacterium* sp. PYR-1 can also mineralize low and high molecular mass PAHs such as anthracene, phenanthrene, fluoranthene, and benzo(*a*)pyrene (Cerniglia, 2003).

In 1991 Water *et al*. described the first strain (*Rhodococcus* sp. UW1) able to grow using pyrene as sole carbon source. Five years later, Schneider *et al*. (1996), identified 4,5-phenanthroic acid as intermediate in the degradation of pyrene by *Mycobacterium* sp. strain RJGII-135, suggesting the *ortho*-cleavage of pyrene instead of the *meta*-cleavage mechanism characteristic of the PAH degradation by Gram-negative bacteria. This same year, Dean-Ross and Cerniglia, published the isolation of a *Mycobacterium flavescens* strain able to grow using pyrene as a sole carbon source which accumulated *cis*-4,5-pyrenediol, 4-phenantroic acid, phtalic acid and 4,5-phenantroic acid. With the identification of these metabolites, the first pyrene degradation pathway was proposed. In 1998, a new pyrene-degrading strain, *Mycobacterium* sp. KR2, was

reported to accumulate some of the previously detected metabolites together with the newly identified 2-carboxybenzaldehid, protochatechuic and 1-hydroxy-2-naphtoic acids (Rehmann *et al*., 1998). Later studies in our laboratory resulted in the isolation of another mycobacterial strain able to grow in pyrene that was identified as *Mycobacterium gilvum* AP1. Strain AP1 accumulated all the previously detected metabolites, including a product reported by several authors but that could not be identified before, and that resulted from the cleavage of both internal rings of pyrene: 6,6'-dihydroxy-2,2'-biphenyl dicarboxylic acid. This confirmed and completed the routes previously proposed (Vila *et al*., 2001). Besides the *ortho*-dioxygenation, a distinctive characteristic of this pathway was the presence of lateral non-productive oxidative branches leading to the *trans*-dihydrodiol and the biphenyl derivative as possible dead-end products (Figure 3).

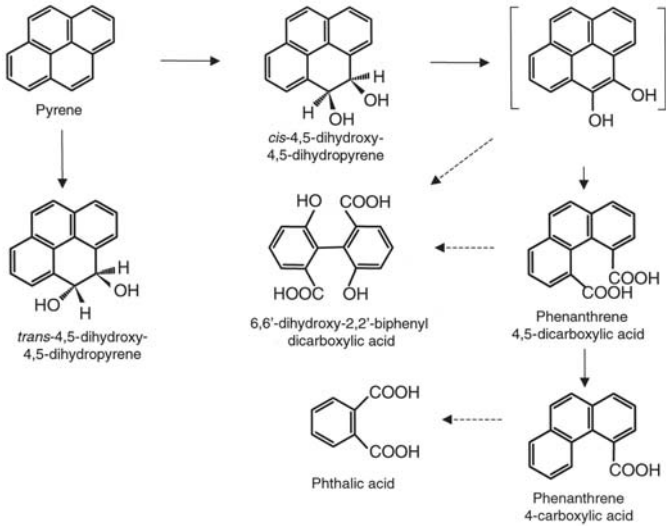

Figure 3. Schematic pathway proposed for the degradation of pyrene by *Mycobacterium* sp. strain AP1. The product in brackets has not been isolated. Dotted arrows indicate two or more successive reactions. Source: Vila *et al*., 2001.

In the last decade, the dioxygenases involved in the initial oxidation of pyrene have been characterized (Khan *et al*., 2001; Krivobok *et al*., 2003; Kim *et al*., 2006), and, more recently, the complete genome sequencing of *Mycobacterium vanbaaleenii* PYR-1 and *Mycobacterium gilvum* PYR-GCK has allowed new advances in the establisment of the genetic basis of the degradation pathways of the pyrene molecule (Kim *et al.*, 2007).

b) Microbial community analysis

With the introduction of hydrocarbon mixtures in a specific environment and their subsequent degradation, the initial microbial community structure experiments numerous shifts. Analyzing the microbial community structure and its dynamics during hydrocarbon biodegradation is the first step to identify the key populations, their interactions, and to ascertain the conditions for their stimulation. There is no single method to study microbial community structure and its dynamics, only the combination of different methods may give a broad overview. Those may be classified in culture-dependent and culture independent methods.

Culture-dependent methods. These techniques require the cultivation of microorganisms for a further characterization. In this group we find the classic isolation and culture in solid or liquid media, the most probable number (MPN) (Rowe *et al*., 1977; Wrenn and Venosa, 1996), and the Biolog® method. The Biolog® method is a rapid method to characterize the microbial communities based on the growing pattern in 95 different carbon sources of the direct sample (Garland and Mills, 1991; Wünsche *et al*., 1995; Berthe-Corti and Burns, 1999; El Fantroussi *et al*., 1999). The main advantage of these techniques is the possibility to obtain pure cultures, useful for further analysis and for biotechnological applications. However, the main limitation of these techniques is that only 0.001%-0.1% of the marine microorganisms or 0.3% of the soil microorganisms are culturable in the laboratory (Kogure *et al*., 1980; Ferguson *et al*., 1984; Torsvik *et al*., 1998; 2003).

Culture-independent methods. These techniques are more representative of whole communities and reduce biases from the necessity of microorganism cultivation (Amann *et al*., 1995; Head *et al*., 1998). They do not need the previous cultivation of the sample and permit to study the microbial populations at different levels. Among them we find fluorescent *in situ* hybridization (FISH), phospholipid fatty acid analysis (PLFA), methods based on polymerase chain reaction (PCR) and methods that allow to link specific microbial populations to physiological processes.

FISH. Fluorescent *in situ* hybridization (FISH) provides a rapid phylogenetic identification and quantification of microorganisms. Intact fixed cells are hybridized with fluorescently labelled oligodeoxynucleotides complementary to their 16S ribosomal RNA (DeLong *et al*., 1989) and the microorganisms are visualized in fluorescence microscopy. The obtained images may then be compared with the whole population using different staining such as DAPI (4',4'-diamidino-2-phenylindole), INT-formizan (iodonitrotetrazolium-formizan) or CTC (5-cyano-2,3-tolyl-tetrazolium chloride). The main drawback of this technique is the need of optimization of the hybridization conditions, the potentially limited permeability of the cell surface to the specific probes and, moreover, the fact that inactive cells may be not detected.

Analysis of PLFA. Phospholipid fatty acids (PLFA) extracted from samples can be used to quantify and characterize viable biomass and community components. Specific PLFAs may indicate the phylogeny and physiology of some group of microorganisms. However, it can not distinguish individual strains (Lechevalier, 1989; Hellman *et al*., 1997, White *et al*., 1997)

Methods based on the use of PCR. Many community analysis techniques use the polymerase chain reaction (PCR) to amplify specific DNA sequences. These methods are based on the 16S rRNA gene or on functional genes. The 16S rRNA gene has both highly conserved and species-dependent variable regions, and is the molecule used in phylogenetic. On the other hand, functional genes, that may be significantly divergent in phylogenetically similar microbial strains, provide important specific information about the degradative pathways present.

Molecular techniques to evaluate microbial community profiles include PCR in combination with clone libraries, or with denaturing and temperature gradient gel electrophoreses (DGGE and TGGE), reverse sample genome probing (RSGP), ribosomal intergenic spacer analysis (RISA), single-strand-conformation polymorphism (SSCP), terminal-restriction fragment length polymorphism (T-RFLP), random amplified polymorphic DNA (RAPD), and amplified ribosomal DNA restriction analysis (ARDRA). DGGE and TGGE are based on the analytical separation of DNA fragments of identical or near identical length based upon their sequence composition. DGGE has been used successfully in many investigations of community structure (Viñas *et al*., 2005, Jiménez *et al*., 2007; Vila *et al*., 2010) and it is now one of the most widespread and well-

established methods to obtain community profiles. RSGP use the entire genome of a microorganism as a specific probe that allows its detection in the environment. This technique has been used successfully in the detection of specific strains of *Mycobacterium*, *Mycoplasma*, *Chlamydia*, *Bacteroides*, *Pseudomonas*, *Sphingomonas* and *Campylobacter* species (Voordouw *et al*., 1992). T-RFLP is used to study the community profile by sizing variable length restriction fragment digest patterns of amplified 16S rDNA (Liu *et al*., 1997). The choice of enzyme for digests is critical and requires some previous knowledge of the bacterial groups presented in the samples.

The results obtained from the PCR technique have some limitations such as the different extractability of the nucleic acids among the distinct microorganisms, the selective amplification of the 16S genes, the genetic recombination and the chimerical sequence formation during PCR, and the differences in number of gene copies or operons of 16S rDNA in different organisms (Liesack *et al*., 1991; Cole and Girons, 1994; Amann *et al*., 1995; Wang and Wang, 1996).

Methods to correlate specific microbial populations with physiological processes. During the last decades, stable isotopes have been used successfully as an indicator of microbial activity in environmental samples. For a given pollutant, the ratio ^{12}C:^{13}C in non biodegraded environmental samples is constant and remain unaffected by abiotic processes. Microbial activity can be isotopically selective, causing shifts in the ^{12}C:^{13}C ratio in substrates and products, depending on the type of molecule and microbial activity. These methods do not provide information on the microbial populations involved in biodegradation processes. However, recently developed ^{13}C-labeled substrate procedures combined with community profiling methods, may determine exactly which organisms are involved in the breakdown of specific molecules. Some of these methods are PLFA-stable isotope probing (SIP), DNA-SIP and RNA-SIP.

i) PLFA-SIP*:* ^{13}C-labeled substrate is pulsed into the microbial community, resulting in the labelling of polar lipid-derived fatty acids which once extracted, separated and analyzed for ^{13}C enrichment may reveal which organisms were dominating the metabolism of the labelled substrate (Boschker *et al*., 1998).

ii) DNA-SIP*:* Stable isotope-labelled DNA can be isolated from mixed microbial communities on the basis of the increase in density. Density centrifugation in cesium chloride gradients is used to separate heavy DNA from natural DNA. Then, 16S rDNA clone libraries from heavy DNA are constructed and sequenced to identify the organisms assimilating the ^{13}C-labeled substrate applied. This technique has been used to determine the populations involved in the degradation of pyrene in a PAH polluted soil (Jones *et al*., 2008b) and also in naphthalene degradation (Jeon *et al*., 2003).

iii) RNA SIP: The use of this technique instead of DNA-SIP avoids the limitations derived from the fact that the DNA synthesis is associated only with cell replication. The procedure is performed as DNA-SIP, but applying reverse transcription from RNA in order to identify active organisms. This has been used in the identification organisms responsible for the metabolism of phenol (Manefield *et al*., 2002).

Another method to correlate specific microorganisms to specific functional processes is the *in situ* fluorescence hybridization associated to microautoradiography (FISH-MAR), and is the most widely applied tool for revealing physiological properties of microorganisms in their natural environment with single-cell resolution. This technique has been used in marine systems and wastewater to describe the functional properties of the present species (Lee *et al*., 1999; Wagner *et al*., 2006). The radio-labelled-substrate is applied into the sample, which is later fixed, hybridized with specific probes, and visualized by fluorescence microscopy. The disadvantage of this technique is the limitation in the number of fluorochromes available.

To overcome the difficulties and problems associated with cultivation techniques, during the last years another approach widely used is metagenomics. This method is

based on the direct genomic analysis of entire microorganism populations (Schmeisser *et al*., 2003; Tyson *et al*., 2004; Venter *et al*., 2004; Tringe *et al*., 2005). Another technique recently used is the DNA microarrays. A DNA microarray is a high-density matrix of hundreds or thousands of individual probes for specific genes (16S rRNA genes or catabolic genes). This technique has served to monitor biodegrading populations and to detect biodegradation genes (Rhee *et al*., 2004).

1.3.3 Strategies to enhance microbial degradation of PAHs during bioremediation.

In the previous paragraphs, the biphasic kinetic observed in PAH bioremediation strategies was attributed to numerous factors such as the limited concentration of nutrients or the lower availability of the PAHs. Numerous strategies have been developed in order to enhance the microbial biodegradation and reduce the residual fraction of the pollutants. Some of these strategies are studied in this Thesis such as the use of biostimulation with oleophilic fertilizers and rhizoremediation.

a) Biostimulation

The success of bioremediation experiences depends on the concentration and availability of some nutrients. Generally, the addition of nutrients is beneficial. However, the supply of these essential elements as water-soluble salts presents several problems. These include the rapid dilution of water-soluble salts of N and P introduced into waters or soils due to lixiviation and turbulence, as well as their utilization by bacterial populations that do not degrade the pollutant. In addition, by increasing the concentration of mobile nitrogen further environmental problems could potentially be created. Therefore, a number of oleophilic organic fertilizers (Atlas and Bartha, 1973; Xu and Obbard, 2003; Nikolopoulou *et al*., 2007) have been developed that, after addition, remain associated with the hydrophobic pollutants (i.e. NAPLs) and locally stimulate hydrocarbon-degrading bacteria. These oleophilic fertilizers have two effects: they apparently help by reducing the PAH viscosity and interfacial tension, and they encourage PAH biodegradation by supplying nutrients at the oil-water interface (Swannell *et al*., 1996). An example is Inipol EAP® 22, successfully used during the bioremediation of Exxon-Valdez oil-spill in Alaska (Lindstrom *et al*., 1991; Pritchard and Costa, 1991; Prince, 1993). This product was composed by oleic acid (26.2%), lauryl phosphate (23.7%), 2–butoxy-1-ethanol (10.8%), urea (15.7%) and water (23.6%) (Xu and Obbard, 2004). The urea is provided as nitrogen source, lauryl phosphate as a phosphorous source, 2-butoxy-1-ethanol as a surfactant and oleic acid to give the material its hydrophobicity. Nowadays, this formulation has been modified due to the toxicity of 2-butoxy-1-ethanol (Hoff, 1993) and the new product is called S200. This product has been applied successfully in numerous experiences of bioremediation such as the *Prestige* oil-spill (Díez *et al*., 2005, Gallego *et al*., 2006, Jiménez *et al*., 2006).

Another possibility to stimulate biodegradation is by increasing the bioavalability of the pollutants with surfactants. Surfactants are organic molecules that usually consist of a hydrophobic part and a hydrophilic part (amphiphilic molecules). The hydrophilic part makes these products soluble in water, while the hydrophobic part makes them tend to concentrate in interfaces. As a result the presence of surfactant molecules at air-water interfaces results in a reduction of the surface tension. On the other hand, the presence of surfactants in non-aqueous phase liquid (NAPL), results in a reduction of the interfacial tension (Volkering *et al*., 1998). Depending on the nature of the hydrophilic group, the surfactants can be distinguished as ionic, cationic, zwitterionic and non-ionic. There are synthetic surfactants such as paraffins, oleofins, alkylbenzenes, alkylphenols, and alcohols and biosurfactants such as glycolipids, phospholipids, fatty acids, lipopeptides/lipoproteins and biopolymeric surfactants.

Surfactants are produced on large scale and have numerous applications as cleaning solvents in bioremediation experiences, enhancing the mobility and the solubility (Ellis *et al*., 1985; Falatko, 1991; Abdul *et al*., 1992; Mulligan *et al*., 2001) and also as biodegradation enhancers (Joshi and Lee; 1995). Introduction of a surfactant in the environment will always lead to contamination and consequently the toxicity of the surfactant and its potential degradation products are the most important criteria for the selection. The toxic effects of surfactants on bacteria may include the disruption of cellular membranes by interaction with lipid components and reactions of surfactant molecules with proteins essential to the functioning of the cell. Non-ionic surfactants are in general less active against bacteria. Biologically surfactants occur naturally and the use in bioremediation experiences may be more acceptable in comparison with synthetic surfactants. (Déziel *et al*., 1996; Zhang *et al*., 1997; Makkar and Rockne, 2003). However, some biosurfactants can be as toxic as synthetic surfactants (Lang and Wagner, 1993).

b) Rhizoremediation

Another strategy to enhance biodegradation efficiency in soils is rhizoremediation. Soil pollutants remediated by this method are generally organic compounds which can not enter the plant because their high hydrophobicity. In this technique, plants do not play the main role in the biodegradation process, but they create the niche for rhizosphere microorganisms to degrade the pollutants. The use of plants has a number of advantages such as a root system that can be used as a vector for the root-colonizing bacteria and for penetration of layers normally not permeable to bacteria. Plants can also attract water with their root system. Moreover, roots can exude up to 35% of their photosynthate as exudate carbon and release oxygen or provide better redox conditions. Plant root exudates contain sugar, organic acids, and amino acids as main components (Weissman, 1964; Jones, 1998; Bertin *et al*., 2003; Chen *et al*., 2007). In addition, the mucigel secreted by root cells, lost root cap cells, or the decay of complete root provides nutrients for microbial populations present in roots. As a result, microbial populations in the rhizosphere are much larger and present different composition than those in rootless bulk soil. Furthermore, the colonization or inoculation of pollutant-degrading bacteria on plant seed may be an important additive to improve the efficiency of phytoremediation or bioagmentation (Child *et al*., 2007; Kuiper *et al*., 2001; 2002). Numerous studies show the beneficial interaction between plants and microbes in bioremediation experiences (Kuiper *et al*., 2004). However little is known about the microbial populations involved.

1.4. Scope of this Thesis

The research presented in this Thesis was part of two projects funded by the Spanish National Program and generally focussed on biodegradation of PAH in polluted environments. Both projects were in cooperation with the research group of Dr. Jose Julio Ortega-Calvo from IRNASE, CSIC (Seville), specialized in bioavailability, while our group at the University of Barcelona, lead by Dr. Magdalena Grifoll, has wide experience in bacterial metabolism and community analysis. The first project was *Bioavailability and microbial metabolism of polycyclic aromatic hydrocarbons present in marine oil spills. Implications in natural attenuation and bioremediation* (VEM2004-08556). The overall objectives of this project were to study the bacterial processes involved in the removal of PAHs as components of marine oil spills, using the fuel of the *Prestige* as model, and evaluate different bioremediation strategies to decontaminate the affected shoreline. In fact, the first two articles presented in this Thesis were the continuation of the works “Trials of bioremediation on a beach affected by the heavy oil spill of the *Prestige*” (Fernández-Álvarez *et al*., 2006), and “Evaluation

of biodiesel as bioremediation agent for the treatment of the shore affected by the heavily oil spill of the *Prestige*" (Fernández-Álvarez *et al*., 2007) and "Microbial community structure of a heavy fuel oil-degrading marine consortium. Linking microbial dynamics with PAH utilization" (Vila *et al*., 2009). The two first papers presented in this Thesis further investigate the marine populations involved in the degradation of the four-ring PAH pyrene. Part of the work of the first article was developed in the laboratory of Dr. Dirk Springael, in the Department of Soil and Water Managament of the Catholic University of Leuven (Belgium) during a short stay at the group of Dr. Dirk Springael.

The second project was *Soil bioremediation: Prospection of the microbial and vegetal diversity for a better bioavailability and mineralization of polycyclic aromatic hydrocarbons*. This project investigated the degradation of PAHs in soil and its possible stimulation by additives such as biosurfactans and vegetable oils or by rhizoremediation with different plants. As part of this project, the third article of this Thesis investigates the effect of oleophilic fertilizers, while the fourth constitutes the first work done in collaboration of the group in the IRNASE on rhizoremediation with creosote polluted soils, subject on which the groups are deepening on their current research.

Objectives

The work presented in this Thesis has focussed on the study of the microbial populations and processes involved in the removal of PAHs from polluted marine and soil environments. As a continuation of previous studies conducted in our group, the first half of the Thesis is aimed at characterize the marine bacterial communities that participate directly or indirectly in the degradation of PAHs after oil spills affecting coastal environments. The second part is centred in soils. Since low biavailability is the main factor that limits the biodegradation of PAHs in soils, we carried out several strategies in order to enhance the biodegradation, i.e., the addition of fertilizers and rhizoremedation, and investigate the mechanisms involved.

The specific objectives of this Thesis have been to:

- Analyze the community structure and function of a marine microbial consortium that degrades pyrene, as as a model high molecular PAHs.
- Characterize taxonomically and phylogenetically one of the most abundant populations in the pyrene-degrading marine consortium and propose a new genus and species.
- Assess possible nutritional deficiencies for the biodegradation of PAH present in fuel at the NAPL/water interface, determine the role of oleophilic fertilizers as biostimulants, and examine the potential production and mobilization of partially oxidized metabolites.
- Evaluate the effect of sunflower rhizosphere in promoting the biodegradation of PAHs present in an aged soil, characterize the sunflower root exudates, and identify the shifts on soil microbial community structure

3.1. Bacterial populations involved in pyrene biodegradation in marine shorelines contaminated by petroleum hydrocarbons: Community structure and function analyses of microbial consortium UBF-Py

Marine oil spills cause great concern due to the associated risk to public health and ecological damage to shorelines. Among the different components of petroleum, polycyclic aromatic hydrocarbons (PAHs) are of special interest because of their persistence, toxicity and potential carcinogenicity. Pyrene, with four fused benzene rings, is, together with its alkyl derivatives, one of the most abundant high molecular PAHs in crude oils and derivatives (Vila and Grifoll, 2009).

It is widely accepted that pyrene degradation in contaminated soils is mainly carried out by bacteria belonging to the group *Actinobacteria* (Kanaly and Harayama, 2010). Metabolite identification studies with mycobacterial strains isolated by their capacity to degrade or grow on pyrene have produced key information on the degradation pathways for its removal (Heitkamp *et al*., 1988a; 1988b; Vila *et al*., 2001; Cerniglia, 2003), while later genomic studies have permitted to identify some of the enzymes involved and their genetic organization (Khan *et al*., 2001; Krivobok *et al*., 2003; Kim *et al*., 2006; Kim *et al*., 2007). However, little is known about pyrene degradation in marine environments, where less than 0.3% of the microbial populations are considered culturable (Amann *et al*., 1995). Only recently, studies realized with pyrene degrading marine consortia and later isolation of the microbial components, have demonstrated that members of the genera *Cycloclasticus* (Wang *et al*., 2008) and *Ochrobactrum* (Arulazhagan and Vasudevan, 2011), both *Proteobacteria*, were capable of degrading pyrene in the absence of other substrates, suggesting that these new genera play a key role in PAH degradation in marine environments. Interestingly, no associations have been established between pyrene degradation and actinobacteria in marine environments.

In previous studies of our group on the bioremediation of marine shorelines affected by the *Prestige* oil spill, an autoctonous fuel-oil degrading microbial consortium (UBF) was developed and used as inoculum (Fernández-Álvarez *et al*., 2006, 2007). Consortium UBF was a stable mixed culture that caused an almost complete removal of all the linear and branched alkanes and an extensive degradation of the three to five-ring PAHs present in the fuel, including a 75% depletion of pyrene (Vila *et al*., 2010). In order to investigate the microbial populations causing this pyrene removal, a subculture of consortium UBF was established and maintained growing in pyrene as the sole carbon source ($0.2 gL^{-1}$).

The HPLC analysis of organic extracts from consortium UBF-Py cultures showed a 34% depletion of pyrene ($0.2\ gL^{-1}$) in 30 days. No other peaks were detected, indicating a complete degradation of the molecule. In fact, subsequent mineralization studies using ^{14}C-pyrene revealed that most of the depleted pyrene (24%) was mineralized to CO_2. Parallel studies realized with phenanthrene showed that UBF-Py was also able to mineralize phenanthrene (31% and 24% at $0.5 mgL^{-1}$ and $0.2 gL^{-1}$ concentrations respectively). This result is consistent with the fact that most pyrene-degrading strains from soils are able to also grow on phenanthrene (Vila *et al*., 2001) (Figure 4).

According to results obtained with the MPN (most probable number) technique, during incubation on pyrene, the pyrene-degrading populations increased from 10^4 to 10^7 during the first 15 days, to remain constant until day 21, and decrease in one order of magnitude by the end of incubation. This descent was attributed to a reduced bioavailability of the residual pyrene. By contrast, the heterotrophic populations increased during the first 21 days (from 10^6 to 10^8), to remain invariable until the end of the experiment. This indicates an accompanying heterotrophic subpopulation directly (pyrene metabolites) or indirectly sustained by the carbon furnished by pyrene.

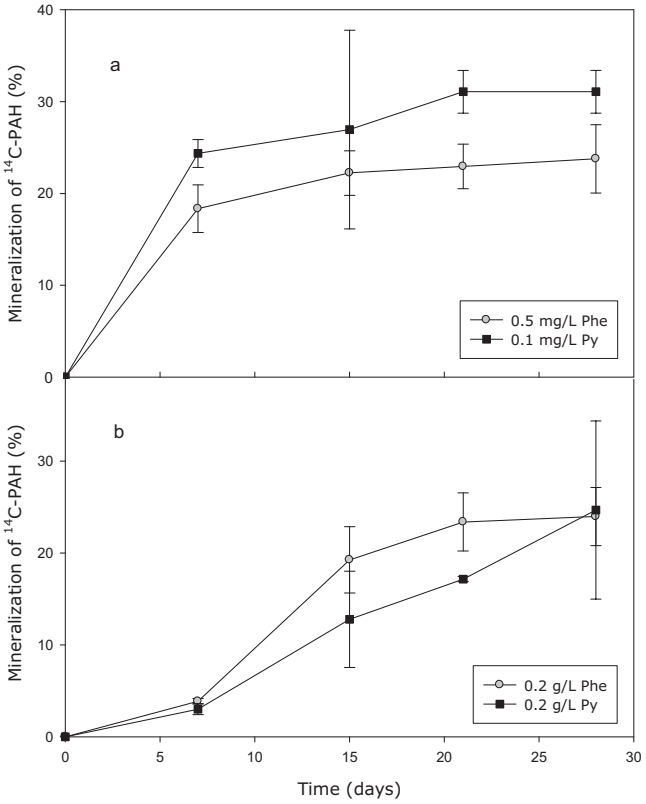

Figure 4. Mineralization of ^{14}C-phenanthrene (Phe) and ^{14}C-pyrene (Py) (as $^{14}CO_2$) by UBF-Py consortium in a final concentration of 0.5 mgL-^{1} of phenanthrene and 0.1 mgL^{-1} of pyrene (a) and in a final concentration of 0.2gL^{-1} (b).

16S rDNA PCR-DGGE fingerprints (Figure 5) from duplicate cultures of UBF-Py with pyrene (0.2 gL^{-1}) or phenantrene (0.2 gL^{-1}) showed highly similar banding profiles indicating a high stability of the consortium. In cultures with pyrene ten major bands were distinguished (S1-S10). Most of them were also detected in cultures with phenanthrene, but their relative abundances were substantially different (i.e. bands S2, S5 and S9), suggesting different catabolic capabilities for each member of consortium.

To determine the community structure of microbial consortium UBF-Py a 16S rDNA clone library was obtained, and, after analyzing 45 clones, 5 distinct OTUs (Operational Taxonomic Units) were found. Table 2 shows the results of comparing the almost complete 16S rDNA sequences with the genetic databases and their correspondence with the DGGE bands.

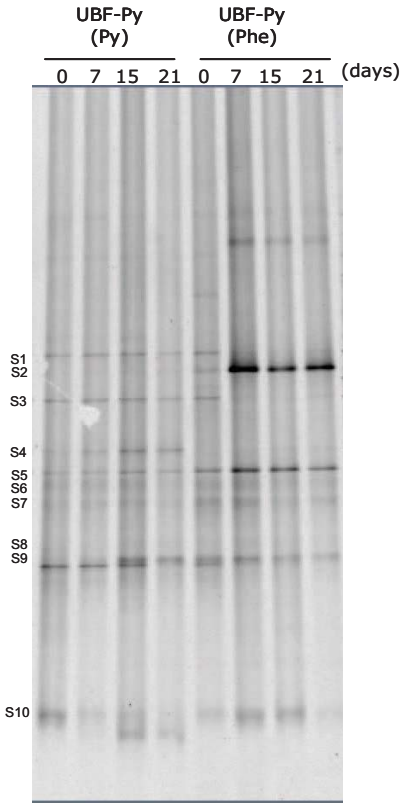

Figure 5. DGGE profile of PCR-amplified 16S rRNA gene fragments from two independent replicate cultures of the microbial consortium UBF-Py after 7, 15 21 and 30 days of incubation in pyrene and phenanthrene.

In an attempt to recover the bacterial populations observed in the molecular analysis as pure cultures, a variety of media were assayed including diluted complex media and minimal media with different hydrocarbons with and without supplements. The 16S rDNA sequences from the obtained isolates were then compared with the genetic databases and with those found in the clone library (Table 2).

The molecular analysis revealed a community mainly composed by *Alphaproteobacteria* (84%) and *Actinobacteria* (16%). The most frequently detected sequence in the clone library (Band S5, 38% of clones) corresponded to the isolate UBF-P1, and presented a low similarity with the databases. Detailed taxonomical and phylogenetic analyses summarized in article 2 revealed that this strain did not fall in any of the described bacterial genus, then, the new genus was thoroughly described and given the name *Breoghania.* Another abundant bacterium detected in the clone library was *Thalassospira* (S6, 34%) (*Rhodospirillaceae*, *Alphaproteobacteria*). The rest of the clones corresponded to *Gordonia* (S10, 16%) (*Nocardiaceae, Actinobacteria*), *Martelella* (no band observed, 7%; UBF-P8) (*Aurantimonadaceae, Alphaprotebacteria*) and *Paracoccus* (S9, 5%; UBF-P7) (Rhodobactereaceae, Alphaproteobacteria). The members of *Thalassospira* and *Gordonia* could not be recovered by culture techniques, although a variety of media were assayed, including a modified M3 medium (Rowbotham and Cross, 1997) and an amended variant of sorbitol medium (Brezna *et al*., 2003), that has been successfully used to culture environmental mycobacteria

Table 2. Sequence analysis of the clones and isolates selected from the consortium UBF-Pyr correlated to corresponding DGGE band.

DGGE Band	Clone (freq %)	Isolates	Fragment length (bp)	Sim (%)	Closest relative in GenBank database (accession no.)	Phylogenetic group
S1=S3		**UBF-P2** [a,b,c,d,e]	**1379**	**99**	***Alcanivorax* dieselolei Qtet3 (GU370129)**	***Alcanivoracaceae* (γ)**
S2		**UBF-P3** [d,e]	**1282**	**100**	***Novosphingobium* sp. TVG9-VII (JF706227)**	***Sphingomonadaceae* (α)**
S4		**UBF-P4** [e]	**1328**	**99**	***Novosphingobium* sp. 2PR51-13 (EU440981)***	***Sphingomonadaceae* (α)**
S5	**38**	**UBF-P1** [a,b,d,e]	**1481**	**92**	***Mycoplana* sp. G1100 (GU199002)**	**(α)**
S6	34		1452	99	*Thalassospira* sp. MAI8 (AB257194)	*Rhodospirollaceae* (**α**)
S7		**UBF-P5** [a]	**1350**	**99**	***Micrococcus* sp. MOLA 73 (AM990848)**	***Micrococcaceae (Actinobacteria)***
S8		**UBF-P6** [b]	**1211**	**99**	***Aurantimonas* sp. 5C.5 (HQ427427)**	***Aurantimonadaceae* (α)**
S9	**5**	**UBF-P7** [a,c,d]	**1413**	**99**	**Uncultured *Paracoccus* S-5m-1 (GU061852)**	***Rhodobacteraceae* (α)**
S10	16		1477	99	*Gordonia* sp. PETBA11 (JQ658415)	*Nocardiaceae (Actinobacteria)*
ND	**7**	**UBF-P8** [a,b,c,d,e]	**1433**	**100**	**Uncultured *Martelella* clone ctg_NISA120 (DQ396149)**	***Aurantimonadaceae* (α)**

Components of the microbial communities that have been isolated are indicated in bold. Isolation media: [a]natural seawater supplemented with LB, artificial seawater supplemented with [b]LB, [c]phenanthrene, [d]pyrene and [e]sorbitol.* All the closest relatives to this strain belong to genus *Sphingopyxis*. α and γ correspond to *alpha* and *gammaproteobacteria*, respectively

Additionally, five isolates were obtained that were not detected during the molecular analysis and were identified as *Novosphingobium* sp. UBF-P3 (Sphingomonadaceae), *Sphingopyxis* sp. UBF-P4 (*Sphingomonadaceae*), *Aurantimonas* sp. UBF-P6 (*Aurantimonadaceae*) (all *Alphaproteobacterial* strains), *Alcanivorax* sp. strain UBF-P2 (*Alcanivoraceae*, *Gammaprotebacteria*) and *Micrococcus* sp. UBF-P5 (*Micrococcaceae, Actinobacteria*).

Representatives of almost all the detected genera have been related with oil or PAH degradation, but there is little evidence on pyrene utilization for growth by these bacteria. Members of the genera *Novosphingobium*, *Thalassospira* and *Paracoccus* have been identified in microbial consortia from marine environments growing on pyrene, phenanthrene or fuel (Zhang *et al*., 2004; Guo *et al*., 2005; Cui *et al*., 2008; Yuan *et al*., 2009; Guo *et al*., 2010; Teng *et al*., 2010; Jiménez *et al*., 2011). *Martelella*, a recently described genus, has also been found in oil degrading consortia but their action on PAHs was not demonstrated (Wang *et al*, 2010). *Alcanivorax* has been widely reported as an obligate hydrocarbon-degrading bacterium able to only use linear alkanes and isoprenoids for growth (Yakimov *et al*., 1998; Dutta and Harayama 2001), however, in the present work the strain of *Alcanivorax* was recovered in diluted LB agar plates, which indicates that its spectrum of substrates may not be as reduced as reported. On the other hand, *Micrococcus* and *Gordonia* belong to the *Actinobateria,* typical soil HMW PAH-degraders (Kanaly and Harayama, 2010).

To investigate the degrading capabilities of the isolates, a variety of representative alkanes and PAHs was screened as carbon sources or transformation substrates using the plate spraying method (Kiyohara *et al*.,1982). Although some of the isolates grew on several types of hydrocarbon solid media, no clearing zones were observed. The strains were also screened for growth in liquid media with pyrene or phenanthrene, but none of the isolate was able to grow significantly respect to controls without carbon source. ^{14}C-pyrene mineralization studies were also conducted for each isolate and for the combination of all of them, but no $^{14}CO_2$ was produced.

It seems plausible that the bacterial group responsible for the attack on pyrene should be one of the most abundant in consortium UBF-Py. According to this, serial dilutions of the mixed culture were subcultured in artificial sea water with pyrene (dilution-to-extinction method) and those showing positive growth were analysed by 16S rRNA PCR-DGGE. All the dilutions showed identical DGGE fingerprints to that of the initial culture, which seemed to suggest that either the non-degrading members of UBF-Py were required for growth of the pyrene degraders (i.e. supplying growth factors) or that the pyrene degraders furnished enough carbon to support of an important accompanying microbiota. In any case, all these results indicated that the populations responsible for pyrene attack could be those not recovered in solid culture, that is the representatives of *Gordonia* and *Thalassospira*.

In order to obtain additional evidence leading to the identification of the pyrene degrading populations, the UBF-Py microbial consortia and each of the isolates were screened for the presence of typical PAH dihydroxilating dioxygenase genes.

The known dihydroxilating dioxygenase genes are grouped in two different clusters corresponding to Gram positive or negative bacteria (Cébron *et al*., 2008). PCR analysis using PAH-ring hydroxilating dioxygenases (PAH-RHD$_\alpha$) primers specific to each cluster of the DNA extracted from isolates resulted negative in all cases, both for Gram positive and Gram-negative ring-hydroxylating genes. However, the same analysis performed on the whole culture was positive for the Gram-positive primers. The PCR product was then cloned and all the clones turned out to correspond to the same sequence. This sequence was compared by BLAST searches in the NR database and NCBI databases and found to be closely related to the NidA3 ring hydroxylating dioxygenase gene found in several pyrene degrading actinobacteria (such as *Mycobacterium vanbaalenii* PYR-1) (Kim *et al*., 2006) (Figure 6). Thus indicating that the detected dioxygenase gene most likely belonged to one of the non-isolated components of UBF-Py, and reassuring the previous catabolic analyses

performed on isolates. These results indicate that the non culturable population belonging to genus *Gordonia* is most likely involved in the initial attack to the pyrene molecule.

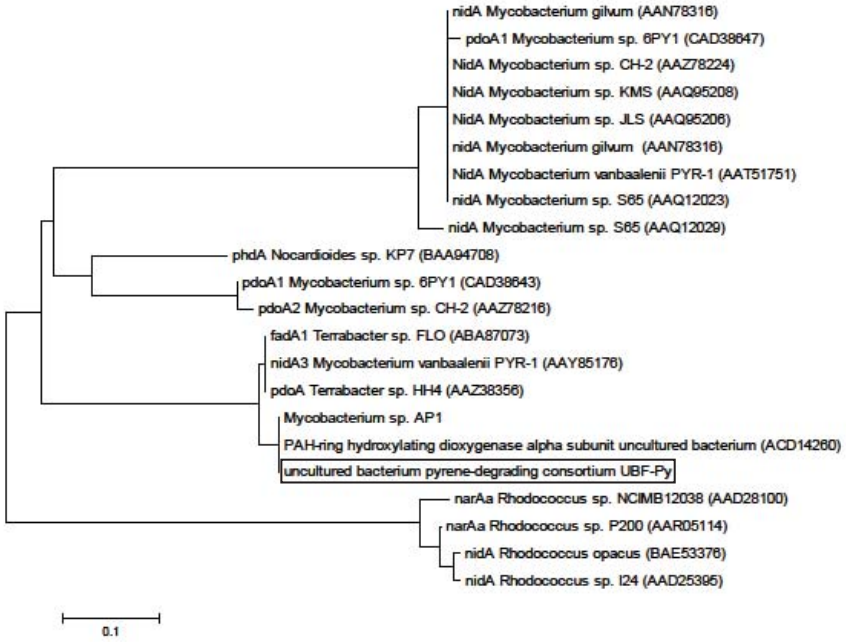

Figure 6. Phylogenetic neighbor-joining tree of ring hydroxylating dioxygenase amino acid sequences of UBF-Py consortium and reference strains taken from GenBank.

This study provides new insights on the degradation of pyrene by marine shoreline microbial communities, providing the first evidence on the role of mycobacteria in the removal of pyrene from polluted environments as well as their connection with other populations. More efforts will be needed to obtain the until now non-culturable pyrene degraders in order to characterize them and make them available for potential biotechnological applications. In fact, recent studies report the successful recovery of previously non-cultivated strains after the the addition of specific growth supplements (i.e. siderophores) to the media. (D'Onofrio *et al*., 2010).

3.2. The bacterial component most frequently detected in the pyrene-degrading marine consortium UBF-Py belongs to a new genus within the family *Cohaesibactereacea*. Description of genus *Breoghania.*

The most abundant morphology obtained after platting serial dilutions of microbial consortium UBF-Py in diluted LB-artificial sea water and incubating for one week, consisted on circular, mucoid, cream coloured colonies of about 1 mm in diameter. Five colonies were purified in the same medium, and, to ascertain whether these isolates constituted different populations, a RAPD analysis was performed using primers RAPD 1 and RAPD2 (Peña *et al*., 2005), and their 16S rRNA gene was sequenced. RAPD analysis revealed identical amplification patterns of all five strains, which indicated that they all represented a single clonal population (strain UBF-P1), probably selected through the enrichment procedure, while the 16S rDNA

corresponded to the most abundant sequence in the clone library of microbial consortium UBF-Py (38%).

The almost complete 16S rRNA gene sequence of strain UBF-P1 was compared to the SILVA 16S rDNA gene database (http://www.arb-silva.de/projects/livingtree/), which contains all the high quality sequences found for type strains (Yarza *et al*., 2008), and the alignment and tree reconstructions were performed using the ARB software package (Ludwig *et al*., 2004) (accession number GQ272328). The closest relative sequence, with 92% sequence similarity was in all the cases *Cohaesibacter gelatinilyticus* DSM 18289^T, that affiliated together with UBF-P1 in all neighbour joining reconstructions. However, maximum likelihood reconstructions rendered trees where the new isolate always appeared independently affiliated within the *Alphaprotebacteria*. In no case could UBF-P1 be affiliated with a classified genus. Indeed, the value of 92% identity with its close relative was clearly below the mean (96.4% ± 0.2), or minimum identity (94.9 ± 0.4) used to differentiate a single genus (Yarza *et al*., 2008), thus suggesting that it was plausible to conclude that strain UBF-P1 belonged to a new genus within the *Alphaproteobacteria*. In order to clarify the affiliation of both isolates further phylogenetic, genetic and phenotypic studies were performed on both strains. A multilocus sequence analysis (MLSA) was performed by concatenating the genes *atp*D (ATP synthasa F1, beta subunit), *pyr*G (CTP synthase), *rpo*B (RNA polymerase beta subunit), *fus*A (translation elongation factor G), and 16S rRNA, or, alternatively, by omitting the 16S rRNA gene sequences (accession numbers FN59891 to FN598917). Homologous sequences were retrieved from the public repository MicrobesOnline (Alm *et al*., 2005; Richter *et al*., 2009), aligned by using the program Clustal X 1.83 (Thompson *et al*., 1997) and the alignments were concatenated in a single dataset.

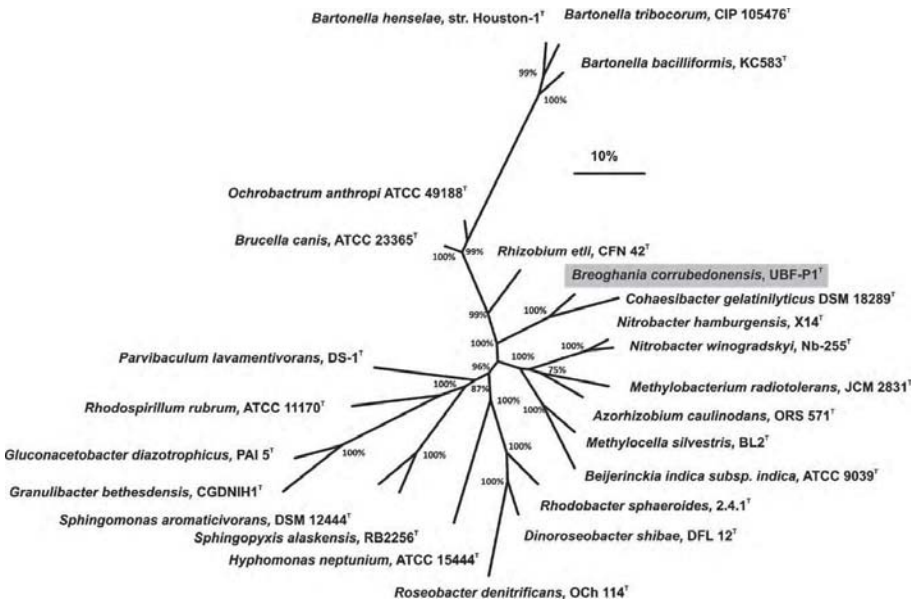

Figure 7. MLSA phylogenetic reconstruction based on concatenated genes *atp*D, *pyr*G, *rpo*B, *fus*A and 16S rRNA of strain UBF-P1 and *Cohaesibacter gelatililyticus* DSM 18289^T, and the selections strains of the *Alphaproteobacteria* for which fully sequenced genomes are available.

The concatenated five partial sequences, using only highly conserved positions, reconstructed a very stable tree (Figure 7). Both bacterial strains, UBF-P1 and *C. gelatiniliyticus* DSM 18289^T affiliated together consistently, with identities that ranged

from 87.8 to 85.6%, depending on whether or not the 16S rRNA gene sequence was included.

Although the new isolate seemed to fall into the recently proposed family *Cohaesibacteraceae* (Hwang and Cho, 2008), the 16S rRNA gene sequence of UBF-P1 did not share the signature positions 194, 678, 712, 1244 and 1293 of that family. The found signatures more closely resembled the majority of other families of the order. Consequently, these observations led to question the reliability of those signature positions for circumscribing families.

Table 3. Distinctive characteristics of strain UBFP1^T and *C. gelatinilyticus* DSM 18289^T. Unless indicated, the tests were simultaneously performed using both strains.

Characteristic	Bacterial strain	
	UBFP1^T	*C. gelatinilyticus* CL-GR15^T
Cell shape	Regular, curved, bulbous rods	Irregular rods[1]
Cell size (mm)	0.6-0.7 x 2.0-3.5	0.2-0.4 x 1.0-3.0[1]
Division type	Binary fission or asymmetric division	Binary fission or asymmetric division[1]
Motility	+	+
Flagella	1 or 2, subpolar	polar[1]
Growth temperature (ºC)		
Optimal	30	25-30
Range	15-40	15-31
NaCl range for growth(%)		
Range	1%-10%	2%-5%
Growth pH		
Optimal	7.5	8.0
Range	5.0 - 8.5	6.0-9.0
Oxigen requirement	Aerobic	Facultatively anaerobic
Catalase	+	+
Oxidase	+	+
Nitrate reduction	+	+*
Nitrite reduction	-	+*
Gelatin hydrolysis	-	+
ADH test	+	-
Urease	+	-
G + C content (mol%)	63.9	52.8 – 53
Major quinone	Q-10	Q-10
Major fatty acid(s) (>10%)	$C_{18:1}\omega7c$ (75.3%)	$C_{18:1}\omega7c$ (58.8); Summed feature 3†(20.8)
Major polar lipids	Diphosphatidylglycerol (DPG)	Diphosphatidylglycerol (DPG)
	Phosphatidylglycerol (PG)	Phosphatidylglycerol (PG)
	Phosphatidylethanolamine (PE)	Phosphatidylethanolamine (PE)
	Phosphatidylmonomethylethan-olamine (PME)	Phosphatidylmonomethylethan-olamine (PME)
	Phosphatidylcholine (PC)	Phosphatidylcholine (PC)

[1] Data reported by Hwang and Cho.
† feature 3 contains one or more of $C_{16:1}$ ω 7c and/or $C_{15:0}$ ISO 2-OH.
* Our results differ from the previously reported data by Hwang and Cho, 2008.

Morphological and physiological tests for both studied strains (Table 3) indicated that strain UBF-P1 could be differentiated from its closest relative genus by means of the nitrite reduction, gelatine hydrolysis, ADH, ODC and urease tests. On the other hand, some of the results found for *C. gelatinilyticus* CL-GR15^T differed from those initially reported and used to describe the family. In consequence, an amendment of this description is proposed below. The cellular fatty acid profile of UBF-P1 (Table 4) shows a predominance of octadecenoic acids together with cyclic $C_{19:0}$ fatty acid, which is typical of the *Rhizobiales* (Moreno *et al*., 1990; Jarvis *et al*., 1996; Kämpfer and Kroppenstedt, 1996; Wilkinson, 1998; Dunfiled *et al*., 1999; Tighe *et al*., 2000). The polar lipid content (Table 4) was similar in both strains, including diphosphatidylglycerol

(DPG), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylmonomethylethanolamine (PME), and phosphatidylcholine (PC) as major components. The respiratory lipoquinone was Q-10, which is present in all the families of the order. The DNA G+C content of UBF-P1 was 63.9 mol%, which is more than 10% higher than that of *Cohaesibacter*, but falls within the variability of the members of the order (Hwang and Cho, 2008).

Table 4. Fatty acid composition of strain UBF-P1^T and *C. gelatinilyticus* DSM 18289^T. Values are percentages of total fatty acids. Cells were grown on Marine Agar at 25ºC. The analysis were simultaneously performed using both strains.

Fatty acid	Content (%)	
	UBF-P1^T	*C. gelatinilyticus* CL-GR15^T
Summed feature 2*	0.9	2.5
Summed feature 3*	2.5	20.8
$C_{16:0}$	4.4	0.7
$C_{17:1}\omega 6c$	0.5	-
$C_{17:0}$	0.9	-
$C_{18:1}\omega 7c$	75.3	58.8
$C_{18:0}$	2.2	3.0
$C_{19:0}$ cyclo$\omega 8c$	6.4	0.6
$C_{19:0}$ 10 methyl	-	0.7
$C_{18:0}$ 3-OH	2.4	2.5
$C_{20:1}$ $\omega 7c$	-	9.2
$C_{20:1}$ $\omega 9c$	1.3	-
Other†	3.3	1.1

* Summed features contain one or more of the following: feature 2, $C_{14:0}$ 3-OH and/or $C_{16:1}$ ISO I; feature 3, $C_{16:1}$ ω 7c and/or $C_{15:0}$ ISO 2-OH.
† Sum of identified components that made up less than 0.5% or unknown (less than 2%).

To complete the comparison between both strains, a matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis was performed revealing that both strains had a completely different profile for the major detectable macromolecules. Those profiles have been stored in the SARAMIS reference spectra database for future identification (Kallow *et al*., 2010).

All the phylogenetic, genetic and phenotypic data analysed indicated that UBF-P1 clearly isolated within the order *Rhizobiales*, and affiliated with the family *Cohaesibacteraceae* (Hwang and Cho, 2008). However, the dissimilarities with the closest genus *C. gelatinilyticus* DSM 18289^T, confirms that our isolate should be circumscribed to a new genus and species that is described below.

In an attempt to identify the role that strain UBF-P1 may play in the degradation of pyrene by microbial consortium UBF-Py, several hydrocarbons and bacterial metabolites from polycyclic aromatic hydrocarbons were assayed as carbons sources. Strain UBF-P1 was not able to grow on hexadecane, pyrene, phenanthrene or phenanthrene metabolites (4-phenanthrene carboxylic acid, phthalic acid and diphenic acid). However, growth was positive on protocatechuic acid. These results suggested that strain UBF-P1 did not participate in the initial attack of pyrene, but could grow on acidic compounds resulting from the degradation of pyrene or other hydrocarbons furnished by other bacterial populations present in the consortium.

Description of *Breoghania* gen. nov.

Breoghania (Bre.o.gha'ni.a. N.L. fem. n. *Breoghania*, named after *Breoghan*, according to Celtic mythology (*Leabhar Ghabhala*, XII century), the first Celtic king of *Gallaecia* (actual Galicia), founder of the city of *Brigantia* (probably A Coruña) that built a tower on the coast from where *Eire* (Ireland) could be seen.

Cells are Gram-negative, aerobic rods, motile by polar flagella. Cells divide by binary fission or asymmetric division. Oxidase and catalase positive, and the G+C content of the DNA is 63.9%mol. The respiratory quinone is ubiquinone 10 (Q10) and the predominant cellular fatty acid is $C_{18:1}\omega7c$ (75.3%). The major polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylmonomethylethanolamine, and phosphatidylcholine. On the basis of 16S rRNA gene sequences, UBF-P1^T affiliates with the Class *Alphaproteobacteria* and the type species is *B. corrubedonensis*.

Description of *Breoghania corrubedonensis* sp. nov.

Breoghania corrubedonensis (Co.ru.be.do.nen'sis N.L. fem. adj. corrubedonensis, of or belonging to Corrubedo, northwest Spain, isolated from the beach of Corrubedo, the location where the sand sample used to inoculate the enrichment cultures from which strain UBF-P1^T was isolated).

In addition to the characteristics reported for the genus, cells are regular, irregular or bulbous rods, motile by one or two subpolar flagella and approximately 0.6-0.7 µm wide and 2-3.5 µm long. They reproduce by asymmetric division. After 5 days of growth on LB ASW (artificial sea water), LB 3% NaCl, or Marine Agar, colonies present a diameter of aprox. 1mm and are are circular, entire, convex, mucoid, and creamy white in colour. After incubation for 5 days at optimal growth conditions, colonies are approximately 1mm in diameter. The strain grows at temperatures in the range of 15–40 ◦C (optimum 30 ◦C) and at pH 5–8.5. Growth occurs with NaCl concentrations of 1–10%. Hydrolysis of gelatine or aesculin does not occur. Aminopeptidase, arginine dihydrolase, ornithine decarboxylase and urease activities are present. Nitrate is reduced to nitrite. Negative for nitrite reduction, indole production, and H_2S formation. β-Glucosidase and β-galactosidase are not produced. Starch, DNA or Tween 80 are not degraded. Acid is only weakly produced from l-arabinose. Growth occurs on glucose, arabinose, mannitol, D-sorbitol, adipate, gluconate (weak), phenyl acetate, acetate, succinate, malate, pyruvate, casaminoacids (Difco), acetone, and Tween 20. No growth occurs on D-xylose, mannose, maltose, starch, caprate, *N*-acetyl-glucosamine, citrate, lactate, oxalacetate, propionate or methanol. The fatty acids are $C_{18:1}\omega7c$ (75.3%), $C_{19:0}$ cyclo ω8c (6.4%), $C_{16:0}$ (4.4%), $C_{16:1}$ ω7c and/or $C_{15:0}$ iso 2-OH (summed feature 3, 2.5%), $C_{18:0}$ 3-OH (2.4%), $C_{18:0}$ (2.2%), and $C_{20:1}$ ω9c (1.3%). Other minor (<1%) fatty acids are $C_{14:0}$ 3-OH or $C_{16:1}$ iso I (summed feature 2), $C_{17:1}$ ω8c, $C_{17:1}$ ω6c, and $C_{17:0}$. Major polar lipids are diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylmonomethylethanolamine (PME), and phosphatidylcholine (PC). The DNA content is 63.9 mol%. The discriminative traits of the species are listed in diagnostic Table 3 and in supplementary material of article 2. The type strain of the species UBF-P1^T has been deposited in three culture collections under the following numbers: CECT 7622, LMG 25482, and DSM 23382.

Emended description of the family *Cohaesibacteraceae,* Hwang and Cho 2008.

The description of the family *Cohaesibacteraceae* was mainly based on the signature sequence and the taxonomic information of the two described members (Hwang and Cho, 2008). However, the signature positions given as indicative of the family are not longer valid. For instance, the 16SrRNA gene sequence of *B corrubedonensis* does not

share the signature positions 194, 678, 712, 1244 or 1293. The signatures found more closely resemble the majority of other families of the order. In addition, reduction of nitrate is positive. The DNA composition ranges from 52 to 64 mol% G+C.

Emended description of *Cohaesibacter gelatinilyticus* Hwang and Cho 2008

The description is as proposed by Hwang and Cho (2008) with the following modifications: nitrate and nitrite reduction are positive. It does not utilize citrate or *N*-acetyl-glucosamine. Oxidative acids from sugars are not produced. Aesculin is not hydrolysed. It does not utilize the hydrocarbons hexadecane, pyrene or phenanthrene, nor acetone or methanol for growth. The type strain is *C. gelatinilyticus* CL-GR15^T (=DSM 18289^T).

3.3. Oleophilic fertilizers promote biodegradation of polycyclic aromatic hydrocarbons present in Nonaqueous-phase liquids by compensating nutrient limitation in the interphase

In the environment PAHs are found as part of non-aqueous phase liquids (NAPLs) such as crude oil, coal tar, and derivatives. This results in a slow partitioning into the water phase that may dramatically reduce their bioavailability and, in consequence, slow down their biodegradation rates and increase their environmental persistence (Ortega-Calvo *et al*., 1995; Peters *et al*., 1999; López *et al*., 2008). Microorganisms present specific mechanisms to overcome the limited transfer of lipophilic substrates to the aqueous phase, such as attachment to the NAPL/water interface, biosurfactant production, chemotaxis, and biofilm formation (Ortega-Calvo and Alexander; 1994; Law and Aitken, 2003; García-Junco *et al*., 2003). It has been reported that microbial growth in the interphase may also cause local nutrient shortages when the most abundant and degradable components of the NAPL are degraded simultaneously with the chemical of interest, in this case PAHs, even if the overall concentration of the nutrients in the system is not limiting (Alexander, 1999). Oleophilic biostimulants are nutrient formulations that were developed with the assumption that they will remain associated with the NAPLs avoiding their dilution in marine environments. They have been applied successfully in numerous bioremediation experiences, being mainly recommended for use on rocky shores or where wave action hinders the effectiveness of slow release and water-soluble biostimulants (Díez *et al*., 2005). However, little is known on how nutritional limitations operate with attached bacteria and how their biodegradation activity can be enhanced during NAPL biodegradation.

The objectives of this work were to assess the possible nutritional deficiencies during PAH biodegradation at the NAPL/water interface and to examine the potential accumulation of partially oxidized metabolites, more available and toxic than the parent PAHs, in the presence or absence of the oleophilic fertilizer S200. In order to attain this goal a laboratory experiment was set up employing a NAPL/water system that maintained the integrity of the organic phase (García-Junco *et al*., 2003) in which the biostimulant was added and that was submerged in mineral media supplemented with overall non-limiting concentrations of inorganic nutrients as water soluble salts. This study was carried out in collaboration between the group of Dr. Ortega-Calvo (IRNASE, CSIC, Sevilla) and our group at the UB. The groups at CSIC set up the experiments and analyzed the mineralization rates, while our group analyzed the biodegradation of the fuel components.

The culture system consisted on sterile biometric flasks equipped with an open-ended glass tube placed vertically in each flask that contained the NAPL. This NAPL was a heavy fuel fluidized with heptamethylnonane HMN (1:1), in order to reduce viscosity and allow reproducible results. The biphasic NAPL/water system used maintained the integrity of the NAPL, thus avoiding potential interferences resulting from emulsion of the NAPL caused by the surfactant component of the biostimulant.

The flasks were inoculated with *Mycobacterium gilvum* VM552, a strain isolated obtained from a PAH-polluted soil capable to use phenanthrene, fluoranthene and pyrene as sole source of carbon and energy. This strain could not grow on HMN. Replicate cultures were supplied with ^{14}C-PAHs to measure specific mineralization rates, but here we will center our discussion of the biodegradation results obtained in our laboratory.

The cultures were incubated for 1500 h, and then, the residual fuel/HMN NAPL from inoculated flasks and abiotic controls was carefully removed, and both the NAPL phase and the aqueous phase were solvent extracted and analyzed by GC-MS

The results obtained from the analyses of the NAPL phase in culture flasks and abiotic controls indicated that the oleophilic biostimulant enhanced the biodegradation of all the families of hydrocarbons analyzed. The total alkanes were significantly degraded (60% ± 12.8%, $p<0.05$) only when the oleophilic stimulant was present. Similar results were found when the degradation percentages were calculated on the basis of the C_{17}/pristane and C_{18}/phytane ratios (results not shown). Interestingly, high molecular weight saturated hydrocarbons (C_{24}-C_{35}) were more extensively degraded (64% - 67%) than smaller alkanes with higher solubility in water (C_{18}-C_{20}, 49%-59%; C_{14}-C_{17}, not significantly degraded). These results show that although short-chain alkanes are in general more biodegradable in the environment, this general rule is not always followed when single bacterial species are considered.

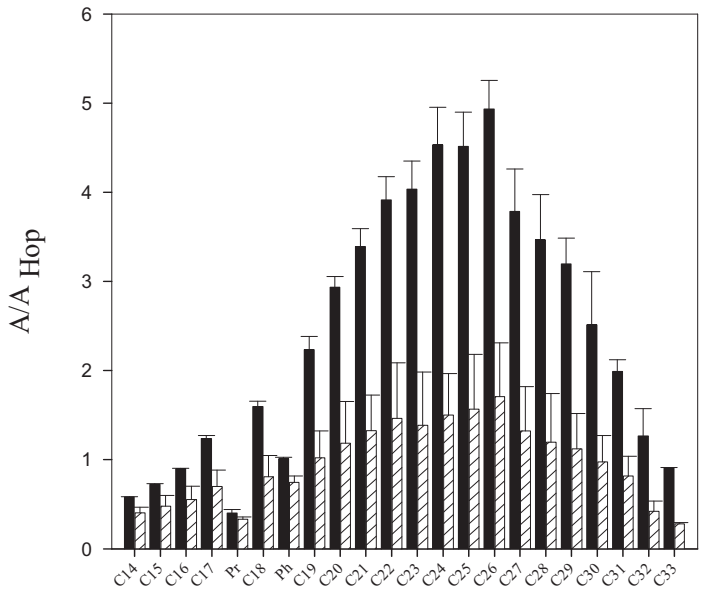

Figure 8. Hopane normalized areas (*A*/*A*HOP) of the *n*-alkanes (*m*/*z* 85) detected by GC-MS analysis in the fuel/HMN residue of the abiotic controls (black bars) and cultures (striped bars)

PAHs and alkyl derivatives were biodegraded about 22% in the absence of S200, while in the presence of the biostimulant this percentage increased to 60%. Only fluorene (43% ± 4.7%) and phenanthrene (38% ± 15%) were significally degraded in the absence of biostimulant. However, when the biostimulant was present a more extensive removal of those PAHs was achieved (84% ± 6.4% for fluorene, 99% ± 0.3% for phenanthrene) in addition to an important degradation of anthracene (40% ± 12.9%), fluoranthene (68% ± 3.4%) and pyrene (43% ± 4.7).

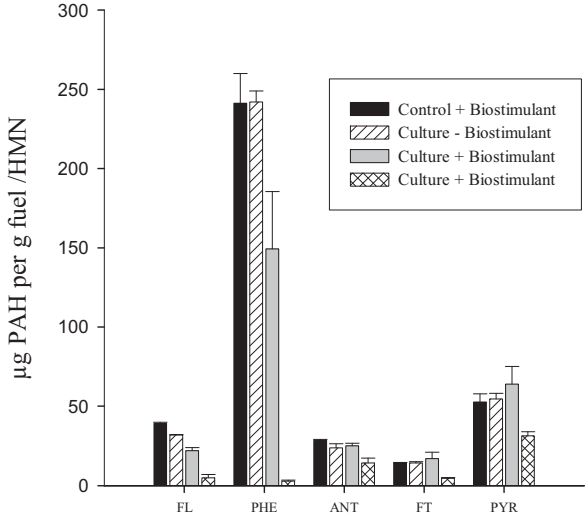

Figure 9. Concentrations of PAHs recovered from NAPL phase from controls and inoculated flasks, both without and with biostimulant. Only PAHs with significative degradation ($p<0.05$) are shown. The bars show the ratio between the peak area and the area hopane for each type of sample. FL, fluorene; PHE, phenanthrene; ANT, Antharecene; FLT, fluoranthene; PYR, pyrene.

Methylated PAHs, more abundant than their nonalkylated counterparts, were attacked in a lesser extent, showing only a light but significant degradation in the presence of the biostimulant. Indeed, the corresponding fragmentograms showed a selective degradation of mono- and dimethylnaphthalenes and monomethylphenanthrenes, following the patterns described in the literature (Kostecki and Calabrese, 1992; Wang *et al*., 1998; Vila *et al*., 2001; Vila and Grifoll, 2009).

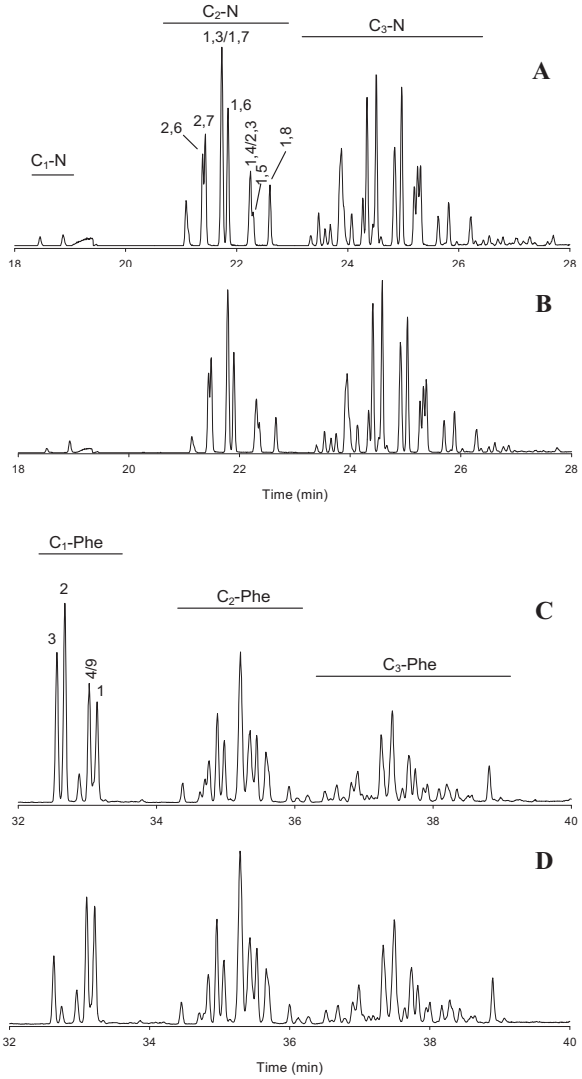

Figure 10. GC-MS fragmentograms showing the selective degradation of the alkyl naphthalene (A and B) and alkyl phenanthrene (C and D) families in flasks inoculated with *Mycobacterium* sp. VM522 (B and D) in the presence of biostimulant, as compared with non-inoculated controls (A and C).

The GC-MS analysis of the neutral and acidic extracts from the aqueous phase with and without biostimulant presented similar profiles of metabolite accumulation. However, the concentration in the flasks with biostimulant was about 10-fold higher (values went from about a tenth to several micrograms per milliliter, i.e., 120 $ng \cdot mL^{-1}$ for 1-indanone, 140 $ng \cdot mL^{-1}$ for diphenic acid, 690 $ng \cdot ml^{-1}$ for 2-carboxycinnamic acid, or 3.6 $\mu g \cdot mL^{-1}$ for 4-methylphtalic acid).The identification of metabolites detected was based on authentic standards, fragmentation patterns and a match higher than 90% with the NIST library (Table S1 and S2, supporting information of article 3). Neutral metabolites corresponded to alkyl oxidized or phenolic naphthalenes, while the acidic compounds were mainly dicarboxylic acids, typically accumulated during the

degradation of PAHs and their methyl derivatives by *Mycobacterium* strains (López *et al*., 2008; Vila *et al*., 2001; Vila and Grifoll, 2009). Specifically, phthalic acid is a common intermediate in the degradation of naphthalene, phenanthrene, fluoranthene and pyrene, while carboxycinnamic and diphenic acid are naphthalene and phenanhtrene metabolites respectively. The presence of metabolites with methyl groups indicates similar biodegradation pathways for the corresponding alkyl PAHs.

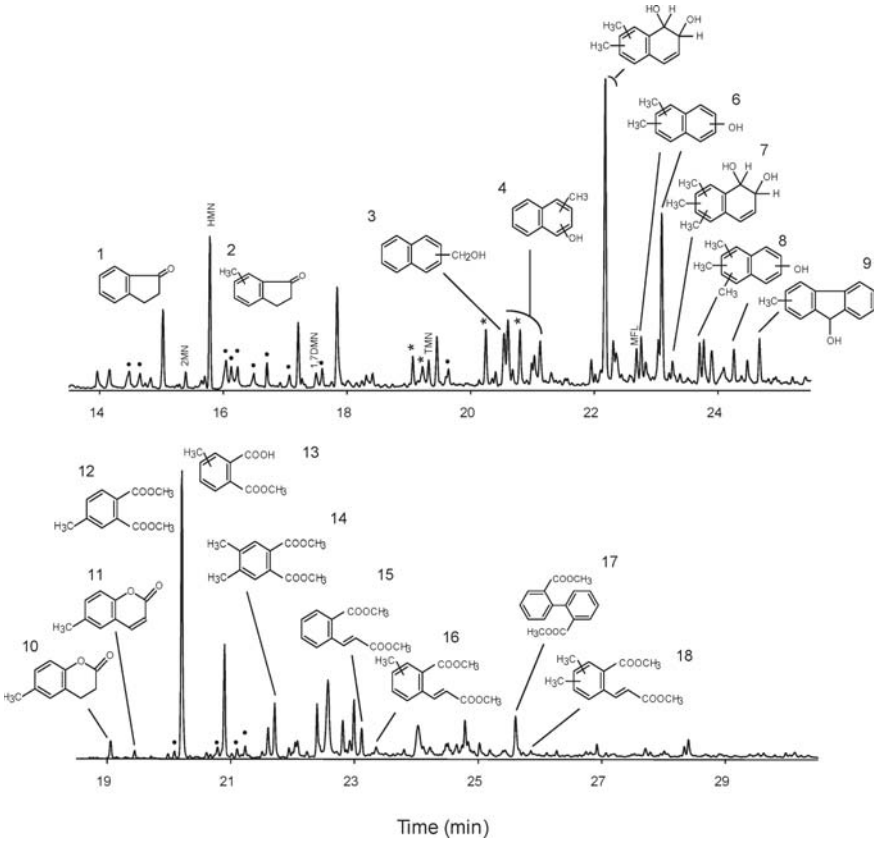

Figure 11. Typical GC-MS chromatograms of the neutral (top) and acid (bottom) extracts from the aqueous phase of the *Mycobacterium gilvum* VM522 culture in the presence of biostimulant indicating the identified peaks.

As previously stated, all the cultures contained non-limiting concentrations of inorganic water soluble nutrients, and the overall contribution of N and P by the oleophilic fertilizer was negligible. On the other hand, results obtained by the CSIC research group indicate that the oleophilic fertilizer did not increase the bioavalability of the individual PAHs (i.e. their NAPL/water partition coefficient). Therefore, the obtained results demonstrate that the oleophilic fertilizer caused an enhancement of PAH biodegradation by locally supplying nutrients in the the NAPL/water interface and promoting the growth of the attached degrading bacteria. This nutrient supply was necessary because the growth of the bacterial strain using the more abundant and ready degradable alkanes caused a limitation on this nutrients in the interface. In addition, it was demonstrated that the enhanced biodegradation produced by this biostimulant, widely used in bioremediation, may be accompanied by an increase in the

production of partially degraded metabolites that partition into the aqueous phase, being more mobile and bioavailable than the parent compounds.

3.4. Effect of sunflower rhizosphere on PAH removal and microbial community structure in an aged creosote polluted soil

There is a considerable interest in the use of biological procedures to remediate contaminated soils, both in terms of pollutant elimination efficiency and preservation of the natural environmental conditions. Bioremediation procedures are based on the ubiquitous ability of microorganisms to degrade pollutants such as PAHs. However, the main limiting factor that bioremediation experiences show is the reduced bioavailability of these contaminants, which results in their persistence into the environment for long periods. Rhizoremediation, the use of the plant rhizosphere as ecosystem to decontaminate polluted soils, has recently gained attention due to it feasibility and contribution to the elimination of organic pollutants. Among these experiences, sunflower plants (*Helianthus annuus*, L) have been reported as highly beneficial in PAH removal from polluted soils (Maliszewka-Kordybach and Smereczak, 2000). However, their role in promoting the biodegradation in aged polluted soils has not been assessed.

The research presented in this study uses a soil that has been affected by creosote pollution for a long period of time, and its PAH content and bioavailability are similar to those found in polluted soils that had undergone extensive bioremediation. The objectives of our work were to determine the viability of using sunflower plants to reduce the residual concentration of PAHs, characterize the root exudates that could play a potential stimulating effect, and determine the shifts in microbial community structure caused by the rhizosphere effect.

This study was a collaboration between the group of Dr. Cantos and Dr. Ortega-Calvo from IRNASE (CSIC, Seville), that set-up the experiment, developed the method to produce root exudates in sterile conditions, and performed PAH mineralization and degradation experiments in the laboratory.

The greenhouse experiments showed that all of the sunflower seeds germinated in creosote-contaminated and uncontaminated soils, with those planted in contaminated soil being significantly taller and presenting higher dry weight. These differences may be attributed to the activity of the microorganisms introduced with the creosote polluted soil that may have been beneficial for the plants due to mobilizing soil nutrients.

The biodegradation experiments showed that the rhizosphere reduced the PAH levels by 93% in 90 days, representing an improvement of 16% compared to contaminated soil without plants. The concentrations of anthracene, fluoranthene, pyrene and chrysene decreased significantly below the levels detected in the unplanted controls after 45d and 90d. A positive effect of planting on the dissipation of fluorene was only observed after 45d, while the levels of these PAH reached concentrations below the detection limit in both conditions at 90 days. The presence of sunflower plants had no significant effect on the dissipation of phenanthrene in any of the sampling periods, presenting a significant decrease after 45 days in both treatments.

The culture dependent analysis of the degrading and heterotrophic microbial populations using the MPN (most probable number) technique (Figure 12) showed a general two order increase in the heterotrophic populations between days 0 and 45. Thereafter, these populations remained constant in the planted soil while decreased slightly in the controls without plant. This was probably due to the depletion of the carbon sources in the controls, which would have been compensated in the treated soil by the rhizosphere. The low molecular weight (LMW) PAH-degrading populations showed a similar behavior, with also an increase of two orders of magnitude between 0 and 45 days in all conditions followed by a slightly decrease in the non-planted soil, but remaining constant in the treated soil. Interestingly, HMW PAH degraders experienced

a substantial increase by 45 d, especially in the plant treatment, and remained at high levels until the end of the experiment. Since at 90 days, the ratio between the HMW PAH degraders and total heterotrophic populations was substantially higher in the treated than in the untreated soil, it could be concluded that in addition to stimulating the general growth of the heterotrophic populations (including that of PAH degraders), the rhizosphere treatment had an additional selective effect on enhancing the growth of the HMW PAH-degrading populations. These results were consistent with those obtained in the PAH analysis and confirm the results obtained by other authors (Miya and Firestone, 2000; Parrish *et al.*, 2005).

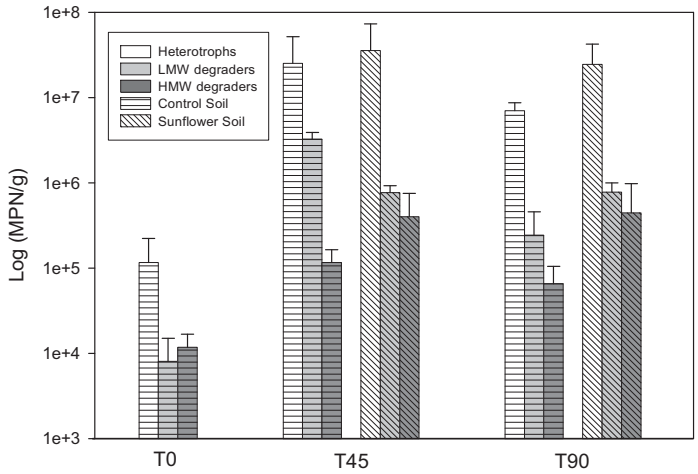

Figure 12. Counts of heterotrophic and PAH-degrading microbial populations in the soil under the different treatments applied in the greenhouse experiment. MPN, most probable number. LMW, low-molecular-weight PAHs. HMW, high-molecular-weight PAHs.

It is known that the microbial communities in the rhizosphere can be considerably different than those in nearby soil that grow without the direct influence of roots. The first approach that we used to observe specific changes in the microbial populations was a 16S rDNA PCR-DGGE analysis (Figure 13). The DGGE profiles from replicate samples for each treatment were very similar, thus indicating a high homogeneity within the pots for each condition. During the incubation time, specific populations increased in relative abundance in both the non-treated and planted soil. A number of bands detected coincided in both treatments, however, their abundance differed from the non-planted soil and the rhizosphere soil, confirming that the rhizosphere may have an effect in promoting the growth of specific populations different than the simple effect of potting and watering the polluted soil.

Table5. Sequence analysis of the 16S rRNA gene clone libraries from the PAH-polluted control soil (without plants) and sunflower rhizosphere soil.

Clone	Frequency (%)[a]		Fragment length (bp)	Sim %	Closest relative in GenBank database [b] (accession no)	Phylogenetic group
	CS	SFS				
CS1		2.4	1364	99	Uncultured *Acidobacteria* bacterium clone HEG_08_216 (HQ597545)	*Acidobacteriaceae (Acidobacteria)*
CS2		2.4	1373	98	Uncultured bacterium clone 60C1 (EU676416)	*Acidobacteriaceae (Acidobacteria)*
CS3		2.4	1422	99	Uncultured *Acidobacteria* bacterium clone SEG_08_603 (HQ729829)	*Acidobacteriaceae (Acidobacteria)*
CS4		2.4	1404	97	Uncultured *Acidobacteria* bacterium clone HG-J02120 (JN409027)	*Acidobacteriaceae (Acidobacteria)*
CS5		2.4	1421	99	Uncultured bacterium clone p6h2ok (FJ478980)	*Acidobacteriaceae (Acidobacteria*
CS6		2.4	1386	97	*Acidobacteria* bacterium IGE-018 (GU187039)	*Acidobacteriaceae (Acidobacteria)*
CS7	2.3		1466	99	Uncultured bacterium clone p27d24ok (FJ478675)	*(Actinobacteria)*
CS8		2.4	1384	99	*Lentzea waywayandensis* strain 173629 (EU570362)	*Pseudonocardiaceae (Actinobacteria)*
CS9	4.7		1444	98	Uncultured bacterium clone 125 (FM209343)	*Cytophagaceae (Bacteroidetes)*
CS10	7.0		1453	99	Uncultured bacterium clone p8c07ok (FJ479495)	*Chitinophagaceae (Bacteroidetes)*
CS 11	2.3		1412	98	Uncultured bacterium isolate 1112864242247 (HQ120332)	*Ohtaekwangia (Bacteroidetes)*
CS 12	2.3		1314	98	Uncultured *Bacteroidetes* bacterium clone HG-J01164 (JN408934)	*Ohtaekwangia (Bacteroidetes)*
CS 13	2.3		1443	95	Uncultured bacterium clone TX2_4C19 (JN178178)	*Rhodothermaceae (Bacteroidetes)*
CS 14	2.3		1329	92	Uncultured bacterium clone p27c17okm (FJ479173)	*Ohtaekwangia (Bacteroidetes)*
CS 15	4.7	4.9	1445	98	Uncultured *Bacteroidetes* bacterium clone g31 (EU979040)	*Ohtaekwangia (Bacteroidetes)*
CS 16	2.3	2.4	1393	99	Uncultured bacterium clone 224T (EU676412)	*Chitinophagaceae (Bacteroidetes)*
CS 17		2.4	1394	98	Uncultured soil bacterium clone UA2 (DQ298006)	*Chitinophagaceae (Bacteroidetes)*
CS 18	2.3		1475	99	Uncultured *Anaerolinae* bacterium clone AMAG11 (AM935836)	*(Choloroflexi)*
CS 19		2.4	1353	99	Uncultured bacterium clone S-Rwb_62 (DQ017911)	*(Choloroflexi)*
CS 20		2.4	1353	99	Uncultured bacterium clone H3-26 (JF703479)	*(Choloroflexi)*
CS 21		4.9	1373	99	*Phormidium autumnale* CCALA 143 (FN813344)	*(Cyanobacteria)*
CS 22		2.4	1324	99	Uncultured diatom clone H-101 (HM565019)	*Bacillariophyta (Cyanobacteria)*
CS 23	4.7		1455	96	Uncultured bacterium isolate 1112864242286 (HQ120393)	*Trueperaceae (Deinococcus)*
CS 24	2.3		1423	99	*Bacillus* sp. M71_D96 (FM992837)	*Bacillaceae (Firmicutes)*
CS 25	9.3		1440	99	*Bacillus* sp. R-36493 (FR682744)	*Bacillaceae (Firmicutes)*
CS 26	2.3		1486	98	*Virgibacillus carmonensis* (T) LMG 20964 (NR_025481)	*Bacillaceae (Firmicutes)*
CS 27	4.7		1438	99	*Bacillus* sp. BF149 (AM934692)	*Bacillaceae 2 (Firmicutes)*
CS 28		4.9	1399	96	Uncultured bacterium clone 15-4-139 (JN609373)	*Gemmatimonadaceae(Gemmatimonadetes)*
CS 29		2.4	1429	98	Uncultured bacterium clone TX5A_120 (FJ152828)	*(Gemmatimonadetes)*
CS 30	2.3		1368	94	Uncultured bacterium clone B6 (FJ660498)	*Planctomycetaceae (Planctomycetes)*

CS 31	2.3		1285	99	*Chelatococcus asaccharovorans* CP141b (AJ871433)	*Methylobacteriaceae (α)*
CS 32	2.3		1276	99	Uncultured bacterium clone HDB_SIOP800 (HM186473)	*Bradyrhizobiaceae (α)*
CS 33	2.3	2.4	1410	97	Uncultured soil bacterium clone F6-154 (EF688392)	*Sphingomonadaceae (α)*
CS 34		2.4	1342	99	*Rhizobium* sp. AC93c (JF970343)	*Rhizobiaceae (α)*
CS 35		2.4	1355	97	Uncultured bacterium clone FCPS478 (EF516121)	*Rhizobiales (α)*
CS 36		2.4	1353	99	Uncultured alpha proteobacterium clone QZ-J4 (JF776915)	*Sphingomonadaceae (α)*
CS 37		2.4	1355	99	*Altererythrobacter* sp. JM27 (GU166344)	*Sphingomonadales (α)*
CS 38	2.3		1425	98	Uncultured bacterium clone SNR65 (AB608675)	*Burkholderiales (β)*
CS 39	4.7	7.3	1418	99	Uncultured bacterium clone HC18-11B13 (JF417848)	*Commamonadaceae (β)*
CS 40	2.3	4.9	1410	99	*Naxibacter suwonensis* (T) 5414S-25 (FJ969487)	*Oxalobacteraceae (β)*
CS 41		2.4	1389	99	*Variovorax* sp. RA8 (AB513921)	*Commamonadaceae (β)*
CS 42		2.4	1396	99	Uncultured beta proteobacterium clone E2006TS6.19 (GU983311)	*(β)*
CS 43		2.4	1394	99	Uncultured beta proteobacterium clone C173 (JF833705)	*Methylophilaceae (β)*
CS 44		2.4	1400	99	Uncultured beta proteobacterium clone C173 (JF833705)	*Methylophilaceae (β)*
CS 45		2.4	1391	99	Uncultured beta proteobacterium clone G2-50 (JF703344)	*(β)*
CS 46		2.4	1391	99	Uncultured ammonia-oxidizing bacterium clone FQ-13C-HF-1 (HQ678202)	*Nitrosomonadaceae (β)*
CS 47	2.3		1434	97	Uncultured bacterium clone 0-99 (GU444064)	*Xanthomonadaceae (γ)*
CS 48	9.3		1458	99	*Pseudomonas* sp. JQR2-5 (DQ124297)	*Pseudomonadaceae (γ)*
CS 49		2.4	1412	99	*Lysobacter niabensis* (AB682414)	*Xanthomonadaceae (γ)*
CS 50		2.4	1391	99	Uncultured bacterium clone Kas172B (EF203204)	*Sinobacteraceae (γ)*
CS 51		2.4	1409	99	*Pseudoxanthomonas* sp. XC21-2 (JN247803)	*Xanthomonadaceae (γ)*
CS 52		2.4	1410	99	Uncultured *Lysobacter* sp. clone T302B2 (HM438520)	*Xanthomonadaceae (γ)*
CS 53		2.4	1399	99	Uncultured bacterium clone BR121 (HQ190468)	*Pseudomonadaceae (γ)*
CS 54		2.4	1419	97	Uncultured bacterium clone RH1020 (AB511013)	*(δ)*
CS 55	7.0		1415	86	Uncultured candidate division OD1 bacterium clone AKYH1067 (AY922093)	Candidate division OD1
CS 56	2.3		1396	94	Uncultured bacterium clone B03-05G (FJ542974)	Candidate división OP11
CS 57	2.3		1368	97	Uncultured bacterium clone N1903_34 (EU104291)	Candidate división TM7
CS 58	2.3		1250	93	Uncultured bacterium clone FF_-aag84c04 (EU469637)	Candidate division WS6

[a] Frequencies in clone libraries obtained from CS (control soil) and SFS (sunflower soil). [b]In sequences showing an identical match to uncultured and to isolated strains, only the latter are listed. Sequences with more than 94% identity are grouped. α, β, γ, δ correspond to *alpha-*, *beta-*, *gamma-* and *deltaproteobacteria*, respectively.

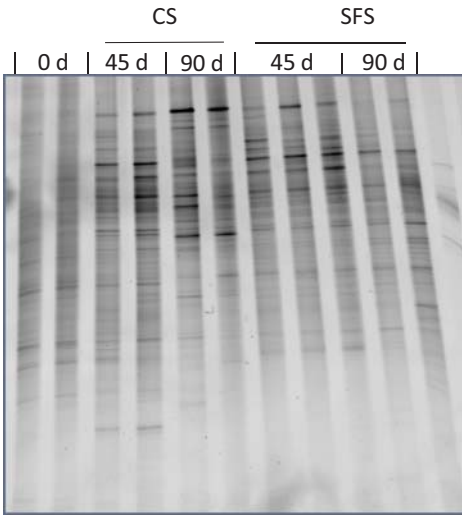

Figure 13. DGGE profile of PCR-amplified 16S rRNA gene fragments from independent replicate samples from control soil (CS) and rhizosphere sunflower soil (SFS) samples after 0, 45 and 90 days. Each lane was loaded with an identical amount of DNA.

In order to analyze the specific structure of the microbial communities present in each soil, 16S rRNA gene libraries were obtained. Soil samples were taken at 90 days and a total of 84 clones were sequenced. The phylogenetic affiliations of each eubacterial clone are detailed in Table 5, while figure 14 summarizes the different bacterial phyla detected and their relative abundances.

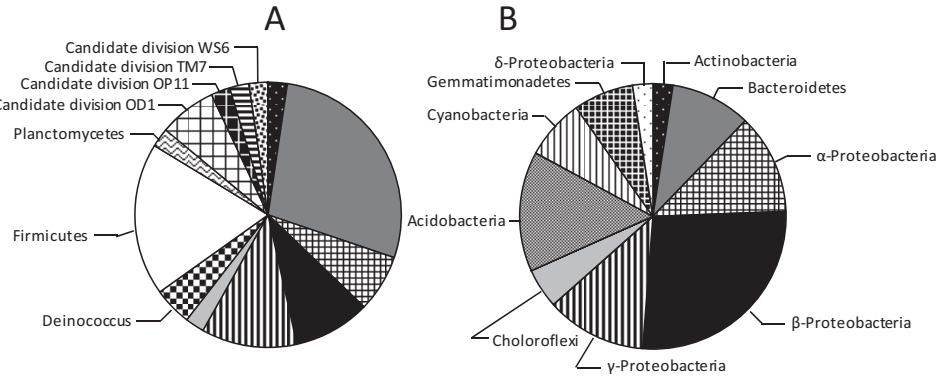

Figure. 14. Relative abundance of eubacterial phylogenetic groups identified in control soil (A) and sunflower rhizosphere soil (B) samples after 90 days of incubation in the greenhouse experiment.

The α-, β-, and γ-*Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, and *Chloroflexi* phyla were represented in both, the non-planted and the planted soil (60% and 68% respectively), however, with the exception of the *Actinobacteria*, their relative abundance varied substantially, confirming that the sunflower rhizosphere effected a dramatic shift in the soil community structure.

The rhizosphere promoted the appearance of new populations within the three *Proteobacteria* subphyla, some of which had been previously reported as PAH-

degraders (Kanaly and Harayama, 2010). Within the α-*Proteobacteria,* the increase of *Sphingomonas* group was interesting because it includes numerous members isolated from plant root systems and members with a versatile degrading capability, allowing them to attack 2-,3- and 4-ring PAH (Fernández-Luqueño *et al*., 2011). Within the β-*Proteobacteria* (from 9% to 27%), the appearance of the *Commamonas* group with members of *Variovorax*, it is also noticeable, since e species of this genus has been isolated from PAH- and xenobiotic-contaminated soils. In addition, the rhizophere promoted the appearance of members of the *Oxalobacteriaceae*, a recently described but uncharacterized family with root colonizing members (Green *et al*., 2007) that are closely related to the *Burkholderia*, which include important soil PAH degraders of both single compounds and creosote mixtures (Grifoll *et al*., 1995). The increase observed in members of the *Methylophillus* group was interesting because in a recent study, we identified a methylotrophic bacterial species as one of the most abundant components of a heavy fuel-degrading consortium (Vila *et al*., 2010). Methylotrophic bacteria are more widely distributed than previously thought, but their roles in natural habitats remain unknown (Lidstrom, 2006; Chistoserdova *et al*., 2009). The *Xanthomonas* group within the γ-*Proteobacteria* was also favored by the rhizosphere, with several of the detected representatives of this group corresponding to bacteria previously detected in polluted sites and identified as PAH degraders. For example, a *Pseudoxhantomonas* strain was recently described as being able to degrade the 4-ring PAH chrysene (Nayak *et al*., 2011). The reduction in the abundance of *Bacteroidetes* in the rhizosphere soil could be a direct consequence of the presence of nutrients from the exudates because this phylum has often been associated with non-nutrient environments (Viñas *et al*., 2005).

In the non-planted soil, 40% of the detected microorganisms belonged to seven phylogenetic groups not detected in the sunflower planted soil. Interestingly, among those , we found members of the Candidate divisions OD1, OP11, TM7 and WS6, which are lineages of non culturable prokaryotic organisms (Hugenholtz *et al*., 1998; Chouari *et al*., 2005), and a relatively high abundance of *Firmicutes*, while the *Planctomycetes* and *Deinococcus* groups were represented with lower proportions.

In contrast, the sunflower rhizosphere soil promoted the presence of four phylotypes (*Acidobacteria, Gemmatimonadetes,* δ-*Proteobacteria* and *Cyanobacteria*) that were not detected in the non-treated soil. The most abundant, the *Acidobacteria* (14.6%) and *Gemmatimonadete*s (7.3%), constitute recently described new phyla (Ludwig *et al*., 1997; Zhang *et al*., 2003) broadly distributed in soils but poorly represented in cultures, which makes it difficult to ascertain their role in nature. The *Acidobacteria* have been observed previously in planted soil (Sipila *et al*., 2008; Yrjala *et al*., 2010), are usually found in non-polluted environments, and generally decrease in the presence of pollutants. Therefore, their higher abundance here after 90 days of treatment may be explained by both the rhizosphere effect and the high degree of removal of PAHs attained in this condition.

To further determine the effect of the sunflower root exudates, several experiments were conducted. After obtaining the sunflower root exudates, a radiorespirometry/residue analysis was carried out to determine the mineralization of ^{14}C-pyrene and the biodegradation of the PAHs present in a soil sample resuspended in sunflower root exudates or mineral medium. At the end of the experiment (250h), the pyrene mineralization in the presence of sunflower root exudates reached 40%, while in cultures containing mineral medium was only 29%. Moreover, the residual contents of total PAHs were not significantly different than those reached in the greenhouse experiments, reassuring that the exudates play a key role in the degradation-promoting effect of the rhizosphere on the dissipation of PAHs.

The evolution of the authocthonous microbiota was also assessed (Figure 15). Cultures were sampled at the end of the incubation time (10d) and their microbial composition analysed by DGGE. During the 10 days of the experiment both treatments showed an increase in the number of microorganisms changing the banding profile as

a result of exposure to exudates, which indicates that the enhanced PAH degradation was accompanied by growth of specific microbial populations.

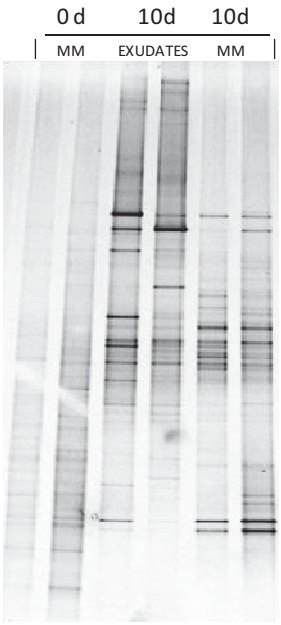

Figure 15 . DGGE profile of PCR amplified 16S rRNA gene fragments in samples from soil suspensions in the bioaccessibility experiment

To gain insight in the nature of the effect of the exudates a representative sample was chemically characterized. (Table 3 of article 4). Among the several components identified, the major carbohydrates were fructose and galactose, while the main amino acids were asparagine and glutamine. These substances have been reported as chemotaxis inductors and chemoattractans (Heinrich and Hess, 1985; Pandya *et al*., 1999; Zheng and Sincalir, 1996).The main fatty acids present were palmitic and stearic acid, whereas others, including the most abundant component of sunflower oil, linoleic acid were detected at lower concentrations. These substances may enhance the bioaccesibility of PAHs in soil by acting as surfactants (Yi and Crowley; 2007).Interestingly, several aromatic acids were identified, being the most abundant phthalic and protocatechuic. These compounds are typical intermediates in the PAH metabolism. These secondary plant metabolites may stimulate PAH degradation by rhizosphere microorganisms and broaden the spectrum of their activity by inducing and promoting the development of organic pollutant-degrading enzymes (Singer *et al*., 2003) or acting as cosubstrates in cometabolic reactions.

The results presented here suggest that the sunflower plants could be effective in promoting the bioaccesibility of PAHs in contaminated soils that have previously undergone extensive bioremediation but still contain unacceptable PAHs levels, due to bioaccesibility restrictions. Our results demonstrate that this strategy seems to represent a promising alternative for increasing bioaccesibiity in a sustainable and low-risk manner. This study is the first to analyze the effect of the rhizosphere on autochthonous bacterial community structure. The exact contribution of the direct effects of the sunflower exudates and the effects related to the ecology of soil microorganisms will be the subject of future research.

Conclusions

1. Culture independent analysis revealed that enrichment of natural marine coastal populations with fuel and then pyrene as carbon sources, produced a stable microbial degrading community mainly composed by *α-Proteobacteria* (84%*)* and *Actinobacteria* (16%). The most abundant population affiliated separately from existing genera within the α-*Proteobacteria,* while other identified members of this group were *Thalassospira*, *Martelella*, *Paracoccus*, *Novosphingobium*, *Sphingopyxis* and *Aurantimonas.* The only γ-*proteobacteria* was *Alcanivorax*, and the detected actinobacteria classified within *Gordonia* and *Micrococcus.*

2. A throughout screening of specific culture media permitted to recover almost all the detected bacteria as pure cultures, with the exception of *Thalassospira* and *Gordonia*. However, none of those or other additional bacterial strains isolated (*Novosphingobium*, *Sphingopyxis*, *Aurantimonas*, *Micrococcus* and *Alcanivorax*) were capable of attacking pyrene or phenanthrene.

3. Functional analysis on the consortium revealed the presence of a single Gram-positive dihydroxilating dioxygenase, not detected in the culturable members, that was closely related to the NidA3 ring hydroxilating dioxygenase found in pyrene-degrading actinobacteria. This strongly suggests that the non-culturable *Gordonia* plays a key role in the initial attack to the pyrene molecule, while the rest of the bacterial components are supported by pyrene-derived carbon molecules furnished by this strain. Conversely, the non-culturable *Gordonia* would need the presence of the other members for growth.

4. The most abundantly detected component of the marine pyrene-degrading consortium has been classified within the new genus and species described in this Thesis *Breoghania corrubedonensis*.

5. The oleophilic fertilizer S200 enhances the bacterial degradation of all the PAHs present in fuel by compensating a nutrient deficiency in the interphase NAPL/water caused by the simultaneous utilization of other components of the mixture (i.e., alkanes).

6. Biostimulation with oleophilic fertilizers increases the production of partially degraded PAH-metabolites that are easily mobilized towards aqueous phases, and whose toxicity and environmental fate remains unknown.

7. Sunflower rhizosphere had a significant effect in reducing the levels of residual PAH of an aged creosote polluted soil in respect to unplanted soil, causing a selective increase of the PAH-degrading microbial populations. Molecular analysis revealed a dramatic shift in the community structure, incrementing the relative abundance of α-, β-, *δ*- and γ-*Proteobacteria, Acidobacteria*, *Cyanobacteria*, and *Gemmatimonadetes,* most of them with PAH degrading representatives. The relative abundance of *Actinobacteria,* known high molecular PAH soil degraders was not affected by the rhizosphere.

8. Sunflower root exudates are composed of carbohydrates, mainly fructose and galactose, a variety of amino acids, and fatty acids such as palmitic and stearic. In addition, the typical PAHs metabolites phthalic acid and protocatechuic were detected. These components may act by enhancing the bioaccesibility of the PAHs

by favouring chemotaxis mechanisms, inducing PAH-degrading enzymes, and acting as cosubstrates.

9. The effect of the rhizosphere observed in soils was reproduced in cultures with exudates, indicating that the exudates are mainly responsible for the stimulation observed.

Introducción

Contaminación Ambiental por Hidrocarburos Aromáticos Policíclicos

Los hidrocarburos aromáticos policíclicos (HAPs) son un grupo de compuestos formados por dos o más bencenos fusionados en disposición angular, lineal o agrupada. La estructura más simple consta de 2 anillos (naftaleno) y las más complejas pueden estar formadas por más de 6 anillos y grupos alquilo. (Wilson y Jones, 1993). Debido a su toxicidad, amplia distribución ambiental y persistencia, los HAPs son considerados contaminantes prioritarios. En 1979, la Agencia de Protección Medioambiental (US EPA, en inglés) de Estados Unidos, incluyó los "16 EPA HAPs" en la lista de contaminantes de investigación prioritaria (Keith y Telliard, 1979).

Las propiedades físico-químicas más importantes de los HAPs son su baja solubilidad acuosa y el coeficiente de partición octanol-agua, que hacen que se adsorban fácilmente a los componentes del suelo y no estén biodisponibles. Estos parámetros físico-químicos cambian con la temperatura y son usualmente medidos a 20-25º C.

Los HAPs pueden causar diferentes efectos tóxicos dependiendo del compuesto, el tipo de exposición (aguda o crónica), los organismos, y las condiciones ambientales. En general, se diferencian cuatro mecanismos de toxicidad: narcosis no-polar, fototoxicidad, activación bioquímica y alteraciones en la regulación hormonal.

La producción de HAPs tiene lugar mediante tres procesos diferentes: origen diagenético (como la petrogénesis de materia orgánica y la formación de carbón), origen pirolítico (combustión incompleta de materia orgánica), y biogénesis. Generalmente, los HAPs se encuentran en forma de FLNA (fases líquidas no acuosas) como crudos de petróleo y derivados (gas-oil, fuel oil) y productos de carbón mineral (alquitrán de carbón, creosota…). Esos compuestos son mezclas muy complejas en la que los diferentes constituyentes exhiben una amplia variedad de propiedades químicas, físicas y toxicológicas.

Generalmente, los procesos que dan lugar a contaminación por HAPs se clasifican en fuentes naturales y antropogénicas. Las fuentes naturales de HAPs incluyen erupciones volcánicas, fuegos forestales, reacciones del metabolismo de plantas y bacterias (Borneff *et al*., 1968; Suess, 1976), y reacciones geológicas térmicas asociadas con la producción de fósiles de fuel y minerales. Las fuentes antropogénicas son las más significativas ,e incluyen la combustión de combustibles fósiles, la pirólisis del carbón y posterior destilación, la creosota, vertidos accidentales durante el transporte, producción y almacenaje de combustibles, procesos industriales, incineración de residuos y lodos urbanos, y efluentes residuales industriales (Cerniglia, 1992).

El destino medioambiental de los HAPs es importante puesto que son la mayor clase de de compuestos químicos carcinogénicos conocidos actualmente. Los posibles destinos de los HAPs en el medio ambiente incluyen volatilización, fotooxidación, oxidación química, bioacumulación, adsorción a partículas, lixiviación y degradación microbiana (Cerniglia, 1992; Kästner, 2000b).

Biodegradación y Biorremediación de HAPs

Actualmente, hay un considerable interés en el uso de sistemas biológicos para remediar ambientes contaminados con HAPs. Los procedimientos biológicos de remediación han sido desarrollados para algunos contaminantes orgánicos peligrosos, y han mostrado ser muy exitosos, tanto desde el punto de vista de la eliminación de contaminantes como en viabilidad económica. Los costes asociados a tratamientos térmicos y fisicoquímicos son mucho más elevados que los tratamientos biológicos y la mayoría de las veces el medio ambiente como sistema idóneo para la vida de

microorganismos es alterado (Wilson y Jones, 1993). Los tratamientos de biorremediación están basados en la ampliamente distribuida habilidad de los microorganismos para degradar contaminantes, siempre que las condiciones medio ambientales sean apropiadas. De hecho, muchas cepas de bacterias, hongos y algas han mostrado la capacidad de degradar una amplia variedad de HAPs que contienen desde 2 a 5 anillos aromáticos (Mueller *et al*., 1996). Sin embargo, la biorremediación de ambientes contaminados con HAPs no siempre produce los resultados deseados. En general los HAPs son rápidamente degradados hasta cierta concentración residual tras la cual apenas se observa degradación. Este fenómeno, conocido como la cinética de palo de jockey (Alexander, 1999) puede estar causado por la limitada concentración de nutrientes, la absorción de algunos HAPs a la matriz orgánica causando una baja disponibilidad, la difusión del compuesto en microporos inaccesibles para la biodegradación, y la ausencia de bacterias eficientes para la degradación de HAPs.

Factores que afectan a la degradación de HAPs

Hay varios factores que pueden afectar al resultado final en las experiencias de biorremediación. Los más importantes son los siguientes: condiciones medioambientales como presencia de tóxicos, pH, temperatura y humedad (en el caso de suelos), presencia de microorganismos adecuados, capaces de degradar los contaminantes, y la estructura molecular del contaminante, su concentración y biodisponibilidad.

Condiciones ambientales.

En algunos casos la contaminación de hidrocarburos puede estar asociada a altos niveles de otros contaminantes. Altas concentraciones de contaminantes tóxicos como metales pesados pueden ralentizar los procesos metabólicos e impedir el crecimiento microbiano.

Por otro lado, la degradación de HAPs es llevada a cabo por microorganismos heterótrofos que requieren un aceptor final de electrones. Generalmente, la degradación de hidrocarburos aeróbica (el oxígeno como aceptor final de electrones) es considerablemente más rápida que los procesos anaeróbicos. Casi todas las experiencias de biorremediación en la práctica son aeróbicas, sin embargo, muchos estudios han descrito la degradación de HAPs en ausencia de oxígeno, a menudo usando un aceptor final de electrones alternativo como el nitrato, sulfato, o hierro ferroso (Lovley *et al*., 1989; Holliger y Zehnder, 1996; Heider y Fuchs, 1997; Macnally *et al*., 1998; Widdel y Rabos, 2001; Jones *et al*., 2008a).

La temperatura también afecta al crecimiento microbiano. A bajas temperaturas la tasa de degradación es más lenta. La mayoría de los estudios de biorremediación han sido realizados a temperaturas mesofílicas (20-30º C) (Menn *et al*., 2000), sin embargo, hay varios ejemplos de experiencias de biorremediación exitosas a temperaturas extremas como en el ártico (Whyte *et al*., 1999) o en localizaciones desérticas (Sorkoh *et al*., 1993).

En general, el pH óptimo para los procesos de biodegradación es el neutro (pH 7,4 -7,8) (Dibble y Bartha, 1979). Verstraete *et al*., (1976) lograron alcanzar mejores resultados en la biodegradación de un suelo contaminado incrementando el pH de 4,5 a 7,4; sin embargo, hay algunas poblaciones de microorganismos degradadores adaptadas a pHs ácidos (Stapleton, 1998).

Es posible que la salinidad en estuarios y océanos pueda ser perjudicial para algunas especies implicadas en la biodegradación, pero hay varios ejemplos de organismos degradadores aislados de ambientes altamente salinos que no degradan en agua dulce (Atlas y Busdosh, 1976).

En los ambientes naturales no contaminados, los nutrientes minerales (N, P y K) raramente son limitantes. Sin embargo, cuando un ambiente es contaminado con

compuestos orgánicos como los HAPs, el cociente C:N puede incrementarse dramáticamente y puede ser un factor limitante en la biorremediación.

Se acepta que por lo general, los microorganismos no crecen en HAPs de elevado peso molecular y por tanto obtienen la energía de otras fuentes de carbono, cometabolizando estos HAPs recalcitrantes. Hay algunas evidencias de que la degradación de HAPs de elevado peso molecular puede ser incrementada mediante la adición de fuentes de carbono no específicas (Keck *et al*., 1989), como por ejemplo materia orgánica como los ácidos húmicos, que se han observado que estimulan la degradación del pireno (Holman *et al*., 2002).

Factores asociados al contaminante

Generalmente, la biodegradación de HAPs disminuye al aumentar el peso molecular y el número de anillos lo que resulta en un correspondiente aumento de la hidrofobicidad y estabilidad electroquímica (Cerniglia, 1992; Kanaly y Harayama, 2000). La recalcitrancia de los HAPs también aumenta con el número de sustituyentes alquilo y la distribución de sus anillos. Los HAPs con configuración angular son más estables que aquellos con una disposición lineal.

La concentración de los contaminantes también afecta a la degradación básicamente pos dos razones, toxicidad e inducción. Los contaminantes poseen propiedades solventes y efectos tóxicos que pueden alterar la estructura de la membrana de los microorganismos. Los efectos tóxicos incluyen la alteración de la estructura de la membrana, resultando en la pérdida de importantes constituyentes celulares, destrucción de los procesos de generación de energía basados en la membrana como disipación de potenciales eléctricos y gradientes de pH, o inhibición de proteínas de membrana (Sikkema *et al*., 1995). Por otro lado, algunos contaminantes sólo son degradados cuando se encuentran por encima de cierta concentración. Esto es debido a que las enzimas implicadas en un proceso particular pueden estar formadas y activas sólo cuando el sustrato, sus metabolitos o posiblemente una molécula relacionada están presentes a cierta concentración (enzimas inducibles) (Chen y Aitken, 1999).

Anteriormente en esta Introducción, se ha mencionado que la biorremediación de los HAPs en suelos y sedimentos muestra a menudo una pérdida bifásica: un periodo rápido de degradación de HAPs, seguido de un periodo largo de lenta degradación (Beck *et al*. 1995; Cornelissen *et al*., 1997; 1988; Williamson *et al*., 1998). Esto resulta en una eliminación incompleta de los HAPs, es decir, una concentración residual de HAPs permanece en la matriz tras la remediación. En muchos casos esto está relacionado con la biodisponibilidad de los HAPs. La biodisponibilidad puede ser definida como la accesibilidad de un compuesto para su asimilación biológica y su posible toxicidad (Alexander, 2000). La biodisponibilidad de un compuesto químico está determinada por la ratio entre la transferencia de masa relativa y la actividad intrínseca de las células microbianas. Está controlada por un número de procesos físico-químicos como la adsorción/desorción, difusión y disolución (Cuypers *et al*., 2002).

Presencia de microorganismos degradadores adecuados

Generalmente, las poblaciones indígenas de microorganismos en ambientes contaminados tienen capacidad para degradar los HAPs. Por esta razón, es importante analizar la estructura de la comunidad y sus cambios durante los procesos de biorremediación para poder mejorar los procesos microbianos de degradación, optimizando las condiciones medio ambientales.

Poblaciones microbianas degradadoras de HAPs

Las bacterias y hongos metabolizan una amplia variedad de contaminantes orgánicos, siendo la degradación microbiana el principal proceso para la eliminación de HAPs del medio ambiente (Prince, 1993; Sutherland *et al*., 1995).

Metabolismo de HAPs

Los HAPs son compuestos reducidos estables y por lo tanto la degradación procede generalmente mediante la oxidación bajo condiciones aeróbicas o anaeróbicas. Hay numerosos estudios que describen una amplia variedad de microorganismos capaces de degradar HAPs (Cerniglia, 1992; 1993; 1997; Kanaly y Harayama, 2000; Prince, 2005; Kanaly y Harayama 2010). La degradación anaeróbica de HAPs es muy lenta comparada con la degradación aeróbica y por lo tanto no juega un papel significativo en las estrategias de biorremediación. Sin embargo, actualmente hay un creciente interés en las poblaciones microbianas implicadas en los procesos de biodegradación anaeróbica (Lovley *et al*., 1989; Langenhoff *et al*., 1996; Heider *et al*., 1999; Widdel y Rabos, 2001; Jones *et al*., 2008a; Meckenstock y Mouttaki, 2011).

La degradación aeróbica de HAPs es generalmente iniciada pos la acción de dioxigenasas multicomponentes que catalizan la incorporación de dos átomos de oxígeno y dos electrones desde el NADH para formar *cis*-dihidrodioles. Estas dioxigenasas multicomponentes generalmente consisten en una reductasa, una ferredoxina y una proteína que contiene hierro y azufre. La subsiguiente deshidrogenación mediante una deshidrogenasa NAD^+-dependiente lleva a la formación de intermediarios dihidroxilados que son posteriormente degradados mediante la ruta de ruptura del anillo y la eventual formación de intermediarios del ciclo del ácido cítrico. Los HAPs pueden ser metabolizados también por monooxigenasas en bacterias para formar los *trans*-dihidrodioles, aunque esta actividad es generalmente menor que la actividad dioxigenasa en el mismo organismo (Heitkamp *et al*., 1988a; 1988b).

Análisis de comunidades microbianas

La introducción de mezclas de hidrocarburos en un ambiente específico y su subsiguiente degradación, produce numerosos cambios en la estructura de la comunidad microbiana inicial. Analizar la estructura de la comunidad microbiana y su dinámica durante la biodegradación de hidrocarburos es el primer paso para identificar las poblaciones clave, sus interacciones, y establecer las condiciones para su estimulación. No hay un único método de estudio de la estructura de la comunidad microbiana y su dinámica, y sólo la combinación de diferentes métodos puede dar una idea amplia general. Estos métodos pueden clasificarse en métodos dependientes de cultivo y métodos independientes de cultivo.

Métodos dependientes de cultivo. Estas técnicas requieren el cultivo de los microorganismos para una posterior caracterización. En este grupo se encuentran el aislamiento clásico y cultivo en medio sólido o líquido, el número más probable (NMP) (Wrenn y Venosa, 1996; Rowe *et al*., 1977), y el método Biolog® (Garland y Mills, 1991; Wünse *et al*., 1995; Berthe-Corti y Burns, 1999; El Fantroussi *et al*., 1999). La principal ventaja de estas técnicas es la posibilidad de obtener las poblaciones microbianas en cultivo puro, útil para posteriores análisis y para aplicaciones biotecnológicas. Sin embargo, la principal limitación de estas técnicas es que sólo el 0,001%-0,1% de los microorganismos marinos o el 0,3% de los microorganismos del suelo son cultivables en el laboratorio (Kogure *et al*., 1980; Ferguson *et al*., 1984; Torsvik *et al*., 1998; Torsvik *et al*., 2003).

Métodos independientes de cultivo. Estas técnicas son más representativas de la comunidad entera y reducen el sesgo de la necesidad de cultivar los microorganismos

(Amann *et al*., 1995; Head *et al*., 1998). No necesitan el previo cultivo de la muestra y permiten estudiar las poblaciones microbianas a diferentes niveles. Entre ellas se encuentra la hibridación fluorescente *in situ* (FISH, en inglés), el análisis de los ácidos grasos de los fosfolípidos (PLFA, en inglés), métodos basados en la reacción en cadena de la polimerasa (PCR, en inglés) y métodos que permiten establecer relaciones directas entre las poblaciones microbianas y los procesos fisiológicos.

Estrategias para aumentar la degradación microbiana de HAPs durante la biorremediación.

Bioestimulación.

El éxito de las experiencias de biorremediación depende de la concentración y disponibilidad de algunos nutrientes. Generalmente, la adición de nutrientes es beneficial. Sin embargo, la adición de estos elementos esenciales como sales solubles en agua presenta varios problemas como la dilución, fenómenos de lixiviación y la utilización por poblaciones bacterianas que no degradan el contaminante de interés. Además, el aumento de la concentración del nitrógeno móvil podría ir asociado a potenciales problemas ambientales. Por lo tanto, se han desarrollado numerosos fertilizantes orgánicos oleofílicos (Atlas y Bartha, 1973; Nikolopoulou *et al*., 2007; Xu y Obrad, 2003) que, tras la adición, permanecen asociados con los contaminantes hidrofóbicos (es decir, FLNA) y estimulan localmente las bacterias degradadoras de hidrocarburos. Un ejemplo es Inipol EAP® 22, exitosamente empleado durante la biorremediación del vertido del Exxon-Valdez en Alaska (Pritchard y Costa, 1991; Prince, 1993; Lindstrom *et al*., 1991). Actualmente, su composición se ha modificado debido a la toxicidad del 2-butoxi-1-etanol (Hoff, 1993) y la nueva formulación se llama S200. Este producto fue aplicado exitosamente en la biorremediación de playas afectadas por el vertido del *Prestige* (Díez *et al*., 2005; Gallego *et al*., 2006; Jiménez *et al.,* 2006).

Otra posibilidad es estimular la biodegradación mediante el aumento de la biodisponibilidad de los contaminantes a través de tensioactivos. Los tensioactivos son moléculas que constan de una parte hidrofóbica y una parte hidrofílica (moléculas amfipáticas o amfifílicas). Los tensioactivos químicos son producidos a gran escala y tienen numerosas aplicaciones como agentes de limpieza en experiencias de biorremediación, aumentando la movilidad y la solubilidad (Mulligan *et al*, 2001; Falatko, 1991; Ellis *et al*, 1985; Abdul *et al*., 1992) y también como potenciadores de la degración (Joshi y Lee, 1995). La introducción de los tensioactivos en el medio ambiente siempre conlleva contaminación y por lo tanto la toxicidad del tensioactivo y de sus potenciales productos de degradación son criterios muy importantes a la hora de su selección. Los tensioactivos biológicos están presentes naturalmente y su uso en experiencias de biorremediación puede ser más aceptable en comparación con los tensioactivos sintéticos (Makkar y Rockne, 2003; Déziel *et al*.,. 1996; Zhang *et al*., 1997). Sin embargo, algunos biotensioactivos pueden ser tan tóxicos como los tensioactivos sintéticos (Lang y Wagner, 1993).

Rizorremediación

Otra estrategia para aumentar la eficiencia de la biodegradación en suelos es la rizorremediación. El uso de plantas tiene numerosas ventajas. El sistema radicular puede ser usado como vector para las bacterias colonizadoras de las raíces y para penetración en capas normalmente no permeables para las bacterias. Las plantas pueden además atraer agua con su sistema radicular. Además, las raíces pueden exudar hasta un 35% de su carbono fotosintetizado y liberar oxígeno, o proporcionar mejores condiciones redox. Los exudados de las raíces de las plantas contienen azúcares, ácidos orgánicos y aminoácidos como principales componentes (Jones ,

1998; Chen *et al*., 2007; Weissman, 1964; Bertin *et al*., 2003). Además el mucílago secretado por las células de la raíz, la pérdida de células de las capas de la raíz o la descomposición de raíces completas proporcionan nutrientes para las poblaciones microbianas presentes. Como resultado, las poblaciones microbianas en la rizosfera son mucho más abundantes y presentan una composición diferente que en los suelos sin plantas. Además, la colonización o inoculación de bacterias degradadoras de contaminantes en semillas de plantas puede ser un importante aditivo para mejorar la eficiencia de la fitorremediación o bioaumento (Chile *et al*., 2007; Kuiper *et al*., 2001; 2002). Numerosos estudios muestran el beneficio de la interacción entre las plantas y los microorganismos en experiencias de biorremediación (Kuiper *et al*., 2004). Sin embargo, se dispone de muy poca información sobre las poblaciones microbianas implicadas.

Objetivos

El trabajo presentado en esta Tesis se ha centrado en el estudio de las poblaciones microbianas y procesos implicados en la eliminación de HAPs de ambientes marinos y suelos contaminados. Como continuación de los estudios realizados en nuestro grupo, la primera parte de esta Tesis se centra en la caracterización de las comunidades marinas bacterianas que participan directamente o indirectamente en la degradación de HAPs tras vertidos marinos que afectan a zonas costeras. La segunda parte se centra en suelos. Debido a que la baja biodisponibilidad es el principal factor que limita las experiencias de biodegradación de HAPs en suelos, llevamos a cabo varias estrategias con el fin de potenciar la biodegradación, por ejemplo mediante la adición de fertilizantes y la rizorremediación, y estudiamos los mecanismos implicados.

Los objetivos específicos de esta Tesis han sido:

- Analizar la estructura y función de un consorcio microbiano marino que degrada pireno, como modelo de HAP de elevado peso molecular

- Caracterizar taxonómicamente y filogenéticamente una de las poblaciones más abundantes en el consorcio degradador de pireno y proponer un nuevo género y especie para su clasificación.

- Evaluar las posibles deficiencias nutricionales en la biodegradación de HAPs presentes en fuel en la interfase FLNA/agua, determinar el papel de los fertilizantes oleofílicos, y examinar la potencial producción y movilización de metabolitos parcialmente oxidados.

- Evaluar los efectos de la rizosfera de girasol en el aumento de la biodegradación de HAPs presentes en suelos envejecidos, caracterizar los exudados de la raíz de girasol e identificar los cambios en la estructura de las comunidades microbianas.

Resultados y discusión

Análisis estructural y funcional de un consorcio microbiano marino degradador de pireno

Los vertidos marinos causan gran preocupación debido al riesgo asociado para la salud pública y daño ecológico en costas. Entre los diferentes componentes del

petróleo, los hidrocarburos aromáticos policíclicos (HAPs) son de especial interés por su persistencia, toxicidad y potencial carcinogenicidad. El pireno, formado por cuatro anillos fusionados de benceno, es, junto con sus alquil derivados, uno de los HAPs más abundantes en crudos y derivados (Vila y Grifoll; 2009).

La degradación del pireno en suelos contaminados es principalmente llevada a cabo por bacterias pertenecientes al grupo *Actinobacteria* (Kanaly y Harayama, 2010). Estudios de identificación de metabolitos con cepas de micobacterias aisladas por su capacidad de degradar o crecer en pireno han producido información clave sobre las vías de degradación (Heitkamp *et al*., 1988a; 1988b; Vila *et al*., 2001; Cerniglia, 2003), mientras que posteriores estudios genómicos han permitido identificar alguna de las enzimas implicadas y su organización genética (Khan *et al*., 2001; Kribovok *et al*., 2003; Kim *et al*., 2006; Kim *et al*., 2007). Sin embargo, la degradación del pireno en ambientes marinos donde menos del 0.3% de las poblaciones microbianas son consideradas cultivables (Amann *et al*., 1995), es poco conocida. Sólo recientemente, estudios realizados con consorcios marinos degradadores de pireno y el posterior aislamiento de sus componentes microbianos han demostrado que miembros del género *Cycloclasticus* (Wang *et al*., 2008) y *Ochrobactrum* (Aruzlazhagan y Vasudevan, 2011), ambos *Proteobacterias*, son capaces de degradar pireno en ausencia de otros sustratos, sugiriendo que estos nuevos géneros juegan un papel en la degradación de HAPs en ambientes marinos. Sorprendentemente no se han establecido asociaciones entre la degradación del pireno y actinobacterias en ambientes marinos.

En estudios previos de nuestro grupo sobre la biorremediación de costas marinas afectadas por el vertido de fuel del *Prestige,* se desarrolló un consorcio microbiano autóctono degradador de fuel que fue usado como inóculo (Fernández-Álvarez *et al*., 2006; 2007). El consorcio UBF era un cultivo mixto que produjo una casi completa eliminación de todos los alcanos lineales y ramificados junto con una extensiva degradación de los HAPs de 3 a 5 anillos presentes en el fuel, incluyendo una eliminación del 75% de pireno (Vila *et al*., 2010). Con el fin de investigar las poblaciones microbianas que causaban esta eliminación del pireno, se estableció un subcultivo a partir del consorcio UBF que se mantuvo creciendo en pireno como única fuente de carbono ($0.2gL^{-1}$).

Los análisis mediante HPLC del extracto orgánico de los cultivos del consorcio UBF-Py mostraron una eliminación del pireno ($0.2gL^{-1}$) del 34% en 30 días. No fueron detectados otros picos, indicando una completa degradación de la molécula. De hecho, subsecuentes estudios de mineralización usando ^{14}C-pireno revelaron que la mayoría del pireno eliminado (24%) era mineralizado a CO_2. Estudios paralelos realizados con fenantreno mostraron que el consorcio UBF-Py era también capaz de mineralizar el fenantreno (31% y 24% para concentraciones de 0.5 mgL^{-1} y 0.2 gL^{-1} respectivamente). Estos resultados son consistentes con el hecho de que la mayoría de las cepas degradadoras de pireno de suelos son capaces de crecer también con fenantreno (Vila *et al*., 2001).

Para determinar la estructura de la comunidad microbiana del consorcio UBF-Py se obtuvo una librería de clones del ADNr 16S y tras analizar 45 clones se encontraron 5 OTUs (Unidades Taxonómicas Operativas, en inglés) distintas. Paralelamente, con el fin de recuperar las poblaciones bacterianas observadas en el análisis molecular en cultivo puro, se realizaron aislamientos en una variedad de medios incluyendo medios complejos diluidos y medios mínimos con diferentes hidrocarburos con y sin suplementos nutricionales. Las secuencias del ADNr 16S de los aislados obtenidos fueron entonces comparadas con las bases de datos genéticas y con aquellos encontrados en la librería de clones (Tabla R1).

Table R1. Análisis de las secuencias de los clones y aislados del consorcio UBF-Py y su correspondiente banda en el DGGE.

Banda DGGE	Clon (freq %)	Aislado	Longitud Fragmento (bp)	Sim (%)	Relativo más cercano en la base de datos GenBank (num. accesso)	Grupo filogenético
S1=S3		**UBF-P2** [a,b,c,d,e]	**1379**	**99**	***Alcanivorax* dieselolei Qtet3 (GU370129)**	***Alcanivoracaceae* (γ)**
S2		**UBF-P3** [d,e]	**1282**	**100**	***Novosphingobium* sp. TVG9-VII (JF706227)**	***Sphingomonadaceae* (α)**
S4		**UBF-P4** [e]	**1328**	**99**	***Novosphingobium* sp. 2PR51-13 (EU440981)***	***Sphingomonadaceae* (α)**
S5	**38**	**UBF-P1** [a,b,d,e]	**1481**	**92**	***Mycoplana* sp. G1100 (GU199002)**	**(α)**
S6	34		1452	99	*Thalassospira* sp. MAI8 (AB257194)	*Rhodospirollaceae* (**α**)
S7		**UBF-P5** [a]	**1350**	**99**	***Micrococcus* sp. MOLA 73 (AM990848)**	***Micrococcaceae (Actinobacteria)***
S8		**UBF-P6** [b]	**1211**	**99**	***Aurantimonas* sp. 5C.5 (HQ427427)**	***Aurantimonadaceae* (α)**
S9	**5**	**UBF-P7** [a,c,d]	**1413**	**99**	**Uncultured *Paracoccus* S-5m-1 (GU061852)**	***Rhodobacteraceae* (α)**
S10	16		1477	99	*Gordonia* sp. PETBA11 (JQ658415)	*Nocardiaceae (Actinobacteria)*
ND	**7**	**UBF-P8** [a,b,c,d,e]	**1433**	**100**	**Uncultured *Martelella* clone ctg_NISA120 (DQ396149)**	***Aurantimonadaceae* (α)**

Los componentes microbianos que han sido aislados están indicados en negrita. Medios de aislamiento: [a]agua de mar natural suplementada con LB, agua de mar artificial suplementeada con [b]LB, [c]fenantreno, [d]pirene y [e]sorbitol.* Todos los relativos más cercanos a esta cepa pertenecían al genero *Sphingopyxis* . α y γ corresponde a *alpha* y *gammaproteobacteria*, respectivamente.

El análisis molecular reveló una comunidad principalmente compuesta por *Alphaproteobacteria* (84%) y *Actinobacteria* (16%). La secuencia detectada más frecuente en la librería de clones (Banda S5, 38% de los clones) correspondió al aislado UBF-P1, que presentó una baja similitud con las bases de datos. Los análisis filogenéticos y taxonómicos detallados en el articulo 2 revelaron que esta cepa no coincidía con ninguna de los géneros bacterianos descritos, por lo que este nuevo aislado fue descrito en detalle y se propuso el género *Breoghania* para su clasificación. Otra bacteria detectada abundantemente en la librería de clones fue *Thalassospira* (S6, 34%) (*Rhodospirillaceae, Alphaproteobacteria*). El resto de los clones correspondieron a *Gordonia* (S10, 16%) (*Nocardiaceae, Actinobacteria*); *Martelella* (no se observó banda, 7%, UBF-P8) (*Aurantimonadaceae, Alphaproteobacteria*), y *Paracoccus* (S9, 5% UBF-P7) (*Rhodobacteraceae, Alphaproteobacteria*). Los miembros de *Thalassospira* y *Gordonia* no pudieron ser recuperados por las técnicas de cultivo, aunque una variedad de medios fueron ensayados.

Adicionalmente, se obtuvieron cinco aislados que no habían sido detectados durante el análisis molecular y fueron identificados como *Novosphingobium* sp. UBF-P3 (*Sphingomonadaceae*), *Sphingopyxis* sp. UBF-P4 (*Sphingomonadaceae*), *Aurantimonas* sp. UBF-P6 (*Aurantimonadaceae*) (todas cepas de *Alphaproteobacteria*), *Alcanivorax* sp. UBF-P2 (*Alcanivoraceae, Gammaproteobacteria*) y *Micrococcus* sp. UBF-P5 (*Micrococcaceae, Actinobacteria*).

Representantes de casi todos los géneros detectados han sido relacionados con petróleo o degradación de HAPs, pero existen pocas evidencias de la utilización del pireno para el crecimiento de estas bacterias. Miembros del género *Novosphingobium*, *Thalassospira* y *Paracoccus* han sido identificados en consorcios microbianos a partir de ambientes marinos creciendo en pireno, fenantreno o fuel (Zhang *et al*., 2004; Guo *et al*., 2005; Cui *et al*., 2008; Yuan *et al*., 2009; Guo *et al*., 2010; Teng *et al*., 2010; Jiménez *et al*., 2011). *Martelella*, un genero descrito recientemente, ha sido también encontrado en consorcios degradadores de fuel pero su acción sobre los HAPs no ha sido demostrada (Wang *et al*., 2010). *Alcanivorax* ha sido ampliamente descrita como un bacteria degradadora de hidrocarburos obligada capaz de usar alcanos lineales e isoprenoides para su crecimiento (Yakimov *et al*., 1998; Dutta y Harayama, 2001), sin embargo, en el presente trabajo la cepa *Alcanivorax* fue recuperada en placas de agar de LB diluido, lo que indica que el espectro de sustratos puede que no sea tan reducido como se consideraba. Por otro lado, *Micrococcus* y *Gordonia*, pertenecientes al grupo *Actinobacteria*, son típicos degradadores de HAPs de elevado peso molecular en suelos (Kanaly y Harayama, 2010).

Con el fin de investigar las capacidades degradadoras de los aislados, se analizó una variedad de alcanos y HAPs como fuentes de carbono o sustratos de transformación usando el método de pulverización en placa (Kiyohara *et al*., 1982). Aunque alguno de los aislados creció en varios tipos de hidrocarburos en medio sólido, no se observaron halos de degradación en ningún caso. La capacidad de crecimiento de las cepas en pireno o fenantreno se analizó también en medio líquido mineral , pero ninguno de ellas creció significativamente respecto a los controles sin fuente de carbono. Se realizaron estudios de mineralización con ^{14}C-pireno para cada aislado y con la combinación de todos ellos, pero en ningún caso se produjo $^{14}CO_2$.

Parecería plausible que el grupo bacteriano responsable del ataque al pireno fuera uno de los más abundantes en el consorcio UBF-Py. De acuerdo con esto, diluciones seriadas del cultivo mixto fueron subcultivadas en agua de mar artificial con pireno, (según el método de la dilución hasta la extinción), y aquellas que mostraron crecimiento positivo fueron analizadas mediante PCR-DGGE del ARNr 16S. Todas las diluciones mostraron idéntico patrón de bandas en el DGGE que el cultivo inicial, lo que parece sugerir que bien todos los miembros del consorcio UBF-Py son requeridos para la degradación de pireno (por ejemplo, suministrándoles factores de crecimiento), o que las degradadoras de pireno suministran suficiente carbono para soportar una importante microbiota acompañante. En cualquier caso, todos estos resultados

indicaban que las poblaciones responsables del ataque del pireno podían ser aquellas no recuperadas en cultivo sólido, esto es, los representantes de *Gordonia* y *Thalassospira*).

Con el fin de obtener una evidencia adicional que conduzca a la identificación de las poblaciones degradadoras de pireno, el consocio microbiano UBF-Py y cada uno de los aislados fueron analizados por la posible presencia de genes de dioxigenasas dihidroxilantes típicos de la degradación de HAPs.

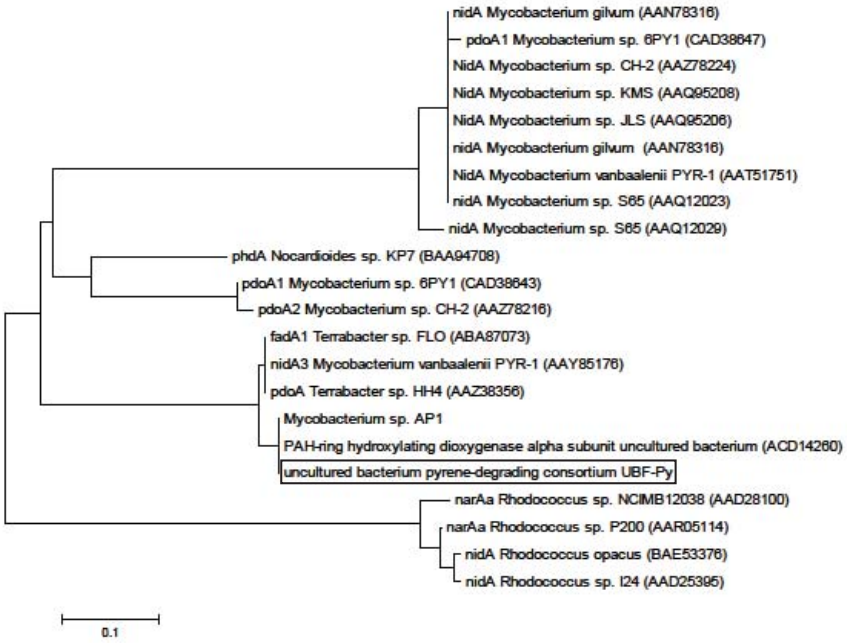

Figura R1. Árbol filogenético construido mediante *neighbour joining* de la secuencias de amino ácidos de la dioxigenasa hidroxilante de anillos del consorcio UBF-Py y de cepas de referencia tomadas del Genbank.

Se sabe que los genes de dioxigenasas dihidroxilantes se agrupan en dos grupos diferentes correspondientes a las bacterias Grampositivas o negativas (Cébron *et al*., 2008). Los análisis de PCR usando cebadores específicos de cada grupo sobre las dioxigenasas hidroxilantes de anillos de HAPs (HAP-RHD$_\alpha$) del DNA extraído de los aislados resultaron negativos en todos los casos, tanto para los genes de Gram-positivos como para los de Gram-negativos. Sin embargo, el mismo análisis realizado sobre el cultivo entero fue positivo con los cebadores de Gram-positivos. El producto de PCR fue entonces clonado y todos los clones resultaron corresponder a la misma secuencia. Esta secuencia fue comparada mediante búsquedas en el BLAST con las bases de datos NR y NCBI y se encontró que estaba cercanamente relacionada con el gen de la dioxigenasa hidroxilante de anillos NidA3 encontrada en varias actinobacterias degradadoras de pireno (como *Mycobacterium vanbaalenii* PYR-1) (Kim *et al*., 2006). Por lo tanto, ello indicaba que el gen de la dioxigenasa detectada pertenecía muy probablemente a uno de los componentes no aislados del consorcio UBF-Py y reafirmaba los análisis catabólicos llevados a cabo previamente sobre los aislados. Estos resultados indican que la población no cultivable perteneciente al

género *Gordonia* está muy probablemente implicada en el ataque inicial de la molécula del pireno (Figura R1).

Este estudio aporta por tanto nuevos indicios sobre la degradación del pireno en costas marinas por comunidades microbianas, proporcionando la primera evidencia sobre el papel de las micobacterias en la eliminación del pireno de ambientes marinos contaminados así como su conexión con otras poblaciones. Se requieren más esfuerzos para obtener degradadores de pireno no cultivados hasta la fecha con el fin de caracterizarlos y hacerlos disponibles para su potencial aplicación biotecnológica. De hecho, recientes estudios han revelado la exitosa recuperación de cepas previamente no cultivadas después de la adición de suplementos de crecimiento específicos al medio (por ejemplo, sideróforos) (D'Onofrio *et al*., 2010).

Breoghania corrubedonensis **gen. nov. sp. nov., una nueva alphaproteobacteria aislada de una playa de Galicia (NO de España) tras el vertido de fuel oil del *Prestige*. Enmienda a la descripción de la familia *Cohaesibacteraceae* y de la especie *Cohaesibacter gelatinilyticus***

La morfología más abundante obtenida tras sembrar diluciones seriadas del consorcio microbiano UBF-Py en placas de agua de mar con LB diluido y incubarlas una semana consistió en colonias circulares, mucosas, de color crema y de alrededor de 1 mm de diámetro. Cinco colonias fueron purificadas en el mismo medio y para averiguar si esos aislados constituían diferentes poblaciones, se realizaron análisis mediante RAPD utilizando los cebadores RAPD1 y RAPD2 (Peña *et al*., 2005), junto con su secuencia del gen 16S ARNr. Los análisis revelaron un idéntico patrón de amplificación para las cinco cepas, lo que indicaba que todas ellas representaban una única población clonal (UBF-P1) probablemente seleccionada a través de las distintas resiembras. Los análisis del gen del ARNr 16S de la librería de clones mostraron que la secuencia más abundante correspondía también al aislado UBF-P1 (38%).

La secuencia casi completa del gen ARNr 16S de la cepa UBF-P1 se comparó con la base de datos del ADNr 16S SILVA (http://www.arb-silva.de/projects/livingtree/), la cual contiene secuencias de cepas de alta calidad (Yarza *et al*., 2008), y el alineamiento y la reconstrucción del árbol fueron desarrollados mediante el paquete de software ARB (Ludwig *et al*., 2004) (número de acceso GQ272328). La secuencia relativa más cercana, con un 92% de similitud fue en todos los casos *Cohaesibacter gelatinilyticus* DSM 18289^T, que se afiliaba junto con UBF-P1 en todos las reconstrucciones mediante *Neighbour-Joining*. Sin embargo, las reconstrucciones de máxima probabilidad producían árboles donde el nuevo aislado aparecía independientemente afiliado dentro de las *Alphaproteobacteria*. En ningún caso se pudo afiliar la cepa UBF-P1 con un género ya descrito. De hecho, el valor del 92% de similitud con su relativo más cercano estaba claramente por debajo de la media (96,4% ± 0,2), o del de la mínima identidad (94,9% ± 0,4) utilizados para diferenciar géneros (Yarza *et al*., 2008), lo que sugería que era plausible concluir que la cepa UBF-P1 pertenecía a un nuevo género dentro de las *Alphaproteobacterias*.

Con el fin de clarificar la afiliación de ambos aislados, se llevaron a cabo posteriores estudios filogenéticos, genéticos y fenotípicos sobre ambas cepas. Se realizó un análisis de secuencias de multilocus (MLSA) concatenando los genes *atp*D (ATP sintasa F1, subunidad beta),*pyr*G (CTP sintasa), *rpo*B (RNA polimerasa subunidad beta), *fus*A (factor G de elongación de traducción), y del ARNr 16S, o alternativamente, omitiendo la secuencia del gen ARNr 16S (números de acceso FN59891 a FN598917). Se obtuvieron secuencias homólogas del depósito público MicobesOnline (Alm *et al*., 2005; Richter *et al*., 2009), que fueron alineadas usando el programa Clustal X 1.83 (Thompson *et al*., 1997) y los alineamientos fueron concatenados en un único conjunto de datos. La concatenación de las cinco

secuencias parciales, usando sólo las posiciones muy conservadas, produjeron un árbol muy estable (Figura R2). Ambas cepas bacterianas, UBF-P1 y *C. gelatinilyticus* DSM 18289^T se afiliaron juntas consistentemente, con identidades que abarcaban desde el 87,6% al 85,6% dependiendo si la secuencia del gen del 16S ARNr era incluida.

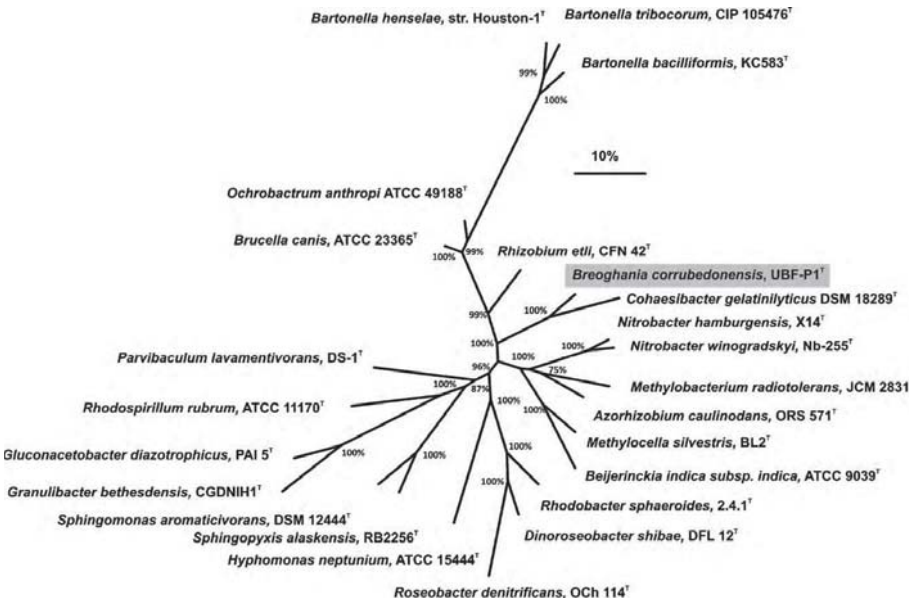

Figura R2. Reconstrucción filogenética mediante MLSA basada en la concatenación de los genes *atp*D, *pyr*G, *rpo*B, *fus*A y 16S ARNr de la cepa UBF-P1 y *Cohaesibacter gelatinilyticus* DSM 18289^T, y de cepas seleccionadas de *Alphaproteobacterias* para cuales su secuencia genómica completa está disponible.

A pesar de que el nuevo aislado parecía recaer en la recientemente propuesta familia *Cohaesibacteraceae* (Hwan y Cho, 2008), la secuencia del gen ARNr 16S no compartía las posiciones clave 194, 678, 712, 1244 y 1293, sino que estas posiciones clave se asemejaban a la mayoría de las otras familias del orden. Consecuentemente, estas observaciones llevan a la cuestión de la fiabilidad de estas posiciones clave para circunscribir familias.

Se realizaron análisis morfológicos y fisiológicos para ambas cepas (Tabla R2) indicando que la cepa UBF-P1 podía ser diferenciada del género relativo más cercano mediante los ensayos de reducción de nitrito e hidrólisis de gelatina, ADH; ODC y test de la ureasa. Por otro lado, algunos de los resultados encontrados para *C. gelatinilyticus* CL-GR15^T diferían de aquellos inicialmente descritos y usados para describir la familia. En consecuencia, sugerimos una enmienda a esta descripción. El perfil de ácidos grasos celulares de UBF-P1, mostró una predominancia de ácidos octadecanoicos junto con ácidos grasos cíclicos $C_{19:0}$, típicos de las *Rhizobiales* (Moreno *et al*., 1990; Jarvis *et al*., 1996; Kämpfer y Kroppenstedt, 1996; Wilkinson, 1998; Dunfiled *et al*., 1999; Tighe *et al*., 2000).

Todos los análisis de datos filogenéticos, genéticos y fenotípicos indicaron que UBF-P1 se aislaba claramente dentro del orden *Rhizobiales* y se afiliaba con la familia *Cohaesibacteraceae* (Hwang y Cho, 2008). Sin embargo, las diferencias con el género más cercano *C. gelatinilyticus* DSM 18289^T, confirman que nuestro aislado podría ser circunscrito como un nuevo género y especie que es descrito más abajo.

Tabla R2. Características distintivas de la cepa UBFP1^T y *C. gelatinilyticus* DSM 18289^T. A menos que se indique, los tests fueron desarrollados simultáneamente usando ambas cepas.

Característica	Cepa bacteriana	
	UBFP1^T	*C. gelatinilyticus* CL-GR15^T
Forma celular	Bacilos regulares, curvados, bulbosos	Bacilos irregulares[1]
Tamaño celular (mm)	0.6-0.7 x 2.0-3.5	0.2-0.4 x 1.0-3.0[1]
Tipo de división	Fisión binaria o división asimétrica	Fisión binaria o división asimétrica[1]
Movilidad	+	+
Flagelos	1 o 2, subpolar	polar[1]
Temperatura de crecimiento (º C)		
Optima	30	25-30
Rango	15-40	15-31
Crecimiento NaCl (%)		
Rango	1%-10%	2%-5%
Crecimiento pH		
Optimo	7.5	8.0
Rango	5.0 - 8.5	6.0-9.0
Requerimiento de Oxígeno	Aeróbico	Anaeróbico facultativo
Catalasa	+	+
Oxidasa	+	+
Reducción de nitrato	+	+*
Reducción de nitrito	-	+*
Hidrólisis de gelatina	-	+
ADH test	+	-
Ureasa	+	-
Contenido G + C (mol%)	63.9	52.8 – 53
Quinona mayoritaria	Q-10	Q-10
Ácidos grasos mayoritarios (>10%)	$C_{18:1}\omega7c$ (75.3%)	$C_{18:1}\omega7c$ (58.8); Suma 3†(20.8)
Lípidos polares mayoritarios	Difosfatidilglicerol (DFG)	Difosfatidilglicerol (DFG)
	Fosfatidilglicerol (FG)	Fosfatidilglicerol (FG)
	Fosfatidiletanolamina (FE)	Fosfatidiletanolamina (FE)
	Fosfatidilmonometiletanolamina (FME)	Fosfatidilmonometiletanolamina (FME)
	Fosfatidilcolina (FC)	Fosfatidilcolina (FC)

[1] Datos descritos por Hwang y Cho (2008) .
† pico 3 contiene uno o más de $C_{16:1}$ ω 7c y/o $C_{15:0}$ ISO 2-OH.
* Nuestros resultados difieren de los datos previamente descritos por Hwang y Cho (2008).

En un intento de identificar el papel que la cepa UBF-P1 podía jugar en la degradación del pireno por el consorcio microbiano UBF-Py, varios hidrocarburos y metabolitos bacterianos de hidrocarburos aromáticos policíclicos fueron ensayados como fuentes de carbono. La cepa UBF-P1 no fue capaz de crecer en hexadecano, pireno, fenantreno o metabolitos del fenantreno (ácido 4-fenantroico, ácido ftálico y ácido difénico). Sin embargo, el crecimiento fue positivo en protocatecuato. Estos resultados sugerían que la cepa UBF-P1 no participaba en el ataque inicial al pireno, pero podía crecer en compuestos ácidos resultantes de la degradación del pireno o de otros hidrocarburos suministrados por otras poblaciones bacterianas presentes en el consorcio.

Descripción de *Breoghania* gen. nov.

Breoghania (Bre.o.gha'ni.a. N.L. fem. N. *Breoghania*, por *Breoghan*, de acuerdo con la mitología celta (*Leabhar Ghabhala*, siglo XII), el primer dios celta de *Gallaecia* (actual Galicia), fundador de la ciudad de *Brigantia* (probablemente A Coruña), que construyó una torre en la costa desde donde podía verse *Eire* (Irlanda).

Las células son bacilos Gram-negativos, aeróbicos, móviles mediante flagelos polares. Las células se dividen mediante división binaria o asimétrica. Oxidasa y catalasa positiva, y con un contenido de G+C del DNA de 63,9%. La quinona respiratoria es ubiquinona 10 (Q10) y el ácido graso celular predominante es $C_{18:1}\omega7c$ (75.3%). Los lípidos polares mayoritarios son difosfatidilglicerol (DFG), fosfatidilglicerol (FG), fosfatidiletanolamina (FEA), fosfatidilmonometiletanolamina (FMEA), y fosfatidilcolina. En base a la secuencia del gen ARNr 16S, la estirpe UBF-P1^T se afilia con la clase *Alphaproteobacteria* y la especie tipo es *B. corrubedonensis*.

Descripción de *Breoghania corrubedonensis* sp. nov.

Breoghania corrubedonensis (Co.ru.be.do.nen'sis N.L. adj. fem. corrubedonensis, de o perteneciente a Corrubedo, noroeste de España, aislada de la playa de Corrubedo, la localización de donde provenía la muestra de arena usada como inóculo en los cultivos de enriquecimiento de los cuales la cepa UBF-P1^T fue aislada.
En adición a las características descritas para el género, las células son regulares, irregulares o bacilos bulbosos, móviles por uno o dos flagelos subpolares y aproximadamente miden 0,6-0,7 µm de ancho y 2-3,5 µm de largo. Se reproducen por división asimétrica. Tras cinco días de crecimiento en AMA (agua de mar artificial) LB, LB 3% NaCl o Agar Marino, las colonias presentan un diámetro aproximado de 1 mm y son circulares, regulares, convexas, mucosas, y de color blanco crema. Tras incubarlas durante cinco días a las condiciones óptimas de crecimiento las colonias son aproximadamente de 1 mm de diámetro. La cepa crece en un rango de temperatura de 15-40 º C (óptimo 30 º C) y a un pH de 5-8,5. El crecimiento tiene lugar con concentraciones de NaCl del 1-10%. No hidroliza la gelatina ni la esculina. La aminopeptidasa, arginina dihidrolasa, ornitina descarboxilasa y actividad ureasa están presentes. El nitrato es reducido a nitrito. Negativa para la reducción de nitritos, producción de indol, y formación de H_2S. No se produce β-Glucosidasa ni β-galactosidasa. No degrada almidón, DNA o tween 80. Producción de ácido (débilmente) a partir de L-arabinosa. Crecimiento en glucosa, arabinosa, manitol, D-sorbitol, adipato, gluconato (débil), fenil acetato, acetato, succinato, malato, piruvato, casaminoácidos (Disco), acetona, y Tween 20. No crece en D-xilosa, manosa, maltosa, almidón, caprato, *N*-acetil-glucosamina, citrato, lactato, oxalacetato, propionato o metanol. Los ácidos grasos son $C_{18:1}\omega7c$ (75.3%), $C_{19:0}$ ciclo ω8c (6.4%), $C_{16:0}$ (4.4%), $C_{16:1}$ ω7c y/o $C_{15:0}$ iso 2-OH (suma total 3, 2.5%), $C_{18:0}$ 3-OH (2.4%), $C_{18:0}$ (2.2%), y $C_{20:1}$ ω9c (1.3%). Otros ácidos grasos minoritarios (<1%) son $C_{14:0}$ 3-OH o $C_{16:1}$ iso I (suma total 2), $C_{17:1}$ ω8c, $C_{17:1}$ ω6c, y $C_{17:0}$. Los lípidos polares mayoritarios son difosfatidilglicerol (DFG), fosfatidilglicerol (FG), fosfatidiletanolamina (FE), fosfatidilmonometiletanolamina (FEA), y fosfatidilcolina (FC). El contenido del ADN en G+C es del 63,9%. Los rasgos discriminativos de la especie están listados en la tabla R3. La cepa tipo de la especie UBF-P1^T ha sido depositada en tres colecciones de cultivos bajo los siguientes nombre CECT 7622, MG25482 y DSM 23382.

Enmienda a la descripción de la familia *Cohaesibacteraceae* Hwang y Cho 2008

La descripción de la familia se basaba principalmente en las posiciones clave de la secuencia ARN 16S y la información taxonómica de los dos miembros descritos (Hwang y Cho, 2008). Sin embargo, las posiciones clave dadas como indicativo de la familia ya no son válidas. Por ejemplo, la secuencia del gen 16S ARNr de *B. corrubedonensis* no comparte las posiciones clave 194, 678, 712, 1244 o 1293 y en UBF-P1 estas posiciones clave se parecían a la mayoría de otras familias del orden. Además, la reducción de nitrato es positiva. La composición del ADN abarca del 52 al 64% molar en contenido G+C.

Enmienda a la descripción de *Cohaesibacter gelatinilyticus* Hwang y Cho, 2008.

La descripción se propone como la de Hwang y Cho (2008) con las siguientes modificaciones: la reducción de nitrato y nitrito es positiva. No utiliza citrato o *N*-acetilglucosamina. No produce ácidos de la oxidación de azúcares. No hidroliza la esculina. No utiliza el hidrocarburo hexadecano, pireno o fenantreno, tampoco la acetona o el metanol para su crecimiento. La cepa tipo es *C. gelatinilyticus* CL-GR15^T (=DSM 18289^T).

Efecto de la fertilización de la interfase sobre la biodegradación de HAPs presentes en fases líquidas no acuosas

En el medio ambiente, los HAPs se encuentran formando parte de fases líquidas no acuosas (FLNA) como los crudos de petróleo, alquitrán y derivados. Como resultado se produce una lenta partición hacia la fase acuosa, lo que reduce dramáticamente su biodisponibilidad y como consecuencia, ralentiza las tasas de biodegradación y aumenta su persistencia medioambiental (Ortega-Calvo *et al*., 1995; Peters *et al*., 1999; López *et al*., 2008). Los microorganismos presentan mecanismos específicos para superar la limitada transferencia de sustratos lipofílicos a la fase acuosa, como son la adhesión a la interfase FLNA/agua, producción de biotensioactivos, quimiotaxis y la producción de biofilms (Ortega-Calvo y Alexander, 1994; Law y Aitken, 2003; García-Junco *et al*., 2003). Se ha propuesto que el crecimiento microbiano en la interfase puede estar limitado por la escasez de nutrientes cuando los componentes más abundantes y degradables de la FLNA son degradados simultáneamente con el compuesto químico de interés, en este caso HAPs, incluso si la concentración total de nutrientes en el sistema no es limitante (Alexander, 1999). Los bioestimulantes oleofílicos son formulaciones de nutrientes que fueron desarrolladas en base a la suposición de que permanecerán asociadas con la FLNA evitando su dilución en ambientes marinos. Han sido aplicados exitosamente en numerosas experiencias de biorremediación, siendo principalmente recomendados para su uso en costas rocosas y donde la acción del oleaje puede dificultar la efectividad de los bioestimulantes acuosos de liberación lenta (Díez *et al*., 2005). Sin embargo, se desconoce como operan estos fertilizantes frente a las limitaciones nutricionales de las bacterias adheridas y como pueden aumentar su capacidad degradadora en las FLNA..

El objetivo de este trabajo fue evaluar las posibles deficiencias nutricionales durante la biodegradación de HAPs en la interfase FLNA/agua y determinar la posible acumulación de metabolitos parcialmente oxidados, más disponibles y tóxicos que sus HAPs parentales, en presencia y en ausencia del fertilizante oleofílico S200. Para conseguir este objetivo, se desarrolló un experimento empleando un sistema de FLNA/agua que mantenía la integridad de la fase orgánica (García-Junco *et al*., 2003) a la que se añadía el bioestimulante y todo ello se encontraba sumergido en medio mineral suplementado con concentraciones no limitantes de nutrientes inorgánicos solubles. Este estudio fue llevado a cabo en colaboración entre los grupos del Dr. Ortega-Calvo (IRNASE, CSIC, Sevilla) y nuestro grupo de la UB. El grupo del CSIC desarrolló el experimento y analizó las tasas de mineralización, mientras nuestro grupo analizó la biodegradación de los componentes del fuel y la presencia de metabolitos.

El sistema de cultivo consistió en matraces biométricos estériles equipados con un tubo de vidrio abierto situado verticalmente en cada matraz que contenía la FLNA. Esta FLNA era un fuel pesado fluidificado con heptametilnonano (HMN) (1:1), con el fin de reducir la viscosidad y permitir la reproducibilidad de los resultados. El sistema bifásico FLNA/agua mantenía la integridad de la FLNA, evitando posibles interferencias resultantes de la emulsión de la FLNA causadas por el componente surfactante del bioestimulante. Los matraces fueron inoculados con *Mycobacterium gilvum* VM522, aislada previamente de un suelo contaminado con HAPs que es capaz de usar fenantreno, fluoranteno y pireno como únicas fuentes de carbono y energía.

Esta cepa no podía crecer en HMN. Réplicas del cultivo fueron suplementadas con ^{14}C-HAPs para medir las tasas de mineralización específicas, sin embargo aquí centraremos nuestra discusión en los resultados de biodegradación obtenidos en nuestro laboratorio.

Los cultivos fueron incubados durante 1500 horas, tras las cuales la FLNA fuel/HMN de los matraces fue cuidadosamente retirada y tanto la FLNA como la fase acuosa fueron extraídas y analizadas por GC-MS.

Los resultados obtenidos de los análisis de la FLNA de los cultivos y de los controles abióticos indicaron que el bioestimulante oleofílico potenciaba la degradación de todas las familias de hidrocarburos analizados. Los alcanos totales fueron degradados significativamente (60% ± 12,8%; $p<0,05$) sólo cuando el estimulante oleofílico estaba presente. Cuando los porcentajes de degradación fueron calculados en base a las ratios C_{17}/pristano y C_{18}/fitano (resultados no mostrados) se obtuvieron los mismos resultados. Resulta interesante que los hidrocarburos saturados de elevado peso molecular (C_{24}-C_{35}) fueran degradados más extensivamente (64%-67%) que los alcanos de cadenas más cortas con alta solubilidad acuosa (C_{18}-C_{20}; 49%-59%; C_{14}-C_{17}; no significativamente degradados). Estos resultados muestran que a pesar de que se acepta que en general los alcanos de cadena corta son más biodegradables en el medio ambiente, esta regla no siempre se cumple cuando se trata de una única especie bacteriana.

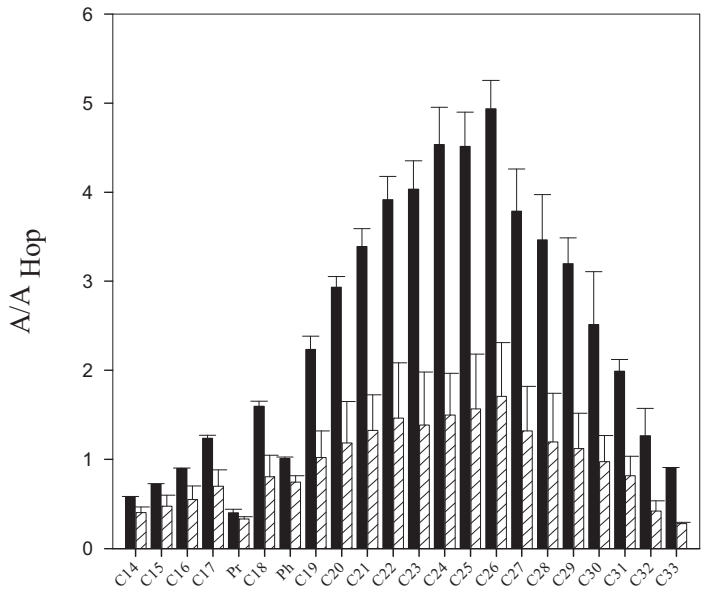

Figura R3. Áreas normalizadas con el hopano (A/AHOP) de los n-alcanos (m/z 85) detectados mediante los análisis de GC-MS en el fuel/HMN residual de los controles abióticos (barras negras) y cultivos (barras ralladas)

Los HAPs y sus alquil derivados fueron biodegradados en un 22% en ausencia de S200, mientras que en presencia del bioestimulante este porcentaje se incrementó al 60%. Solamente el fluoreno (43% ± 4,7%) y el fenantreno (38% ± 15%) fueron significativamente degradados en ausencia del bioestimulante. Sin embargo, cuando el bioestimulante estaba presente se alcanzó una mayor eliminación de estos HAPs (84% ± 6,4% para el fluoreno; 99% ± 0,3% para el fenantreno) junto con una

importante degradación de antraceno (40% ± 12,9%), fluoranteno (68% ± 3,4%) y pireno (43% ± 4,7%).

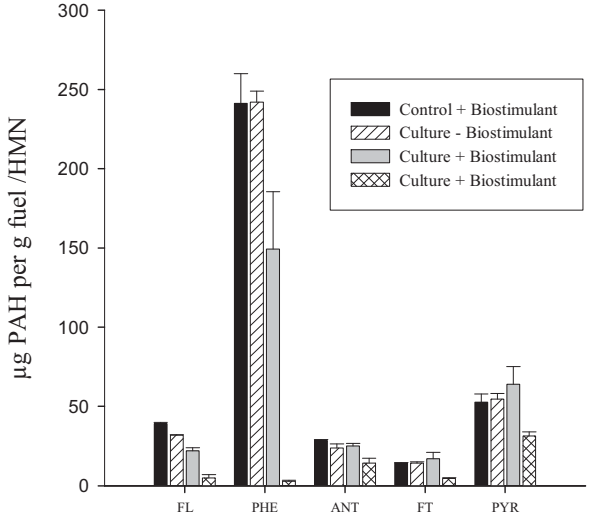

Figura R4. Concentraciones de los HAPs recuperados de la FLNA de los controles y los matraces inoculados, ambos con y sin el bioestimulante. Sólo los HAPs con degradación significativa ($p<0,05$) son mostrados. Las barras mostradas son el cociente entre el pico del área y el área del hopano para cada muestra. FL, fluoreno; PHE fenantreno; ANT, antraceno; FLT, fluoranteno; PYR, pireno.

Los HAPs metilados, más abundantes que sus homólogos no alquilados, fueron degradados en mayor extensión, mostrando una ligera pero significante degradación sólo en presencia del bioestimulante. De hecho, los correspondientes fragmentogramas mostraron una degradación selectiva de los mono- y dimetilnaftalenos ,siguiendo el patrón descrito en la literatura (Kostecki y Calíbrese, 1992; Wang *et al*., 1998; Vila *et al*., 2001; Vila y Grifoll, 2009).

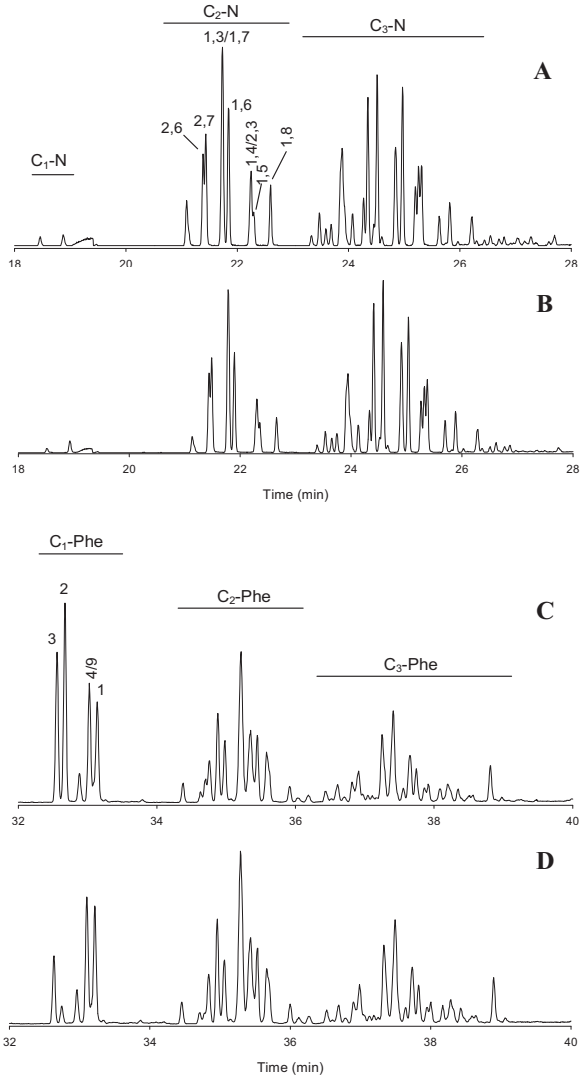

Figura R5. GC-MS fragmentogramas mostrando la degradación selectiva de las familias de alquil naftalenos (A y B), y de alquil fenantreno (C y D) en los matraces inoculados con *Mycobacterium* sp. VM522 (B y D) en la presencia de bioestimulante, en comparación con los controles no inoculados (A y C).

Los análisis mediante GC-MS de los extractos ácidos y neutros de la fase acuosa con y sin bioestimulante presentaron perfiles similares de acumulación de metabolitos. Sin embargo, la concentración en los matraces con bioestimulante fue alrededor de 10 veces superior (los valores van de aproximadamente una décima a varios microgramos por mililitro, por ejemplo, 120 $ng \cdot mL^{-1}$ para 1-indanona, 140 $ng \cdot mL^{-1}$ para ácido difénico, 690 $ng \cdot ml^{-1}$ para ácido 2-carboxicinámico, o 3,6 $\mu g \cdot mL^{-1}$ para ácido 4-metilftálico). La identificación de los metabolitos detectados se hizo en base a estándares de productos auténticos, patrones de fragmentación o de una similitud superior al 90% con la librería del NIST (Tabla 1 y 2 de la información suplementaria

del artículo 3). Los metabolitos neutros correspondieron a alquil-HAPs oxidados en su grupo metil o a naftalenos fenólicos, mientras que los compuestos ácidos fueron principalmente ácidos dicarboxílicos, típicamente acumulados durante la degradación de HAPs y sus metil derivados por cepas de *Mycobacterium* (López *et al*., 2008; Vila *et al*., 2001; Vila y Grifoll, 2009). En concreto, el ácido ftálico es un intermediario común en la degradación del naftaleno, fenantreno, fluoranteno y pireno, mientras que el ácido carboxicinámico y el ácido difénico son metabolitos del naftaleno y fenantreno respectivamente. La presencia de estos metabolitos con grupos alquilo indica una ruta de biodegradación similar para los correspondientes alquil-HAPs.

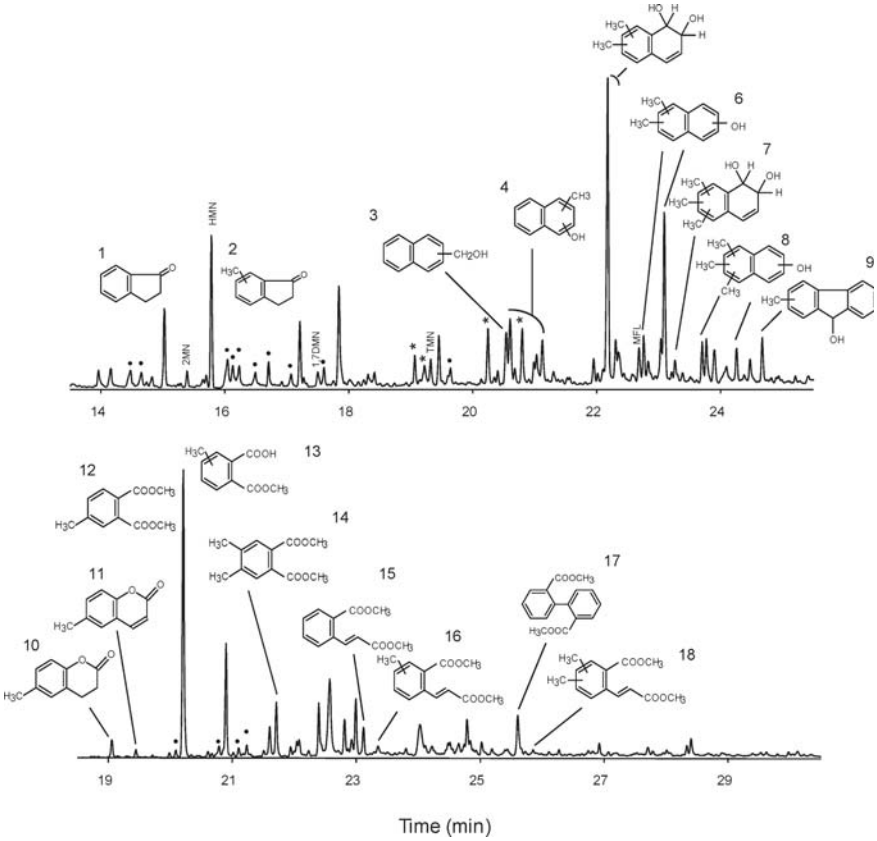

Figura R6. Típico GC-MS cromatograma de los extractos neutros (arriba) y ácidos (abajo) de la fase acuosa de los cultivos de *Mycobacterium gilvum* VM522 en presencia del bioestimulante indicando los picos identificados.

Como se ha comentado previamente, los cultivos contenían concentraciones de nutrientes inorgánicos no limitantes, y la contribución total de N y P debida al fertilizante oleofílico era insignificante. Por otro lado, los resultados obtenidos por el grupo de investigación del CSIC indican que el fertilizante oleofílico no aumenta la biodisponibilidad de los HAPs individuales (es decir, su reparto de la FLNA a la fase acuosa). Por lo tanto, los resultados obtenidos demuestran que el fertilizante oleofílico estimula la biodegradación de HAPs localmente suministrando nutrientes en la interfase FLNA/agua y promoviendo el crecimiento de las bacterias degradadoras adheridas. Este suministro de nutrientes es necesario porque el crecimiento de la cepa bacteriana usando los alcanos, más abundantes y rápidos de degradar, causaba una

limitación de nutrientes en la interfase. Además, se demostró que el aumento en la biodegradación producido por este bioestimulante ampliamente usado en la biorremediación puede estar acompañado de un incremento en la producción de metabolitos de degradación parcial que van a la fase acuosa, siendo más móviles y biodisponibles que sus compuestos parentales.

Influencia de la rizosfera de girasol en la bioaccesibilidad y biodegradación de HAPs en suelos

La rizorremediación, o utilización de la rizosfera de las plantas como ecosistema para descontaminar suelos, se presenta como un estrategia prometedora dada su demostrada viabilidad y contribución en la eliminación de una variedad de contaminantes orgánicos. Las plantas de girasol (*Helianthus annuus*, L) han sido descritas como altamente beneficiales en la eliminación de HAPs de suelos contaminados (Maliszewka-Kordybach y Smereczak, 2000). Sin embargo, su papel en el aumento de la biodegradación de suelos contaminados envejecidos no ha sido estudiado.

En el presente estudio se ha utilizado un suelo afectado por contaminación con creosota durante largo tiempo, y cuyo contenido en HAPs y biodisponibilidad de los mismos son similares a los de encontrados para suelos contaminados que han sido sometidos a una extensiva biorremediación. Los objetivos de nuestro trabajo consistieron en determinar la viabilidad del uso de las plantas de girasol para reducir la concentración residual de HAPs del suelo, caracterizando el papel de los exudados radiculares y determinando los cambios en la estructura de la comunidad microbiana causados por el efecto de la rizosfera.

Este estudio fue una colaboración entre los grupos del Dr. Cantos y el Dr. Ortega-Calvo del IRNASE (CSIC, Sevilla), que establecieron el sistema experimental, desarrollaron un método para la producción de los exudados de raíz en condiciones estériles, y llevaron a cabo los experimentos de mineralización y degradación de HAPs en el laboratorio.

Los experimentos llevados a cabo en el invernadero mostraron que todas las semillas de girasol germinaron tanto en el suelo contaminado con creosota como en el suelo no contaminado, siendo las plantas en el suelo contaminado significativamente más altas y presentando mayor peso seco. Las diferencias pueden atribuirse a la actividad de los microorganismos introducidos con el suelo contaminado con creosota, que pueden haber producido un efecto beneficioso para las plantas mediante la movilización de nutrientes.

Los experimentos de biodegradación mostraron que la rizosfera reducía el nivel de los HAPs hasta un 93% en 90 días, representando una mejora del 16% respecto al suelo contaminado sin plantas. Las concentraciones de antraceno, fluoranteno, pireno y criseno disminuyeron significativamente por debajo de los niveles detectados en los controles sin planta tras 45 días y 90 días. En el caso del fluoreno el efecto positivo de las plantas fue observado únicamente a los 45 días, mientras que los niveles de este HAP fueron por debajo de los límites de detección en ambas condiciones tras 90 días. La presencia de las plantas de girasol no tuvo un efecto significativo en la disipación del fenantreno en ninguno de los periodos muestreados, presentando una disminución significativa tras 45 días en ambos tratamientos.

Los análisis mediante técnicas dependientes de cultivo (Número Más Probable, NMP) (Figura R7), mostraron un aumento general de dos órdenes de magnitud en las poblaciones heterótrofas entre los días 0 y 45, para luego permanecer constantes en el suelo con plantas y ligeramente disminuir en el suelo sin plantas. Esto era probablemente debido al agotamiento de las fuentes de carbono en los controles, lo cual podía haber sido compensado en los tratamientos con plantas mediante la rizosfera. Las poblaciones degradadoras de bajo peso molecular (LMW, en inglés)

mostraron un comportamiento similar, con un aumento de dos órdenes de magnitud entre los días 0 y 45 en todas las condiciones seguido de un ligero descenso en el suelo sin plantas pero permaneciendo constantes en el suelo tratado. Resultó interesante que los degradadores de HAPs de elevado peso molecular (HMW) sufrieran un aumento substancial tras 45 días, especialmente en el tratamiento con plantas y permanecieran a altos niveles hasta el final del experimento. A los 90 días, la ratio entre los degradadores de HAPs de elevado peso molecular y las poblaciones heterótrofas totales era substancialmente mayor en los tratamientos con planta que en suelo control, lo que podía indicar que además de la estimulación del crecimiento general en las poblaciones heterótrofas (que incluyen a los degradadores), la rizosfera tenía un efecto adicional aumentando selectivamente el crecimiento de las poblaciones degradadoras de HAPs de elevado peso molecular. Estos resultados eran consistentes con los obtenidos en los análisis de la degradación de los HAPs y confirmaban lo resultados descritos por otros autores (Miya y Firestone, 2000; Parrish *et al*., 2005).

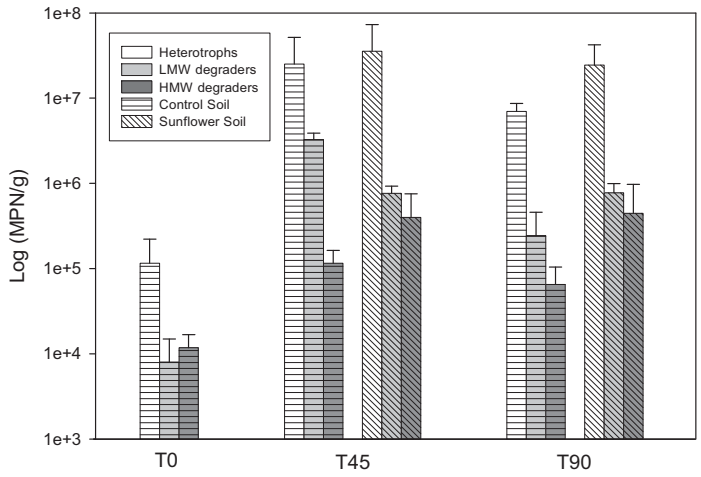

Figura R7. Enumeración de las poblaciones microbianas heterótrofas y degradadoras de HAPs en el suelo en los diferentes tratamientos aplicados en el experimento del invernadero. MPN, (número más probable, en ingles). LMW, bajo peso molecular (en inglés); HMW, elevado peso molecular (en inglés)

Se sabe que las comunidades microbianas de la rizosfera pueden ser considerablemente diferentes a aquellas que crecen en áreas cercanas sin la influencia de las raíces. El primer abordaje que se usó para observar cambios específicos en las poblaciones microbianas consistió en el análisis mediante DGGE del ADNr 16S (Figura R8). Los perfiles de DGGE de réplicas de cada tratamiento fueron muy similares, indicando por lo tanto una alta homogeneidad dentro de cada mesocosmos, para cada tratamiento. Durante la incubación, se produjeron incrementos en las abundancias relativas de poblaciones específicas para cada uno de los tratamientos (con planta y sin planta). Varias de las bandas detectadas en ambos tratamientos coincidían, sin embargo su abundancia difería en el tratamiento sin planta respecto a la del suelo de la rizosfera, confirmando que la rizosfera podía tener un efecto en el aumento de poblaciones específicas más allá que el simple hecho de regar y distribuir la tierra en sus respectivas macetas.

Tabla R3. Análisis de las secuencias del 16S ARNr de la librería de clones a partir del suelo contaminado con HAPs control (sin plantas) y la rizosfera de girasol.

Clon	Frequencia (%)[a]		Long Fragmento (bp)	Sim %	Relativo más cercano en la base de datos GenBank database[b] (número de accesso)	Grupo Filogenético
	CS	SFS				
CS1		2.4	1364	99	Uncultured *Acidobacteria* bacterium clone HEG_08_216 (HQ597545)	*Acidobacteriaceae (Acidobacteria)*
CS2		2.4	1373	98	Uncultured bacterium clone 60C1 (EU676416)	*Acidobacteriaceae (Acidobacteria)*
CS3		2.4	1422	99	Uncultured *Acidobacteria* bacterium clone SEG_08_603 (HQ729829)	*Acidobacteriaceae (Acidobacteria)*
CS4		2.4	1404	97	Uncultured *Acidobacteria* bacterium clone HG-J02120 (JN409027)	*Acidobacteriaceae (Acidobacteria)*
CS5		2.4	1421	99	Uncultured bacterium clone p6h2ok (FJ478980)	*Acidobacteriaceae (Acidobacteria*
CS6		2.4	1386	97	*Acidobacteria* bacterium IGE-018 (GU187039)	*Acidobacteriaceae (Acidobacteria)*
CS7	2.3		1466	99	Uncultured bacterium clone p27d24ok (FJ478675)	*(Actinobacteria)*
CS8		2.4	1384	99	*Lentzea waywayandensis* strain 173629 (EU570362)	*Pseudonocardiaceae (Actinobacteria)*
CS9	4.7		1444	98	Uncultured bacterium clone 125 (FM209343)	*Cytophagaceae (Bacteroidetes)*
CS10	7.0		1453	99	Uncultured bacterium clone p8c07ok (FJ479495)	*Chitinophagaceae (Bacteroidetes)*
CS 11	2.3		1412	98	Uncultured bacterium isolate 1112864242247 (HQ120332)	*Ohtaekwangia (Bacteroidetes)*
CS 12	2.3		1314	98	Uncultured *Bacteroidetes* bacterium clone HG-J01164 (JN408934)	*Ohtaekwangia (Bacteroidetes)*
CS 13	2.3		1443	95	Uncultured bacterium clone TX2_4C19 (JN178178)	*Rhodothermaceae (Bacteroidetes)*
CS 14	2.3		1329	92	Uncultured bacterium clone p27c17okm (FJ479173)	*Ohtaekwangia (Bacteroidetes)*
CS 15	4.7	4.9	1445	98	Uncultured *Bacteroidetes* bacterium clone g31 (EU979040)	*Ohtaekwangia (Bacteroidetes)*
CS 16	2.3	2.4	1393	99	Uncultured bacterium clone 224T (EU676412)	*Chitinophagaceae (Bacteroidetes)*
CS 17		2.4	1394	98	Uncultured soil bacterium clone UA2 (DQ298006)	*Chitinophagaceae (Bacteroidetes)*
CS 18	2.3		1475	99	Uncultured *Anaerolinae* bacterium clone AMAG11 (AM935836)	*(Choloroflexi)*
CS 19		2.4	1353	99	Uncultured bacterium clone S-Rwb_62 (DQ017911)	*(Choloroflexi)*
CS 20		2.4	1353	99	Uncultured bacterium clone H3-26 (JF703479)	*(Choloroflexi)*
CS 21		4.9	1373	99	*Phormidium autumnale* CCALA 143 (FN813344)	*(Cyanobacteria)*
CS 22		2.4	1324	99	Uncultured diatom clone H-101 (HM565019)	*Bacillariophyta (Cyanobacteria)*
CS 23	4.7		1455	96	Uncultured bacterium isolate 1112864242286 (HQ120393)	*Trueperaceae (Deinococcus)*
CS 24	2.3		1423	99	*Bacillus* sp. M71_D96 (FM992837)	*Bacillaceae (Firmicutes)*
CS 25	9.3		1440	99	*Bacillus* sp. R-36493 (FR682744)	*Bacillaceae (Firmicutes)*
CS 26	2.3		1486	98	*Virgibacillus carmonensis* (T) LMG 20964 (NR_025481)	*Bacillaceae (Firmicutes)*
CS 27	4.7		1438	99	*Bacillus* sp. BF149 (AM934692)	*Bacillaceae 2 (Firmicutes)*
CS 28		4.9	1399	96	Uncultured bacterium clone 15-4-139 (JN609373)	*Gemmatimonadaceae(Gemmatimonadetes)*
CS 29		2.4	1429	98	Uncultured bacterium clone TX5A_120 (FJ152828)	*(Gemmatimonadetes)*
CS 30	2.3		1368	94	Uncultured bacterium clone B6 (FJ660498)	*Planctomycetaceae (Planctomycetes)*

CS 31	2.3		1285	99	*Chelatococcus asaccharovorans* CP141b (AJ871433)	*Methylobacteriaceae (α)*
CS 32	2.3		1276	99	Uncultured bacterium clone HDB_SIOP800 (HM186473)	*Bradyrhizobiaceae (α)*
CS 33	2.3	2.4	1410	97	Uncultured soil bacterium clone F6-154 (EF688392)	*Sphingomonadaceae (α)*
CS 34		2.4	1342	99	*Rhizobium* sp. AC93c (JF970343)	*Rhizobiaceae (α)*
CS 35		2.4	1355	97	Uncultured bacterium clone FCPS478 (EF516121)	*Rhizobiales (α)*
CS 36		2.4	1353	99	Uncultured alpha proteobacterium clone QZ-J4 (JF776915)	*Sphingomonadaceae (α)*
CS 37		2.4	1355	99	*Altererythrobacter* sp. JM27 (GU166344)	*Sphingomonadales (α)*
CS 38	2.3		1425	98	Uncultured bacterium clone SNR65 (AB608675)	*Burkholderiales (β)*
CS 39	4.7	7.3	1418	99	Uncultured bacterium clone HC18-11B13 (JF417848)	*Commamonadaceae (β)*
CS 40	2.3	4.9	1410	99	*Naxibacter suwonensis* (T) 5414S-25 (FJ969487)	*Oxalobacteraceae (β)*
CS 41		2.4	1389	99	*Variovorax* sp. RA8 (AB513921)	*Commamonadaceae (β)*
CS 42		2.4	1396	99	Uncultured beta proteobacterium clone E2006TS6.19 (GU983311)	*(β)*
CS 43		2.4	1394	99	Uncultured beta proteobacterium clone C173 (JF833705)	*Methylophilaceae (β)*
CS 44		2.4	1400	99	Uncultured beta proteobacterium clone C173 (JF833705)	*Methylophilaceae (β)*
CS 45		2.4	1391	99	Uncultured beta proteobacterium clone G2-50 (JF703344)	*(β)*
CS 46		2.4	1391	99	Uncultured ammonia-oxidizing bacterium clone FQ-13C-HF-1 (HQ678202)	*Nitrosomonadaceae (β)*
CS 47	2.3		1434	97	Uncultured bacterium clone 0-99 (GU444064)	*Xanthomonadaceae (γ)*
CS 48	9.3		1458	99	*Pseudomonas* sp. JQR2-5 (DQ124297)	*Pseudomonadaceae (γ)*
CS 49		2.4	1412	99	*Lysobacter niabensis* (AB682414)	*Xanthomonadaceae (γ)*
CS 50		2.4	1391	99	Uncultured bacterium clone Kas172B (EF203204)	*Sinobacteraceae (γ)*
CS 51		2.4	1409	99	*Pseudoxanthomonas* sp. XC21-2 (JN247803)	*Xanthomonadaceae (γ)*
CS 52		2.4	1410	99	Uncultured *Lysobacter* sp. clone T302B2 (HM438520)	*Xanthomonadaceae (γ)*
CS 53		2.4	1399	99	Uncultured bacterium clone BR121 (HQ190468)	*Pseudomonadaceae (γ)*
CS 54		2.4	1419	97	Uncultured bacterium clone RH1020 (AB511013)	*(δ)*
CS 55	7.0		1415	86	Uncultured candidate division OD1 bacterium clone AKYH1067 (AY922093)	Candidate division OD1
CS 56	2.3		1396	94	Uncultured bacterium clone B03-05G (FJ542974)	Candidate división OP11
CS 57	2.3		1368	97	Uncultured bacterium clone N1903_34 (EU104291)	Candidate división TM7
CS 58	2.3		1250	93	Uncultured bacterium clone FF_-aag84c04 (EU469637)	Candidate division WS6

[a] Frecuencias en la librería de clones obtenidas a partir de CS (suelo control) y SFS (rizosfera de girasol). [b]Secuencias con idéntica similitud en cepas no cultivadas y aisladas, sólo se muestra la aislada. Secuencias con más de un 94% de identidad están agrupadas. α, β, γ, δ corresponde a *alpha-*, *beta-*, *gamma-* y *deltaproteobacteria*, respectivamente.

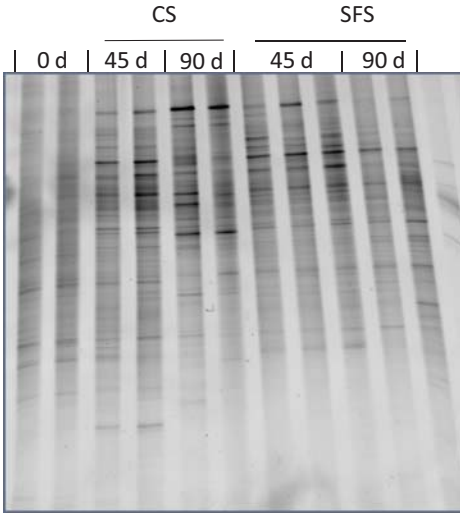

Figura R8. Perfil de DGGE de los genes amplificados mediante PCR del 16S ARNr a partir de réplicas independientes de muestras del control (CS) y del suelo de la rizosfera de girasol tras 0, 45 y 90 días. Cada carril contiene cantidades idénticas de ADN.

Para poder analizar la estructura específica de las comunidades microbianas presentes en cada suelo, se obtuvo una librería de clones del gen del ARNr 16S. Las muestras fueron tomadas a 90 días y se secuenciaron un total de 84 clones. La afiliación filogenética de cada clon de eubacteria se detalla en la tabla R3, mientras que la Figura R9 resume los diferentes filos bacterianos detectados y su abundancia relativa.

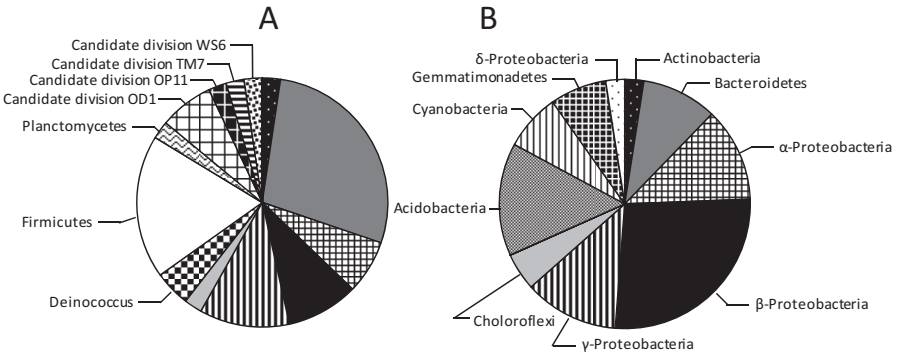

Figura R9. Relativa abundancia de los grupos filogenéticos de eubacterias identificados en el suelo control (A) y en el suelo de la rizosfera de girasol (B) tras 90 días de incubación en el invernadero.

Los subfilos α-, β-, y γ-*Proteobacteria*, y los filos *Actinobacteria*, *Bacteroidetes*, y *Chloroflexi* estaban representados en ambos tratamientos, sin planta y con planta (60% y 68% respectivamente), sin embargo, a excepción de las *Actinobacterias*, su abundancia relativa variaba sustancialmente, confirmando que la rizosfera de girasol provocaba un cambio dramático en la estructura de las comunidades del suelo.

La rizosfera favoreció la aparición de nuevas poblaciones dentro de los 3 subfilos de *Proteobacteria*, algunos de los cuales habían sido previamente identificados como

degradadores de HAPs (Kanaly y Harayama, 2010). Dentro de las α-*Proteobacteria*, el aumento de *Sphingomonas* fue de especial interés puesto que este grupo incluye numerosos miembros aislados de raíces de plantas y miembros con una gran versatilidad en la capacidad de degradar HAPs de 2, 3 y 4 anillos (Fernández-Luqueño *et al*., 2011). Dentro del grupo β-*Proteobacteria* (de 9% a 27%), la aparición del grupo *Commamonas*, con miembros de *Variovorax*, también fue muy notable, puesto que se ha descrito el aislamiento de especies de este género de suelos contaminados con HAPs y xenobióticos. Además, la rizosfera favoreció la aparición de miembros de *Oxalobacteriaceae,* una familia descrita recientemente, pero no bien caracterizada, con miembros colonizadores de raíces (Green *et al*., 2007) que se encuentran muy próximos a *Burkholderia*, que incluye importantes degradadores de HAPs en suelos contaminados tanto con compuestos individuales como de mezclas como la creosota (Grifoll *et al*., 1995). El aumento observado en los miembros de *Methylophillus*, fue también interesante, puesto que estudios recientes en nuestro grupo han identificado especies de bacterias metilotróficas como uno de los componentes más abundantes en un consorcio degradador de fuel pesado (Vila *et al*., 2010). Las bacterias metilotróficas están más ampliamente distribuidas de lo que se pensaba, pero su papel en el medio ambiente natural es todavía desconocido (Lidstrom, 2006; Chistoserdova *et al*., 2009). El grupo *Xanthomonas*, dentro de las γ-*Proteobacteria,* fue también favorecido por la rizosfera, y varios de los representantes detectados pertenecían a bacterias previamente identificadas en emplazamientos contaminados e identificados como degradadoras de HAPs. Por ejemplo, *Pseudoxhantomonas*, ha sido descrita por su capacidad de degradar el HAPs de cuatro anillos como el criseno (Kayak *et al*., 2011). La reducción en la presencia de los *Bacteroidetes* en la rizosfera podía deberse a una consecuencia directa de la presencia de nutrientes de los exudados, puesto que este filo se ha asociado a menudo en ambientes con carencia de nutrientes (Viñas *et al*., 2005).

En el suelo no plantado, el 40% de los microorganismos detectados pertenecía a 7 grupos filogenéticos no detectados en el suelo con plantas de girasol. Entre ellos se encontraron miembros de las divisiones candidatas OD1, OP11, TM7 y WS6, las cuales son linajes de organismos procariotas no cultivados (Hugenholtz *et al*., 1998; Chouari *et al*., 2005), junto con una alta abundancia relativa de *Firmicutes*, mientras que los grupos *Planctomicetes* y *Deinococcus* fueron representados en una menor proporción.

Por el contrario, la rizosfera de girasol promovió la aparición de cuatro filotipos (*Acidobacteria, Gemmatimonadetes,* δ-*Proteobacteria* y *Cyanobacteria*), que no fueron detectados en el suelo no tratado. Los más abundantes, *Acidobacteria* (14,6%) y *Gemmatimonadetes* (7,3%) constituyen filos recientemente descritos (Ludwig *et al*., 1997; Zhang *et al*., 2003) y ampliamente distribuidos en suelos pero escasamente representados en cultivos, lo que dificulta determinar su papel en la naturaleza. Las acidobacterias han sido observadas anteriormente en suelos con plantas (Sibila *et al*. 2008; Yrjala *et al*., 2010) y se encuentran normalmente en ambientes no contaminados, disminuyendo en presencia de contaminantes. Por lo tanto, su alta abundancia aquí tras 90 días de tratamiento podría explicarse por el efecto de la rizosfera y el alto grado de eliminación de HAPs conseguidos en este tratamiento.

Para determinar los efectos de los exudados de girasol, se llevaron a cabo varios experimentos. Tras obtener los exudados de raíz de girasol, se realizaron análisis radiorespirométricos y de residuos para determinar la mineralización de ^{14}C-pireno y la biodegradación de los HAPs presentes en una muestra de suelo resuspendida en exudados de las raíces de girasol y en medio mineral. Al final de la incubación (250h) se observó que en presencia de exudados de girasol el pireno fue mineralizado un 40% mientras que en cultivos que contenían medio mineral fue sólo del 29%. Además el contenido residual de los HAPs totales no fue significativamente diferente aquellos alcanzados en el experimento del invernadero, reafirmando que los exudados juegan un papel en la biodegradación promoviendo la disipación de HAPs.

La evolución de las poblaciones autóctonas fue también analizada (Figura R10). Los cultivos fueron muestreados al final de la incubación (10d) y su composición microbiana fue analizada mediante DGGE. Durante los 10 días de incubación, ambos tratamientos mostraron un incremento en el número de microorganismos, y un cambio en el perfil de bandas como resultado a la exposición a exudados, lo que indica que el aumento en la degradación de HAPS era acompañado de un aumento de poblaciones microbianas específicas.

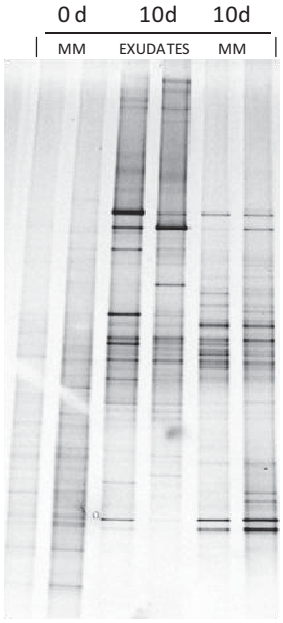

Figura R10. Perfil de DGGE de los fragmentos amplificados del gen del 16S ARNr de muestras de suelo resuspendidas del experimento de bioaccesibilidad.

Con el fin de investigar en mayor profundidad la naturaleza del efecto de los exudados éstos se sometieron a análisis químico (Tabla 3 del artículo 4). Entre los diferentes componentes identificados, los carbohidratos mayoritarios fueron fructosa y galactosa, mientras que los aminoácidos mayoritarios fueron asparagina y glutamina. Estas sustancias han sido descritas como inductores de la quimiotaxis y quimioatrayentes (Heinrich y Hess, 1985; Pandya *et al*., 1990; Zheng y Sinclair, 1996). Los ácidos grasos mayoritarios fueron palmítico y esteárico, mientras que otros, en los que se incluye el principal componente del aceite de girasol, el ácido linoleico, fueron detectados a bajas concentraciones. Estas sustancias pueden aumentar la bioaccesibilidad de los HAPs en suelo actuando como surfactantes (Yi y Crowley; 2007). Es interesante que se identificaran varios ácidos aromáticos, siendo los más abundantes el ftálico y el protocatecuato. Estos compuestos son típicos intermediarios en el metabolismo de HAPs. Los metabolitos secundarios de plantas pueden tener un efecto en la estimulación de la degradación de HAPs por los microorganismos de la rizosfera y ampliar el espectro de su actividad induciendo la producción de enzimas degradadores de contaminantes orgánicos (Singer *et al*., 2003), o bien actuando como cosustratos en reacciones cometabólicas.

Estos resultados sugieren que las plantas de girasol podrían ser efectivas en la eliminación de residuos recalcitrantes de HAPs en suelos que previamente han sido sometidos a una biorremediación extensiva pero que contienen niveles inaceptables de HAPs debido a las limitaciones en la bioaccesibilidad. Su acción se debería a la

estimulación selectiva de las poblaciones degradadoras y el aumento de la bioaccesibilidad de HAPs. Esta estrategia seria una alternativa prometedora para el aumento de la bioaccesibilidad de una manera sostenible y de bajo riesgo. Este estudio es el primero en analizar el efecto de la rizosfera sobre la estructura de las comunidades bacterianas. La exacta contribución de los efectos directos de los exudados de girasol y los efectos relacionados con la ecología de los microorganismos del suelo serán el sujeto de futuras investigaciones.

Conclusiones

1. Análisis independientes de cultivo mostraron que el enriquecimiento de poblaciones marinas de zonas costeras mediante cultivo en fuel y posteriormente en pireno como fuentes de carbono, produjo una comunidad microbiana degradadora estable compuesta principalmente por *α-Proteobacteria* (84%*)* y *Actinobacteria* (16%). La población más abundante se afiliaba separada de los géneros existentes dentro de las *α-Proteobacteria,* mientras otros miembros identificados en este grupo fueron *Thalassospira*, *Martelella*, *Paracoccus*, *Novosphingobium*, *Sphingopyxis* y *Aurantimonas.* La única γ-*Proteobacteria* fue *Alcanivorax*, y las *actinobacterias* detectadas pertenecían a *Gordonia* y *Micrococcus.*

2. La utilización de una gran variedad de medios de cultivo específicos permitió recuperar en cultivo puro casi todas las bacterias detectadas, a excepción de *Thalassospira* y *Gordonia*. Sin embargo, ninguno de estos aislados o los aislados adicionales obtenidos de otras bacterias (*Novosphingobium*, *Sphingopyxis*, *Aurantimonas*, *Micrococcus* y *Alcanivorax*) fueron capaces de atacar el pireno o fenantreno.

3. Los análisis funcionales del consorcio mostraron la presencia de una única dioxigenasa dihidroxilante de HAPs, no detectada en los miembros cultivables, que estaba muy cercanamente relacionada a la dioxigenasa hidroxilante de pireno NidA3 encontrada en actinobacterias degradadoras. Esto sugiere que la *Gordonia* no cultivada juega un papel en el ataque inicial de la molécula del pireno, mientras que el resto de los componentes bacterianos son mantenidos mediante el carbono proporcionado por los derivados del pireno producidos por esta cepa. Por otro lado, los miembros no cultivables de *Gordonia* podrían necesitar la presencia de las otras poblaciones presentes para su crecimiento.

4. El componente microbiano más abundante del consorcio marino degradador de pireno ha sido clasificado dentro de un nuevo género y especie, descritos en esta Tesis bajo la denominación *Breoghania corrubedonensis.*

5. El fertilizante oleofílico S200 aumenta la degradación de todos los HAPs presentes en fuel compensado las deficiencias nutricionales en la interfase FLNA/agua causadas por la utilización simultánea de otros componentes (por ejemplo, alcanos).

6. La bioestimulación con fertilizantes oleofílicos aumenta la producción de metabolitos de HAPs parcialmente oxidados, que son fácilmente movilizados hacia la fase acuosa, y cuya toxicidad y destino ambiental se desconoce.

7. La rizosfera de girasol tuvo un efecto significativo en la reducción de los niveles residuales de HAPs en suelos envejecidos contaminados con creosota respecto a

los suelos no plantados, causando un aumento selectivo de las poblaciones microbianas degradadoras de HAPs. Los análisis moleculares mostraron un cambio drástico en la estructura de la comunidad, aumentando la abundancia relativa de α-, β-, *δ*- y γ-*Proteobacteria, Acidobacteria*, *Cyanobacteria*, y *Gemmatimonadetes,* muchos de ellos con representantes degradadores de HAPs. La abundancia relativa de *Actinobacteria*, conocido por ser un degradador de HAPs de elevado peso molecular no fue afectada por la rizosfera.

8. Los exudados de la raíz de girasol están compuestos por carbohidratos, principalmente fructosa y galactosa y una variedad de aminoácidos, y ácidos grasos como el palmítico y el esteárico. Además, fueron detectados compuestos descritos como metabolitos de HAPs, como el ácido ftálico y el protocatecuato. Estos componentes pueden actuar favoreciendo la bioaccesibilidad de los HAPs (incrementando la biodisponibilidad y favoreciendo mecanismos de quimiotaxis), induciendo enzimas degradadoras de HAPs o actuando como cosustratos.

9. El efecto de la rizosfera observado en suelos fue reproducido en cultivos líquidos con exudados, indicando que los exudados son los principales responsables de la estimulación observada.

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Impact factor and contribution to the papers

I. **Gallego, S**., Vila, J., Tauler, M., Nieto, J.M., Breugelmans, P., Springael, D., Grifoll, M. (2012) Microbial community structure and function analysis of a pyrene-degrading marine consortium. Submitted to *FEMS Microbiology Ecology*.

Sara Gallego performed all of the research of this work, with the exception of the screening of some isolation media, she also discussed the results and wrote the paper. The 2010 impact factor of *FEMS Microbial Ecology* is 3.46, corresponding to the first quarter among the Microbiology indexed publications.

II. **Gallego, S**., Vila, J., Nieto, J.M., Urdiain, M., Rosselló-Móra, R., Grifoll, M. (2010) *Breoghania corrubedonensis* gen. nov. sp. nov., a novel *alphaproteobacterium* isolated from a Galician beach (NW Spain) after the *Prestige* fuel oil spill, and emended description of the family *Cohaesibacteraceae* and the species *Cohaesibacter gelatinilyticus. Systematic and Applied Microbiology* , 33:316–321.

Sara Gallego performed the whole research of this work with the exception of the RAPD, the MLSA, and the MALDI-TOF MS analysis, performed by the group of Dr. Roselló Móra from the IMEDEA, CSIC. She also discussed the results and wrote the paper. The 2010 impact factor of *Systematic and Applied Microbiology* is 3.08, corresponding to the second quarter among both, the Microbiology and the Biotechnology and Applied Microbiology indexed publications.

III. Tejeda-Agredano, M.C., **Gallego, S.**, Niqui-Arroyo J.L., Vila, J., Grifoll, M., Ortega-Calvo, J.J. (2011) Effect of interface fertilization on biodegradation of polycyclic aromatic hydrocarbons present in nonaqueous-phase liquids. *Environmental Science and Technology,* 45:1074–1081.

This work was in collaboration with the group of Dr. Ortega-Calvo, from the IRNAS, CSIC. Sara Gallego performed the whole research carried out at the University of Barcelona, which included all the chemical analyses. She also participated in the discussion and the writing of the manuscript. The 2010 impact factor of *Environmental Science and Technology* is 4.83, corresponding to the first quarter among the Environmental Sciences indexed publications.

IV. Tejeda-Agredano, M.C., **Gallego, S**., Vila, J., Grifoll. M., Ortega-Calvo, J-J., Cantos, M. (2012) Influence of the sunflower rhizosphere on the bioaccessibility and biodegradation of PAHs in soil. Submitted to *Soil Biology & Biochemistry.*

Maria del Carmen Tejeda-Agredano and S. Gallego contributed equally to the achievment of results presented in this publication, performed in collaboration with the group of Drs. Ortega-Calvo and M. Cantos, IRNAS, CSIC, Sevilla. The 2010 impact factor for *Soil Biology & Biochemistry* is 3.34, corresponding to the first place of Soil Science indexed publications.

Barcelona, 15th May 2012

Signed:

Dra. Magdalena Grifoll

Estructura de la comunidad microbiana y análisis funcional de un consorcio marino degradador de pireno

Un consorcio microbiano marino (UBF) altamente eficiente en la eliminación de los hidrocarburos de un fuel oil pesado fue obtenido mediante enriquecimiento a partir de una muestra de arena de una playa contaminada por el vertido del *Prestige*. La subpoblación responsable de la degradación del pireno (UBF-Py) fue seleccionada mediante un subcultivo del consorcio UBF en medio mínimo con pireno. Tras 30 días de incubación, la comunidad microbiana mineralizó el 31% del pireno. La ausencia de intermediarios parcialmente oxidados, indicó la cooperación entre los diferentes componentes en la mineralización del sustrato. La composición de la comunidad microbiana fue determinada mediante técnicas dependientes de cultivo y métodos basados en la reacción en cadena de la polimerasa (PCR-DGGE y librería de clones). Los análisis moleculares mostraron una comunidad altamente estable compuesta principalmente por *Alpha-Proteobacteria* (84%, *Breoghania*, *Thalassospira*, *Paracoccus,* y *Martelella*) y *Actinobacteria* (16%, *Gordonia*). Los miembros de *Thalassospira* y *Gordonia* no fueron recuperados como cultivos puros, pero los métodos de aislamiento produjeron cinco cepas adicionales no detectadas en el análisis molecular, y clasificadas dentro de los géneros *Novosphingobium*, *Sphingopyxis* y *Aurantimonas* (*Alpha-Proteobacteria*s), *Alcanivorax* (*Gamma-Proteobacteria*) y *Micrococcus* (*Actinobacteria*). Ninguno de los aislados fue capaz de degradar pireno u otros HAPs en cultivos puros, y además, las PCRs de los genes de dioxigenasas de Gram-positivos y Gram-negativos no produjeron amplificación en ninguno de las cepas. Sin embargo, cuando el consorcio UBF-Py fue analizado por la posible presencia de dioxigenasas, una dioxigenasa hidroxilante de anillos de HAPs altamente relacionada con la dioxigenasa del pireno NidA3 presente en micobacterias fue detectada. Esto podría señalar al representante de *Gordonia* como el degradador clave del pireno en el consorcio, lo cual es consistente con el papel predominante de las *Actinobacterias* en la eliminación de pireno en ambientes marinos.

Microbial community structure and function analysis of a pyrene-degrading marine consortium

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Abstract

A marine microbial consortium (UBF) highly efficient in removing the different heavy fuel oil hydrocarbons was obtained by enrichment culture from a beach polluted by the *Prestige* oil spill. The subpopulation responsible for pyrene degradation (UBF-Py) was selected by subculturing in pyrene minimal medium. After 30 days of incubation, this microbial community mineralized 31% of pyrene. The absence of accumulation of partially oxidized intermediates indicates the cooperation of different microbial components in the mineralization of the substrate. The microbial community composition was determined by culture dependent and polymerase chain reaction based methods (PCR-DGGE and clone libraries). Molecular analyses showed a highly stable community composed mainly by *Alpha-Proteobacteria* (84%, *Breoghania*, *Thalassospira*, *Paracoccus,* and *Martelella*) and *Actinobacteria* (16%, *Gordonia*). The members of *Thalassospira* and *Gordonia* were not recovered as pure cultures, but isolation procedures produced five additional strains not detected in the molecular analysis that classified within the genera *Novosphingobium, Sphingopyxis* and *Aurantimonas (Alpha-Proteobacteria), Alcanivorax (Gamma-Proteobacteria)* and *Micrococcus (Actinobacteria).* None of the isolates were able to degrade pyrene or other PAHs in pure cultures, and, in addition, the PCR amplification of Gram-positive and Gram-negative dioxygenase genes did not produce results with any of the strains. However, when the UBF-Py consortium was screened for dioxygenases, a PAH ring-hydroxylating dioxygenase closely related to the NidA3 pyrene dioxygenase present in mycobacterial strains was detected. This would point out to the representative of *Gordonia* as the key pyrene degrader in the consortium .and is consistent with a preeminent role of actinobacteria in pyrene removal in coastal environments.

Introduction

Vast amounts of petroleum enter the marine environment each year from both natural and antropogenic sources. Accidental oil spills, accounting for about 12% of the total oil released (NAS, 2003), cause extensive ecological damage to marine shorelines and have an enormous impact on local economic activities due to the associated risk to public health. Physical and chemical procedures frequently used to clean-up coastal areas result in uncontrolled dispersal of oil residues that often persist in sediments (Tansel *et al*, 2011). Microbial biodegradation is the main process for actual removal of contaminants from the environment, and it has been proved that can be stimulated by the addition of nitrogen and phosphorous fertilizers (Prince & Atlas, 2005). The identification of the microbial populations involved in hydrocarbon degradation in polluted shorelines and coastal sediments may provide new efficient and non-disruptive strategies to enhance the removal of oil residues from these environments.

Among petroleum components, high molecular weight polycyclic aromatic hydrocarbons (PAHs) are of special concern due to their toxic and genotoxic potentials and because their chemical stability and low availability confers them a high

environmental persistence (Samanta *et al*., 2002). Pyrene, with four fused aromatic rings, is, together with its alkyl derivatives, one of the most abundant high-molecular weight PAHs in oil and derivatives (Vila & Grifoll,, 2009). The isolation of a number actinobacterial strains (mainly *Mycobacterium*) that use pyrene as a sole source of carbon and energy, has provided valuable information on the metabolic pathways for degradation of pyrene in soils (Kanaly & Harayama, 2010). In fact, early molecular approaches (Kim *et al*., 2006) followed by total genome analysis of *M. vanbaalenii PYR-1* (Kim *et al*., 2007) and *M. gilvum* spyr1 (Kallimanis *et al*., 2011) have contributed to the characterization of key enzymes such as the initial ring-hydroxylating dioxygenases, at a biochemical and genetic level. Conversely, little is known about the organisms and processes involved in the degradation of high molecular weight PAHs in the seas. Since a great portion of marine bacteria are considered non-cultivable (Amann *et al*., 1995), a number of studies have used culture-independent rRNA approaches to analyze changes in the structure of microbial communities from natural marine environments polluted by real oil spills (Mcnaughton *et al*., 1999) or from micro- (Niepceron *et al*., 2010; Mckew *et al*., 2007; Yakimov *et al*., 2005) and mesocosms (Cappello *et al*., 2007) that mimic such environments. Subculturing such communities with oil or oil components as sole carbon source has produced a number of efficient degrading consortia whose community structures have been thoroughly analyzed. These works have identified more than 20 bacterial genera involved in oil degradation in marine environments that classified within the *Alpha-, Beta-,* and *Gamma Proteobacteria* or the *Flexibacter-Cytophaga-Bacteroidetes* groups (Macnaughton *et al*., 1999; Cui *et al*., 2008; Aruzlashagan & Vasudevan 2009; Vila *et al*., 2010; Shao *et al*., 2010; Nipceron *et al*., 2010; Wang & Tam, 2011). These community dynamics analyses and, in a few cases, further isolation techniques, have proven that *Alcanivorax* is a key marine alkane degrader while *Cycloclasticus* seems to be a ubiquitous degrader of 2 and 3-ring PAHs (Yakimov *et al*., 2007). However, most of the studies with PAH-degrading consortia fail to link specific degradative capabilities to their components. Only recently has been demonstrated the capability of attacking pyrene in the absence of other substrates by a few marine bacteria: *Cycloclasticus* sp. P1, isolated from deep sea sediment (Wang *et al*., 2008), and *Ochrobactrum* sp. VA1 (Arulazhagan & Vasudevan, 2011). Interestingly, no associations have been established between pyrene degradation and actinobacteria in marine environments.

In previous studies, a marine fuel-degrading consortium (UBF) was obtained and characterized (Fernández-Alvárez *et al*., 2006, 2007) from a beach contaminated with fuel-oil from the *Prestige* spill. This consortium was proved to highly efficient in degrading a variety of hydrocarbon families within the fuel. UBF cultures showed a complete removal of all the linear and branched alkanes, an extensive attack on three to five-ring PAHs (>99% for fluorene, phenanthrene, anthracene and dibenzothiophene and 75%, 73% and 30% for pyrene, chrysene and benzo(*a*)pyrene, respectively) and a considerable depletion of their alkyl derivatives (Vila *et al*., 2010). In an attempt to link microbial populations to specific catabolic activities, different enrichment subcultures were set up from the original UBF consortium using fuel fractions or single PAHs as carbon sources. Here we combine different non-culture and culture-depending approaches to identify, isolate and characterize the microbial components of the pyrene enrichment subculture, including the screening of the community and the obtained isolates for the presence of PAH hydroxylating dioxygenase genes.

Material and methods

Chemicals

PAH substrates were obtained from Sigma-Aldrich Chemie (Steinheim, Germany). Solvents were obtained from J.T. Baker (Deventer, the Netherlands). 9-^{14}C-

phenanthrene (13.1mCimmol^{-1}, dissolved in methanol, radiochemical purity >98%) and 4,5,6,10-^{14}C-pyrene (58.7mCimmol^{-1}, dissolved in methanol, radiochemical purity >98%) were purchased from Sigma-Aldrich Chemie.

Media and supply of hydrocarbons

Pyrene was added to the sterile artificial seawater (Vila & Grifoll, 2009) dissolved in acetone and the mixture shaken at 200 r.p.m and 30ºC overnight to allow acetone removal. Before inoculation, the sterile seawater was supplemented with the following sterile solutions: nitrogen (NH_4NO_3, final concentration 11mM), phosphorous (K_2HPO_4, 0.7mM); and metals [$MgS0_4 \cdot 7H_2O$, 0.2gL^{-1}; $FeSO_4 \cdot 7H_2O$, 0.012gL^{-1}; $MnSO_4 \cdot H_2O$, 0.003gL^{-1}; $ZnSO_4 \cdot 7H_2O$, 0.003gL^{-1}; $CoCl_2 \cdot 6H_2O$, 0.001gL^{-1}; and nitrilotriacetic acid disodium salt (Sigma-Aldrich Chemie), 0.123gL^{-1}].

Microbial consortium UBF-Py

The microbial consortium UBF was obtained from an enrichment culture established in artificial seawater and *Prestige* fuel oil (5gL^{-1}) inoculated with a composite sand sample collected from the beach of Corrubedo (A Coruña, NW Spain) after the oil spill. After 3 years of transfers, a subculture (1:50) of consortium UBF was established in nutrient-supplemented artificial seawater with pyrene as the sole carbon source (0.2gL^{-1}). This UBF-Py enrichment culture has been transferred monthly for 3 years.

Biodegradation of the pyrene by consortium UBF-Py

The microbial consortium UBF-Py was used to inoculate (0.5ml) duplicate 100-ml Erlenmeyer flasks containing 25 ml of nutrient-supplemented artificial seawater and pyrene (0.2gL^{-1}). Sterile noninoculated flasks were used as controls. Cultures and controls were incubated at 25ºC under fully aerobic conditions (rotary shaking, 200 r.p.m). At 30 days, the entire flask content of duplicate cultures and controls were extracted five times with 10ml of ethyl acetate, acidified to pH 2 and extracted again in the same manner. Neutral and acidic extracts were dried using Na_2SO_4, concentrated under vacuum to a final volume of 1ml, and analysed by reverse-phase HPLC. These analyses were conducted in Hewlett-Packard model 1050 chromatograph equipped with a HP-1040M diode array UV-visible detector set at 254 nm. Separation was achieved on a Chromspher C18 (Chrompack) (25 cm by 4.6mm, 5-µm particle size) (column applying a linear gradient of methanol [10 to 95% (vol/vol) in 20 min] in acidified water (0.6% H_3PO_4). Flow was maintained at 1mlmin^{-1}. Extract injection volume was 10 µl. Residual pyrene concentration was determined from the peak areas of duplicate samples by using standard calibration curves.

Enumeration of heterotrophic and hydrocarbon-degrading microbial populations

Bacterial counts from the UBF-Py consortium were performed using the miniaturized most probable number (MPN) method in 96-well microtiter plates with 8 replicate wells per dilution (Wrenn & Venosa, 1996). Total heterotrophs were counted in 1:10 Luria-Bertani artificial seawater and pyrene-degraders were counted in nutrient-supplemented artificial seawater containing pyrene at a final concentration of 0.5gL^{-1}. The hydrocarbon was added to the plates dissolved in pentane and the media was added after solvent evaporation. MPN plates were incubated at room temperature (25ºC ± 2ºC) for 30 days. Positive wells were detected by turbidity (heterotrophs) and the presence of coloration (brownish/ yellow) for PAH degraders.

Mineralization experiments

Mineralization of 9-^{14}C-phenanthrene and 4,5,6,10-^{14}C-pyrene by consortium UBF-Py was examined in 15ml-Pyrex tubes containing 4.5 ml of supplemented artificial seawater, 0.5 ml of culture in the stationary phase, and unlabeled PAH at a concentration below its water solubility (1.18 mgL^{-1} for phenanthrene and 0.135 mgL^{-1} for pyrene at 25ºC, Miller *et al*., 1985). The labelled and unlabelled substrates were combined in methanol and these solution (300-500 µl) were added to empty sterile flasks. The supplemented artificial seawater and inocula were added after methanol evaporation. The final concentrations of phenanthrene and pyrene were 0.5 mgL^{-1} and 0.1 mgL^{-1}, respectively, while the final radioactivity was of 0.004 and 0.003µCi ml$^{-1.}$ Mineralization experiments were also performed at 0.2 gL^{-1} final concentration and 0.004 µCiml^{-1} of radioactivity for both, phenanthrene and pyrene. In this case, after evaporation of methanol the mixture in supplemented artificial seawater was sonicated for better availability.

The tubes were closed with Teflon-lined stoppers equipped with alkali traps (1ml of 0.5 M NaOH) to measure the $^{14}CO_2$ produced from added ^{14}C- phenanthrene or ^{14}C-pyrene, and incubated at 25ºC on a rotary shaker at 150 r.p.m. At selected incubation times, triplicate cultures and uninoculated controls were withdrawn, acidified with 0.03M HCl to reduce the pH below 5, and incubated overnight to facilitate CO2 release. Then, the NaOH solution was mixed with 5 ml liquid scintillation cocktail (Ultima Gold; PerkinElmer, Boston, MA), and the mixture was kept in darkness for 8 h for the dissipation of chemioluminiscence. Radiactivity was measured with a Packard Tri-Carb 1600CA liquid scintillation counter (PerkinElmer, Boston, MA).

Isolation, identification and catabolic characterization of culturable organisms

Heterotrophic bacterial strains from consortium UBF-Py were isolated after serial dilution and plating on 1:10 diluted Luria-Bertani (LB) agar prepared in artificial seawater or natural filtered seawater. To isolate specific hydrocarbon-utilizing strains, the same dilutions were plated on nutrient-supplemented artificial seawater agar plates containing 0.1gL^{-1} of pyrene or phenanhtrene, as well as in variants of this PAH-mineral medium containing in addition 0.1gL^{-1} of yeast extract, or 9gL^{-1} sorbitol and 0.5gL^{-1} of yeast extract (sorbitol medium, SM, Brezna *et al*., 2003). In order to discriminate against Gram-negative bacteria and facilitate the isolation of supposed Gram-positive pyrene-degrading bacterias, SM was also used with the addition of streptomycin (10mgL^{-1}). The hydrocarbons were added to the medium at 50º in acetone solution and placed uncovered under sterile laminar flow for acetone evaporation. Other media used included modified M3 medium (Rowbotham & Cross, 1977) after dispersion and differential centrifugation technique (DCC) (Maldonado *et al*., 2005) but did not produce additional isolates. All the isolates were identified according to their almost complete 16S rRNA gene sequences.

Growth in, or transformation of, other PAHs was tested in artificial seawater plates supplemented with nutrients or yeast extract (0.1 gL^{-1}), and each PAH as the sole carbon source. Each of these plates was sprayed with an acetone solution (2%) of one hydrocarbon and incubated at 25ºC. After 30 days of incubation, growth was considered positive by a significant increase in bacterial biomass on test plates compared with non-sprayed control plates. Transformation was also considered positive by clearing zones around the bacterial mass, accompanied by accumulation of colored metabolites. As an exception, naphthalene was supplied to the inoculated medium as crystals in the lid, and its transformation was evident only by the accumulation of diffusible yellow-colored metabolites around the bacterial mass.

Growth on pyrene and phenanthrene (0.2 gL^{-1}) was tested in liquid artificial seawater medium supplemented with nutrients or yeast extract (0.25 gL^{-1}). After 30

days of incubation, the concentration of cell protein in cultures was measured. Growth was considered positive if, after this time, the concentration of cell protein in cultures was threefold that obtained for controls without the carbon source. The protein concentration was determined using the entire flask contents of cultures by a modification of the Lowry method (Daniels *et al*., 1994). ^{14}C-pyrene mineralization was also determined for each single isolate and for the combination of the eight isolates obtained together, as described above.

DNA extraction and PCR amplification of the 16S rDNA from UBF-Py consortium and bacterial isolates

Total DNA from UBF-Py consortium was extracted using Power Soil DNA isolation kit (Mobio, Carlsbad, USA). Template DNA from cultured bacterial strains was obtained by boiling a single colony in 0.1 ml of sterile distilled water for 10 minutes. 1 µl of this suspensions and DNA extracts were directly used for PCR amplification reactions.

Eubacterial 16S rDNA fragments of microbial consortia and bacterial isolates were amplified using primers 27f and 1492r (Weisburg *et al*., 1991) and the PCR mixture contained 1 µL of the total extracted DNA as the template, 1.25U *Taq* DNA polymerase (Biotool B&M Labs, Madrid, Spain), 25pmol of each primer (Sigma-Aldrich, Steinheim, Germany), 5nmol of each dNTP (Fermentas, Hanover, MD) and 1 x PCR buffer (Biotool B&M Labs) in a total volume of 25µL. After 10 min of initial denaturation at 94°C, 30 cycles of amplification were carried out, each consisting of 30 sec of denaturation at 94°C, 30 sec of annealing at 56°C and 2 min of primer extension at 72°C, followed by a final primer extension of 10 min at 72°C. For DGGE fingerprinting analysis the primers used were GC40-63f and 518r (El Fantroussi *et al*., 1999) and the PCR mixture was prepared in the PureTaq^{TM}Ready-To-Go^{TM} PCR beads tubes (GE healthcare, United Kingdom) in a final volume of 25 µL containing 1µl of DNA template and 25 pmol of each primer (Sigma-Aldrich, Steinheim, Germany). After 5 min of initial denaturation at 95°C, 30 cycles of amplification were performed, each consisting of 30 sec of denaturation at 95°C, 30 sec of annealing at 55°C and 1 min of primer extension at 72°C followed by a final primer extension of 5 min at 72°C. All the PCR amplifications were performed on an Eppendorf Mastercycler.

16S rRNA gene clone library and RFLP analysis.

Amplified 16S rRNA gene fragments from the UBF-Py microbial consortium after 30 days of incubation were examined on 0.8% agarose gels (Pronadisa, Conda, Madrid, Spain), purified using a Wizard®SV Gel and PCR Clean-Up system (Promega, Madison, USA) and cloned using the pGEM®-T Easy Vector System (Promega, Madison, USA). Transformants were selected by PCR amplification using vector PCR primers, the PCR products were purified, and inserts were digested separately with 2.5U of *Msp*I (Promega, Madison, WI) in 20µL reaction mixtures as recommended by the manufacter. Restriction products were electrophoresed in 1% agarose gels, stained with ethidium bromide and then photographed under UV light. DNA fragmentation profiles were compared and indistinguishable patterns were grouped as one Operational Taxonomic Unit (OTU) for further analyses. Clones corresponding to each different OTU were analyzed by nested PCR, including a first PCR using vector PCR primers and a second PCR using either eubacterial primers GC40-63F and 518R (for DGGE analysis) or eubacterial primers 27f or 1492r (for phylogenetic analysis) as described above.

DGGE analysis

The PCR products from the microbial consortium were examined on 1.5% agarose gels and directly loaded on 8% polyacrylamide gels with denaturing gradients ranging from

45% to 65% (100% denaturant contains 7M urea and 40% formamide). Electrophoresis was performed at a constant voltage of 100 V for 16H in 1X TAE buffer (40mM Tris, 20mM sodium acetate, 1 mM EDTA, pH 7.4) at 60ºC on a DGGE-2001 System (CBS Scientific, Del Mar, CA, USA) machine. The gels were stained for 30 min with 1 x SYBR Gold nucleic acid gel stain (Molecular Probes, Eugene, OR, USA) and photographed under UV light, using a Bio-Rad molecular imager FX Pro Plus multi-imaging system and Quantity-one version 4.5.1 image analysis software (Bio-Rad Laboratories, Hercules, CA, USA).

Sequencing and phylogenetic analysis

The PCR products from clone libraries and isolates were purified and sequenced with ABI Prism Bigdye Terminator cycle-sequencing reaction kit (version 3.1) using amplification primers 27f and 1492r, and, when necessary, internal primers 357f and 1087r (Lane, 1991). Sequencing reactions were obtained with an ABI prism 3700 Applied Biosystems automated sequencer at the Scientific-Technical Services of the University of Barcelona. DNA sequencing runs were assembled, aligned and manually adjusted using the BioEdit Software (Hall, 1999) and, then, they were analyzed with the CHIMERA CHECK program (RDPII) (Cole *et al*., 2003). The resulting DNA sequences were examined and compared with BLAST alignment tool comparison software (Altschul *et al*., 1997) and the classifier tool of the Ribosomal Database Project II at http://rdp.cme.msu.edu/ (Maidak *et al*., 2000).

Detection and phylogenetic analysis of PAH-ring hydroxylating dioxygenase genes

Oligonucleotide primers PAH-RHD$_\alpha$ GN F, PAH-RHD$_\alpha$ GN R, and PAH-RHD$_\alpha$ GP F, PAH-RHD$_\alpha$ GP R (Cébron *et al*., 2008) were used for specific amplification of PAH-ring hydroxylating dioxygenase alpha subunit genes of gramnegative (GN) and grampositive (GP) bacteria, respectively. DNA extracted from the UBF-Py microbial consortium or DNA from isolated strains were used as template for PCR amplification reactions using PureTaq^{TM}Ready-To-Go^{TM} PCR beads tubes, as described previously. After 5 min of initial denaturation at 95°C, 40 cycles of amplification were performed, each consisting of 30 sec of denaturation at 95°C, 30 sec of annealing at 52°C and 1 min of primer extension at 72°C followed by a final primer extension of 10 min at 72°C.

PCR products were examined in 1.5% agarose gels, purified and cloned in pGEM-T vector as described above. DNA sequences were obtained from recombinant plasmids using amplification primers, and then analyzed and compared to GenBank database by BLAST searches as previously described.

Results

Pyrene-degrading microbial marine consortium UBF-Py

The enrichment culture UBF-Py has been maintained by monthly transfers in artificial sea water and pyrene (0.2 g.L^{-1}) during 3 years, therefore it was considered a stable microbial consortium. After 30 days of incubation, UBF-Py reduced the concentration of pyrene in 34% respect abiotic controls. The analysis of the neutral or the acidic extracts from cultures did not show other detectable peaks, which is indicative of a complete degradation of the pyrene molecule.

Table 1. Enumeration of heterotrophic and pyrene degrading bacteria from the UBF-Pyr consortium during 1 month by the most probable number method.

Time (days)	Heterotrophic bacteria (mpn/ml)	Pyrene degrading bacteria (mpn/ml)
0	$3{,}3 \cdot 10^6$	$7{,}5 \cdot 10^4$
7	$5{,}8 \cdot 10^7$	$2{,}0 \cdot 10^7$
15	$3{,}8 \cdot 10^8$	$5{,}0 \cdot 10^7$
30	$1{,}9 \cdot 10^8$	$3{,}8 \cdot 10^6$

As shown in Table 1, the pyrene-degrading bacterial populations increased fast between the first 7 days of incubation (3 orders of magnitude), remaining approximately constant until day 15. Thereafter this population decreased in one order of magnitude, possibly due to exhaustion of available substrate. The sizes of the heterotrophic microbial populations remained 1 to 2 orders of magnitude larger than the pyrene-degrading population except at 7 days, when both populations presented similar sizes. Interestingly, the total heterotrofs remained constant until the end of incubation. This seems to indicate an important heterotrofic population unable to attack pyrene, but that somehow feeds on the carbon furnished by this compound being able to keep a high constant abundance throughout the transfers.

Mineralization of ^{14}C-pyrene and ^{14}C-phenanthrene by consortium UBF-Py and parallel microbial community dynamics

Pyrene-degrading soil bacterial isolates are usually capable of also utilizing the 3-ring PAH phenanthrene as growth substrate (Vila *et al.,* 2001). To determine the capability of degrading phenantrene of consortium UBF-Py and detect possible changes in the microbial community structure during the degradation of both compounds, a mineralization experiment was set-up in which the population dynamics were monitored by DGGE analysis. Generally, mineralization experiments are performed in acidified culture medium (i.e. pH 5) to facilitate the release of the CO_2 produced into the gas phase and the same cultures are monitored in a continuous manner (Tejeda-Agredano *et al.,* 2011). Here, the change in pH could have modified the structure of the microbial community and/or precipitate some of the seawater components, so mineralization was measured by withdrawing separate triplicate flasks for each data point and then acidifying the medium to release the CO_2 produced. The cumulative mineralization curves of ^{14}C-pyrene and ^{14}C-phenanthrene by UBF-Py are shown in Fig. 1. The relatively wide variability observed between some of the replicates (standard deviation) is explained by the method used.

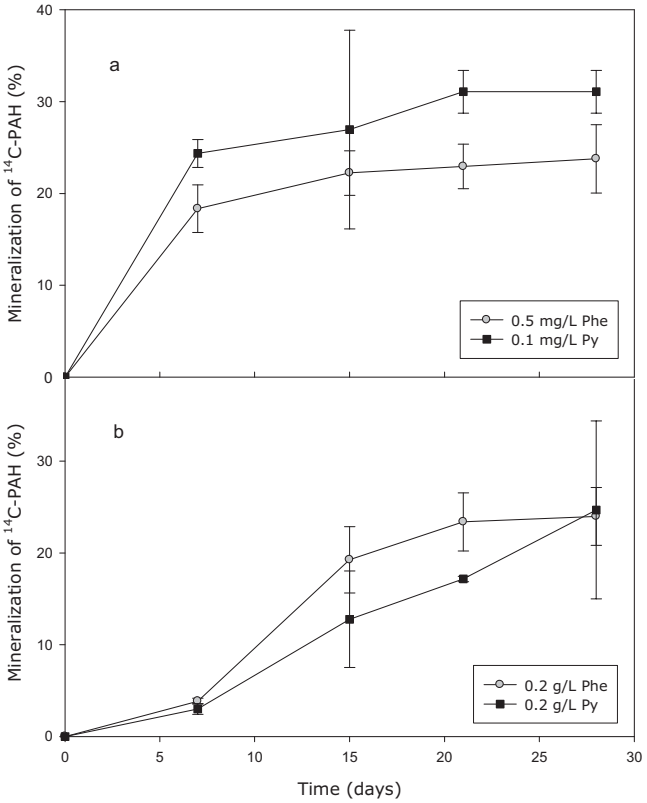

Fig. 1. Mineralization of ^{14}C-phenanthrene (Phe) and ^{14}C-pyrene (Py) (as $^{14}CO_2$) by UBF-Py consortium in a final concentration of 0.5 mgL-^{1} of phenanthrene and 0.1 mgL^{-1} of pyrene (a) and in a final concentration of 0.2gL^{-1} (b).

When the PAHs were supplied at concentrations below their water solubility, i.e. there were no bioavailability limitations and growth was insignificant. The inoculated pyrene grown cells mineralized pyrene and phenanthrene with similar first order-kinetics, although the final mineralization percentages for pyrene (31%) were slightly higher than those observed for phenanthrene (24%). When the PAHs were supplied at 0.2 mg.L^{-1}, the mineralization curves exhibited an S-shape, indicative of microbial growth (Alexander, 1999). In this case the mineralization of phenantrene was faster than that of pyrene, according to the higher bioavalability of the first, but the accumulated mineralization reached at 30 days was very similar for both compounds (24%). No mineralization was detected in sterile controls. Mineralization curves obtained in the same conditions for the known pyrene-degrader *Mycobacterium* sp. strain AP1 (Vila *et al*., 2001) showed similar values and profiles to those observed with UBF-Py (results not shown), thus indicating that the method to measure mineralization was reliable.

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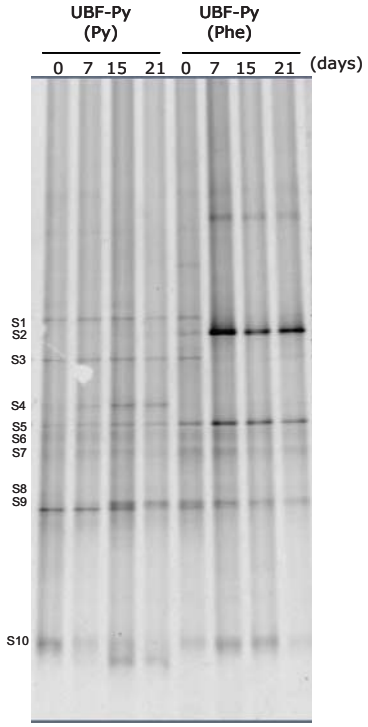

Fig 2. DGGE profile of PCR-amplified 16S rRNA gene fragments from two independent replicate cultures of the microbial consortium UBF-Py after 7, 15 21 and 30 days of incubation in pyrene and phenanthrene.

In parallel with mineralization experiments, replicates of the cultures containing 0.2 $mgL^{-1}$of the PAHs were incubated without the addition of the labeled compounds and were used for microbial community analyses. The DGGE fingerprints obtained from duplicate cultures and from the cultures with each PAH (pyrene or phenantrene) throughout the incubation time showed highly similar banding profiles indicating a high stability of the microbial community (Fig. 2)

The DGGE banding profiles from the UBF-Py cultures incubated with pyrene showed ten major bands (S1-S10) (Fig. 2). Most of these bands were also detected when the consortium was incubated with phenanthene, but their relative abundances presented a substantial shift, with two of them (S2 and S9) increasing dramatically.

Microbial community analysis of microbial consortium UBF-Py

To determine the microbial community structure of consortium UBF-Py, a 16S rDNA clone library was obtained just before its transfer. After analyzing 45 clones by RFLP, those with different restriction pattern were sequenced, and 5 distinct OTUs were found. The rarefaction curve reached a clear saturation, indicating that further sampling of the clone library would not reveal additional diversity (Fig S1). Sequence analysis of the clones, and the comparison of their mobility in DGGE respect the microbial consortium profile is shown in table 2. Table 2 shows the results of comparing the sequences of the different clones obtained with the genetic databases and their correspondence with the bands previously detected by DGGE.

Table 2. Sequence analysis of the clones and isolates selected from the consortium UBF-Pyr correlated to corresponding DGGE band.

DGGE Band	Clone (freq %)	Isolates	Fragment length (bp)	Sim (%)	Closest relative in GenBank database (accession no.)	Phylogenetic group
S1=S3		**UBF-P2** [a,b,c,d,e]	**1379**	**99**	***Alcanivorax* dieselolei Qtet3 (GU370129)**	***Alcanivoracaceae* (γ)**
S2		**UBF-P3** [d,e]	**1282**	**100**	***Novosphingobium* sp. TVG9-VII (JF706227)**	***Sphingomonadaceae* (α)**
S4		**UBF-P4** [e]	**1328**	**99**	***Novosphingobium* sp. 2PR51-13 (EU440981)***	***Sphingomonadaceae* (α)**
S5	**38**	**UBF-P1** [a,b,d,e]	**1481**	**92**	***Mycoplana* sp. G1100 (GU199002)**	**(α)**
S6	34		1452	99	*Thalassospira* sp. MAI8 (AB257194)	*Rhodospirollaceae* (**α**)
S7		**UBF-P5** [a]	**1350**	**99**	***Micrococcus* sp. MOLA 73 (AM990848)**	***Micrococcaceae (Actinobacteria)***
S8		**UBF-P6** [b]	**1211**	**99**	***Aurantimonas* sp. 5C.5 (HQ427427)**	***Aurantimonadaceae* (α)**
S9	**5**	**UBF-P7** [a,c,d]	**1413**	**99**	**Uncultured *Paracoccus* S-5m-1 (GU061852)**	***Rhodobacteraceae* (α)**
S10	16		1477	99	*Gordonia* sp. PETBA11 (JQ658415)	*Nocardiaceae (Actinobacteria)*
ND	**7**	**UBF-P8** [a,b,c,d,e]	**1433**	**100**	**Uncultured *Martelella* clone ctg_NISA120 (DQ396149)**	***Aurantimonadaceae* (α)**

Components of the microbial communities that have been isolated are indicated in bold. Isolation media: [a]natural seawater supplemented with LB, artificial seawater supplemented with [b]LB, [c]phenanthrene, [d]pyrene and [e]sorbitol.* All the closest relatives to this strain belong to genus *Sphingopyxis*. α and γ correspond to *alpha* and *gammaproteobacteria*, respectively

In an attempt to obtain the bacterial populations observed in the molecular analysis in pure culture, a variety of isolation media, including diluted complex media and minimal medium with different hydrocarbons, were assayed. Fifty-two different morphologies were selected and their almost complete 16S rRNA gene sequences were obtained, revealing the presence of only 8 indistinguishable sequences. Those isolates were designated UBF-P1 to UBF-P8 and the result of their comparison with databases is also shown in table 2 together with their correspondence with bands and clones.

The most frequently detected sequence in the clone library (Band S5, 38% of clones) matched that of a bacterial strain (UBF-P1) recovered from most of the complex and mineral media with phenantrene and pyrene assayed. This strain corresponded to a new genus within the *Cohaesibacteriaceae* family (*Alphaproteobacteria*) that has been described separately and named *Breoghania corrubedonensis* (Gallego *et al*., 2010). Following in abundance was a bacterium that would classify within the genus *Thalassospira* (S6, 34%) (*Rhodospirillaceae, Alphaproteobacteria),* and that was not obtained in pure culture. This was also the case of a representative of *Gordonia* (S10, 16%) (*Nocardiaceae, Actinobacteria*) detected likewise with a high frequency in the clone library. Other sequences found with lower frequencies presented best matches with uncultured representatives of *Martelella* (no band observed, 7%) (*Aurantimonadaceae, Alphaproteobacteria)* and *Paracoccus* (S9, 5%) (*Rhodobactereaceae, Alphaproteobacteria)*. The member of *Martelella* (UBF-P8) was recovered in pure culture from most isolation media utilized, while the *Paracoccus* (UBF-P7) was only recovered in seawater with LB1/10 and minimal medium with pyrene or phenanthrene.

Interestingly, five of the isolates obtained from UBF-Py were not observed during the molecular analysis; three were *Alphaproteobacterial* strains identified as *Novosphingobium* sp. UBF-P3 (*Sphingomonadaceae*), *Sphingopyxis* sp. UBF-P4 (*Sphingomonadeaceae*) and *Aurantimonas* sp. UBF-P6 (*Aurantimonadaceae*), whereas the other two belonged to the *Gammaproteobacteria, Alcanivorax* sp. UBF-P2 (*Alcanivoraceae),* and *Actinobacteria*, *Micrococcus* sp. UBF-P5 (*Micrococcaceae)*. As shown in Table 2, the closest relative found in GenBank for strain UBF-P4 was a strain identified as *Novosphingobium* sp., however, the RDP classification tool classified this strain within the genus *Sphingopyxis.*

As expected, none of the sequences detected in the UBF-Py 16S rDNA library had been observed as major components of the original fuel-degrading consortium UBF (Vila *et al*., 2010). However, the sequences of isolates *Paracoccus* sp. UBF-P7 and *Martelella* sp. UBF-P8, also detected as major components of the UBF-Py clone library, were indistinguishable from partial sequences found for previous isolates obtained from consortium UBF in pyrene and hexadecane plates. In addition, the sequence of the abundant clone S6, closely related to *Thalassospira*, was highly similar to that of an isolate recovered initially from a subculture of UBF in the aromatic fraction of fuel, but that was not possible to further maintain in culture (Vila *et al*., 2010).

Additional efforts focused on the isolation of the *Thalassospira* (S6) and *Gordonia* (S10), abundant components of UBF-Py according with the molecular analyses, resulted unsuccessful. The approaches included solid media based in artificial or filtered natural seawater combined with yeast extract, different hydrocarbons (hexadecane, naphthalene, phenanthrene, or pyrene), and single or complex carbon sources. Specific media for actinobacteria included a modified M3 medium (Rowbotham & Cross, 1977) and regular media with streptomycin. Alternatively, the dilution-to-extinction method was used as an attempt to obtain isolates or simpler consortia that allowed a functional study in liquid medium. Serial dilutions of consortium

UBF-Py were inoculated in artificial sea water and pyrene, but after incubation all the dilutions presenting growth showed DGGE fingerprints identical to that of the initial culture (Fig. S2).

Catabolic characterization of the bacterial strains isolated from UBF-Py

The 8 obtained isolates were inoculated in artificial seawater plates with and without yeast extract, and the surface of the agar was coated with a variety of single PAHs (fluoranthene, dibenzotiophene, acenaphthene, anthracene, naphthalene, phenanthrene, fluorene and pyrene), according to a method (Kiyohara *et al*., 1982) that has proven successful in characterizing numerous soil isolates. Although growth was visible for all the isolates, no clearing zones were detected. Since some studies have reported that some PAH degrading strains are unable to produce clearing in these conditions, growth of the strains was screened in liquid supplemented seawater with pyrene or phenanthrene crystals (0.2 gL^{-1}). None of the isolates showed significative growth in respect to controls without carbon source.

^{14}C-pyrene mineralization studies were also conducted with each isolate and the combination of the eight isolates obtained together. After 30 days of incubation, no $^{14}CO_2$ was detected in any of the cultures, indicating that the components of UBF-Py that were not recovered in culture media might be required for pyrene degradation.

PAH-ring hydroxylating dioxygenase (PAH-RHD$_\alpha$) genes analysis

PCR amplification of PAH-RHD$_\alpha$ genes from gramnegative and grampositive PAH-degrading bacteria was performed on DNA extracted from UBF-Py culture after 30 days of incubation. Positive results (presence of a PCR amplification product) were obtained only in reactions with the primers for the PAH-ring hydroxylating dioxygenase genes from grampositive bacteria. The PCR product (286 base pairs) was cloned into pGEMT Easy vector, and 20 clones were sequenced with vector-specific primers flanking the insert. All the DNA sequences obtained turned out to be repetitions of a unique DNA sequence, which was found to be closely related to NidA3 ring hidroxylating dioxygenase genes found in several pyrene degrading grampositive actinobacteria like that of *Mycobacterium vanbaalenii* PYR-1 (Kim *et al*., 2006) and *Mycobacterium* sp. AP1, a pyrene degrading strain isolated and extensively studied by our group. The similarity was even more evident when the deduced aminoacid sequences were compared (Fig. 3).

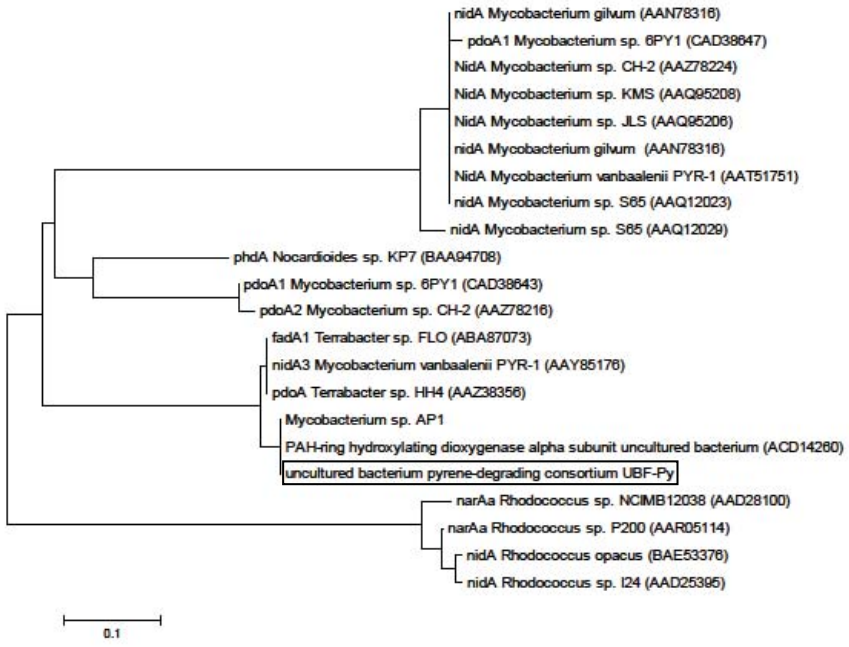

Fig 3. Phylogenetic neighbor-joining tree of ring hydroxylating dioxygenase amino acid sequences of UBF-Py consortium and reference strains taken from GenBank.

PCR amplification of PAH-ring hydroxylating dioxygenase genes from every single bacterial strain isolated from UBF-py consortium produced negative results, thus indicating that the detected dioxygenase gene most likely belongs to one of the non-isolated bacterial components of UBF-Py.

Discussion

Very little is known about the biological processes involved in the clean-up of contaminated marine environments. A great portion of hydrocarbon degraders could remain unknown since a large fraction of bacteria inhabiting marine environments have not been cultivated (Harayama *et al*., 2004). The objective of this work was to identify key players in the removal of pyrene from shorelines after a marine oil spill. The marine microbial consortium UBF-Py, derived from a fuel-degrading consortium inoculated with beach materials affected by the *Prestige* oil spill in Galicia, served as a model. Previous studies with marine pyrene-degrading consortia focussed on mixed cultures obtained from mangrove swamps (Guo *et al*., 2005), seaport, or oily saline wastewater samples (Aruzlazhagan & Vasudevan, 2009), therefore, it was to be expected that the reported microbial components differed considerably from those found here.

Microbial consortium UBF-Py removed 34% of the pyrene supplied as carbon source. This percentage is considerably inferior to that found for fuel-degrading consortium UBF growing on fuel (75%), however the concentration of pyrene in the fuel supplied was substantially lower and its bioavailability was higher due to the effect of the non-aqueous liquid phase (García-Junco *et al*., 2003). Pure cultures growing on pyrene as sole carbon source in similar concentrations accumulate partially oxydated

intermediates (Vila *et al*., 2001). The absence of this type of products in UBF-Py cultures indicates the cooperation of different microbial components in the mineralization of the substrate. The rapid mineralization of phenantrene by pyrene-grown cultures is consistent with the fact that pyrene-degrading strains are usually able to degrade phenanthrene (Vila *et al*., 2001), however, the dramatic change in community structure when UBF grew on phenanthrene suggest different catabolic functions for the discrete members of UBF-Py.

Molecular analyses showed a highly stable community composed mainly by *Alphaproteobacteria* (84%) and *Actinobacteria* (16%). The detected *Alpha-proteobacteria* included representatives of the newly described genus *Breoghania* (Gallego *et al*., 2010)*, Thalassospira*, *Paracoccus,* and *Martelella,* while the observed *Actinobacteria* belonged to *Gordonia*. The members of *Thalasosspira* and *Gordonia* were not recovered as pure cultures, but isolation procedures produced five additional strains not detected in the molecular analysis that classified within the genera *Novosphingobium, Sphingopyxis* and *Aurantimonas (Alphaproteobacteria), Alcanivorax (Gammaproteobacteria)* and *Micrococcus (Actinobacteria).*

Almost all the detected *Alpha-Proteobacteria* genera have been related with oil or PAH-mixtures degradation, but there is little evidence of them utilizing pyrene for growth. *Novosphingobium* and *Thalassospira* have been reported as main components in microbial consortia from deep sea sediment growing on a mixture of phenanthrene and pyrene and the corresponding isolates were apparently able to degrade 2-4 ring PAHs (Cui *et al*., 2008; Yuan, 2009). In fact, the high increase in the relative abundance of *Novosphingobium* when UBF-Py was growing in phenanthrene, initially suggested that this species may trigger the degradation of this PAH. Jimenez *et al*. (2011) identified several members of *Thalassospira* in a fuel degrading marine consortium but related this genus to alkane degradation as other authors did in previous marine studies (Hara *et al*., 2003; McKew *et al*., 2007; Kodama *et al*., 2008). *Paracoccus* has been found in several PAH-degrading microbial communities both in soils and mangrove sediments and its ability to degrade *n*-alkanes and PAHs, including pyrene, has been demonstrated (Guo *et al*., 2010; Guo *et al*., 2005; Teng *et al*., 2010 Zhang *et al*., 2004). Species of *Martelella*, also a recently described genus (Rivas *et al*., 2005) have been isolated from oil degrading consortia from superficial sea waters but their action on PAHs has not been demonstrated (Wang *et al*., 2010).

The *Gammaproteobacteria Alcanivorax* has been reported as an obligate hydrocarbon-degrading bacterium able to only use alkanes (up to C_{32}) and long-chain isoprenoids for growth, and attacking the alkyl groups of *n*-alkylbenzenes and *n*-alkylcycloalkanes (Yakimov *et al*., 1998; Dutta & Harayama, 2001). No PAH-degradation has been reported for this genus. Interestingly, we have recovered the *Alcanivorax* member of UBF-Py in artificial sea water agar with diluted LB, and maintain this strain in the same media, which indicates that his spectrum of substrates may not as reduced as reported. It is also worth to note that this strain was detected as two bands with different mobility in DGGE but with identical sequence, this suggesting that the general attribution of bands with different mobility to different populations should be carefully considered. To resume the discussion on the previously reported capabilities of the identified populations, *Actinobacteria* are typical soil HMW PAH-degraders, with *Mycobacterium* and *Rhodococcus* being the most frequently pyrene-degrading isolates (Kanaly & Harayama, 2010). However, species of *Micrococcus* and *Gordonia* able to degrade a variety hydrocarbons have been isolated from mangrove sediments (Santos *et al*., 2011), a member of *Micrococcus* was detected in an oil-degrading soil consortia able to attack a variety of PAHs (Silva-Castro *et al*., 2011); and a strain of *Gordonia* sp. was isolated from a oil-degrading sludge and proven to grow on pyrene (Jacques *et al*., 2007; Jacques *et al*., 2008). *Gordonia* is also known as a

biosurfactant producer (Franzetti *et al*., 2007) and degrades long-chain alkanes which would favour its presence in a consortium initially enriched with fuel.

None of the isolates were able to degrade pyrene or other PAHs in pure cultures and the PCR amplification of Gram-positive and Gram-negative dioxygenases did not produce results. This could indicate that the capability of attacking pyrene in the UBF-Py microbial consortium relied in the non-culturable members of *Thalassospira* or *Gordonia.* In fact, when the UBF-Py consortium was screened for dioxygenases a PAH ring-hydroxylating dioxygenase closely related to the NidA3 pyrene dioxygenase undistinguishable from that detected in *Mycobacterium* sp. AP1 in the same conditions, was demonstrated. This would point out to the representative of *Gordonia* as the key pyrene degrader in the consortium. The results obtained in the dilution-to-extintion experiments showing identical DGGE fingerprint in all the dilutions and in the original culture revealed that all the components are necessary for the consortium to grow on pyrene. Therefore we could hypothesize that for *Gordonia* to attack pyrene products produced by the other bacterial components (i.e. growth factors or chemical signals) necessary. These other components would grow as secondary degraders obtaining their energy and cell components from carbon furnished by pyrene, the sole carbon source, after been broken down by *Gordonia*. It is true that the presence of other dioxygenases not detectable in the conditions of the experiment could not be ruled out, and that possible PAH catabolic capabilities of some of the isolates could have been missed, especially those of the uncultured *Thalassospira.* However, our results clearly indicate high dependence and cooperation in the removal of pyrene by marine communities, and that the key degrading bacteria (those that initiate the attack on pyrene) do not need to be the most abundant. In support to this is the clear oligotrophy shown by all the isolates.

A number of recent works have lead to the assumption that as *Alcanivorax* is a key alkane-degrader in marine environments, *Cycloclasticus* is a key PAH degrader (Kanaly &Harayama, 2010). An increase in the relative abundance of *Cycloclasticus* has been correlated with PAH degradation in enrichment cultures and natural environments, and strains belonging to this genus have been demonstrated to degrade pyrene. In this work *Cycloclasticus* was not detected and neither was in our previous study on the heavy fuel (50% PAHs) -degrading consortium UBF. More efforts are needed to reveal the catabolic functions held by marine bacteria that remain uncultured and new methods should be developed for isolation of PAH- degrading marine bacteria.

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Supplementary Material

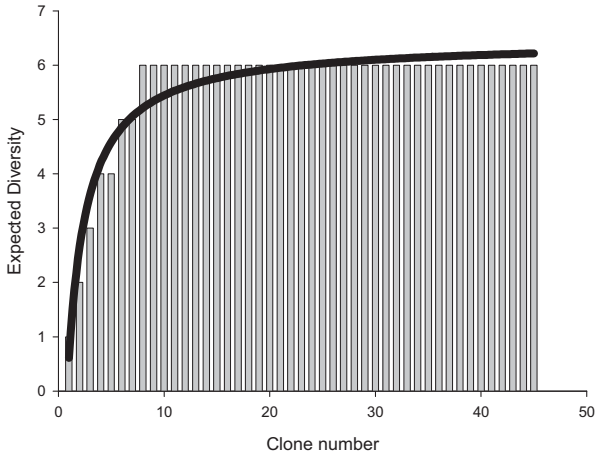

Fig S1. Rarefaction curve for clone library from UBF-Pyr consortium. The expected number of RFLP patterns (OTUs) is plotted vs. the number of clones sampled.

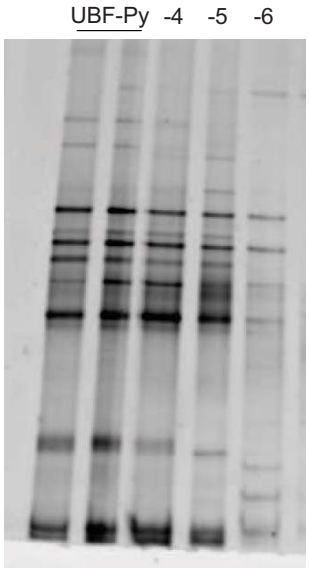

Fig S2. DGGE profile of PCR amplified 16S rRNA gene fragments from original and diluted cultures (dilution -4, -5 and -6) of the microbial consortium UBF-Py after 30 days of incubation in pyrene.

Breoghania corrubedonensis gen. nov. sp. nov., una nueva *alphaproteobacteria* aislada de una playa de Galicia (NO de España) tras el vertido de fuel oil del Prestige, y enmienda a la descripción de la familia *Cohaesibacteraceae* y de la especie *Cohaesibacter gelatinilyticus*

Se ha aislado una bacteria gram-negativa, designada UBF-P1^T a partir de un enriquecimiento establecido en agua de mar artificial, suplementado con nutrientes y con pireno como única fuente de carbono, e inoculado con un consorcio marino degradador de fuel oil obtenido a partir de una muestra de arena recogida en una playa de Corrubedo (A Coruña, Galicia, España) después del vertido accidental del Prestige. Los análisis filogenéticos basados en la secuencia casi completa del gen 16Sr ARNr afiliaron la cepa UBF-P1^T con la familia *Cohaesibacteraceae*, siendo *Cohaesibacter gelatinilyticus* (DSM 18289^T) la especie más cercana con un 92% de similitud en su secuencia. Las células fueron bacilos irregulares, móviles, estrictamente aerobios, catalasa y oxidasa positivos. La lipoquinona respiratoria más abundante fue la ubiquinona 10. Entre los lípidos polares, los más abundantes fueron difosfatidilglicerol (DFG), fosfatidilglicerol (FG), fosfatidiletanolamina (FE), fosfatidilmonometiletanolamina (FME), y fosfatidilcolina (FC). Los ácidos grasos más abundantes detectados fueron $C_{18:1\omega7_c}$, $C_{19:0\ cyclo\omega_{8c}}$, y $C_{16:0}$. El contenido de G+C de la cepa UBF-P1^T fue el 63.9% molar. La comparación taxonómica con el relativo más cercano basada en características genotípicas, fenotípicas y quimiotaxonómicas confirmaron que la cepa UBF-P1^T podía ser clasificada como un género nuevo y especie, para el cual el nombre *Breoghania corrubedondnesis* gen. nov. sp. nov. fue propuesto. La cepa tipo de este nuevo taxón es UBF-P1^T (CECT 7622, LMG 25482, DSM 23382).

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Breoghania corrubedonensis gen. nov. sp. nov., a novel *alphaproteobacterium* isolated from a Galician beach (NW Spain) after the Prestige fuel oil spill, and emended description of the family *Cohaesibacteraceae* and the species *Cohaesibacter gelatinilyticus*

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ABSTRACT

A Gram-negative bacterium designated UBF-P1^T was isolated from an enrichment culture established in nutrient supplemented artificial sea water with pyrene as a carbon source, and inoculated with a marine fuel oil-degrading consortium obtained from a sand sample collected from the beach of Corrubedo (A Coruña, Galicia, Spain) after the Prestige accidental oil spill. Phylogenetic analysis based on the almost complete 16S rRNA gene sequence affiliated strain UBF-P1^T with the family *Cohaesibacteraceae*, *Cohaesibacter gelatinilyticus* (DSM 18289^T) being the closest relative species with 92% sequence similarity. Cells were irregular rods, motile, strictly aerobic, catalase and oxidase positive. Ubiquinone 10 was the major respiratory lipoquinone. The major polar lipids comprised diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylmonomethylethanolamine (PME), and phosphatidylcholine (PC). The major fatty acids detected were $C_{18:1}\omega7c$, $C_{19:0}$ cyclo$\omega8c$, and $C_{16:0}$. The G + C content of strain UBF-P1^T was 63.9 mol%. The taxonomic comparison with the closest relative based on genotypic, phenotypic and chemotaxonomic characteristics supported that strain UBF-P1^T could be classified as a novel genus and species, for which the name *Breoghania corrubedonensis* gen. nov., sp. nov. is proposed. The type strain of this new taxon is UBF-P1^T (CECT 7622, LMG 25482, DSM 23382).

Bergey's Manual describes the order *Rhizobiales* as a phenotypically heterogeneous assemblage of Gram-negative bacteria, solely based on 16S rRNA gene sequence analysis [13]. The family *Cohaesibacteraceae* [8] has been recently proposed, and includes a single genus and species, *Cohaesibacter gelatinilyticus*, formed by strains from a coastal marine environment. The description of the novel family was mainly based on the fact that phylogenetic analysis (16S rRNA) revealed a distinct lineage that did not cluster with any of the previous families. In addition, *Cohaesibacter* could be differentiated from members of other families by a combination of chemotaxonomic characteristics, including a DNA content of 53% G + C, Q-10 as the sole major respiratory quinone, polar lipid composition, and a few minor phenotypic characters (e.g. presence of gelatinase activity, nitrate reduction, urease activity and lack of arginine dihydrolase). In this study, we propose a new genus within the family *Cohaesibacteraceae* for a bacterial strain obtained from a beach in the northwest of Spain after the Prestige oil spill in 2002, and additionally investigate its catabolic capabilities for hydrocarbon degradation.

Following the oil spill, an autochthonous fuel oil-degrading microbial mixed culture (UBF) was obtained that was used in a series of studies that evaluated the efficacy of different microbial inocula in the bioremediation of the contaminated shoreline [5,6]. To obtain this culture, a sand sample from the beach of Corrubedo (A Coruña, Galicia, Spain), heavily polluted with fuel from the Prestige incident, was used to inoculate an enrichment medium based on artificial sea water (ASW) (per litre: NaCl, 24.53 g; Na_2SO_4, 4.11 g; $MgCl_2$, 11.2 g; $CaCl_2$, 1.16 g; $SrCl_2$, 0.042 g; KCl, 0.7 g; $NaHCO_3$, 0.2 g; KBr, 0.1 g; H_3BO_3, 0.0029 g; NaF, 0.003 g) supplemented with nutrients (NH_4NO_3 10 mM and Na_2HPO_4 1 mM) and metals (per litre: $MgSO_4{\cdot}7H_2O$, 0.2 g; $FeSO_4{\cdot}7H_2O$, 12 mg; $MnSO_4{\cdot}H_2O$, 3 mg; $ZnSO_4{\cdot}7H_2O$, 3 mg; $CoCl_2{\cdot}6H_2O$, 1 mg; nitrilotriacetic acid disodium salt, 0.123 g), and containing fuel recovered from the sea as a sole source of carbon and energy (5 g L^{-1}). Incubation was at 25 °C and 200 rpm. According to a recent study [30], after several years of biweekly transfers the UBF culture comprises

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a stable microbial consortium capable of extensive degradation of all the neutral components of fuel, including high molecular weight alkanes, PAHs and alkylated derivatives. To further characterize its components, UBF was used to inoculate (500 μL) a subculture in the same marine mineral medium (25 mL) containing the polycyclic aromatic hydrocarbon pyrene as a carbon source (0.2 g L^{-1}), and, after 2 years of monthly transfers (1:50) serial dilutions of this pyrene degrading subculture were spread onto plates containing a 1/10 dilution of Luria Bertani medium (final concentration per litre; peptone 1 g, yeast extract 0.5 g) prepared in artificial sea water (LB 1/10 ASW) and solidified with agar (15 g L^{-1}). The plates were incubated at 25 °C for 15 days. Five colonies corresponding to one of the most abundant morphologies were purified in the same medium. To visualize whether the isolates constituted different populations, a RAPD analysis was performed using primers RAPD1 and RAPD2, according to Peña et al. [18], and also their 16S rRNA gene was sequenced as explained below. RAPD analysis revealed identical amplification patterns of all five strains, which indicated that they all represented a single clonal population, probably selected through the various reinoculation steps. A single strain that was named UBF-P1 was selected for further taxonomic characterization. After 1 week of incubation, the colonies were circular, mucoid, cream coloured, and measured about 1 mm in diameter. The different clonal variants of the strain were maintained in culture under the same conditions, but were also stored at −80 °C (in LB 1/10 ASW with 20% glycerol). Strain UBF-P1 grew on full strength LB medium with either ASW or 3% NaCl, R2A medium (Difco) with 3% NaCl, and Marine Agar (Difco). A separate study, including 16S rDNA PCR-DGGE and clone library analysis of the pyrene degrading mixed culture from where strain UBF-P1 was isolated, confirmed that UBF-P1 was the most abundant component [30].

The almost complete 16S rRNA gene of strain UBF-P1 was amplified by using *Taq* DNA polymerase (Biotools B&M Labs, Madrid, Spain) and the bacterial universal primers 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1521r (5′-AAGGAGGTGATCCAGCCGCA-3′) [31]. The PCR product was purified by using the Qiaex II Extraction kit (Qiagen, Hilden, Germany), and sequenced with the ABI Prism BigDye Terminator cycle-sequencing reaction kit (version 3.1), following the manufacturer's instructions. The reactions were performed using the amplification primers 27f and 1521r, but also internal primers 357f (5′-ACTCCTACGGGAGGCAGCAG-3′) and 1087r (5′-CTCGTTGCGGGACTTAACCC-3′). Sequencing reactions were obtained with an ABI Prism 3700 Applied Biosystems automated sequencer at the Scientific-Technical Services of the University of Barcelona. DNA sequencing runs were assembled using the BioEdit software [7]. The new sequence with the accession number GQ272328 was added to the updated and prealigned 16S rRNA gene database (LTP_s100, http://www.arb-silva.de/projects/living-tree/) compiling all sequences of all type strains for which an entry of high quality has been found [33]. The sequence was aligned by using the ARB software package [14] (http://www.arb-home.de), and manually improved. The tree reconstructions (Supplementary Figures S1–S4) were performed by either using the neighbour joining program, as implemented in the ARB software package, or by using the RAxML algorithm version 7.0 with the GTRGAMMA model [22]. In all cases, the tree reconstructions were performed by using a sequence conservation filter of 40% for all *Bacteria*. For the reconstructions, the closest alphaproteobacterial type strains with a fully sequenced genome available in the public repositories (Supplementary Figures S1 and S3) were used. Alternatively, the sequences with highest similarity not present in the genome repositories were included (Supplementary Figures S2 and S4). The closest relative sequence, with 92% sequence identity, was in all cases that of *Cohaesibacter gelatinilyticus* DSM 18289^T, that affiliated together with our isolate in all neighbour joining reconstructions. However, maximum likelihood reconstructions rendered trees where the new isolate always appeared independently affiliated within the *Alphaproteobacteria*. In no case could UBF-P1 be affiliated with a classified genus. The value of 92% identity with its close relative was clearly below the mean (96.4% ± 0.2) or minimum identity (94.9 ± 0.4) used to differentiate a single genus [33], thus it appeared plausible to conclude that strain UBF-P1 belongs to a new genus within the *Alphaproteobacteria*.

Further phylogenetic, genetic and phenotypic studies on strain UBF-P1 were performed in parallel with its closest relative, the type strain of *C. gelatinilyticus* (DSM 18289^T) obtained from DSMZ (Braunschweig, Germany). To clarify the affiliation of both isolates, a multilocus sequence analysis (MLSA) was performed by concatenating the genes *atp*D (ATP synthase F1, beta subunit), *pyr*G (CTP synthase), *rpo*B (RNA polymerase beta subunit), *fus*A (translation elongation factor G), and 16S rRNA, or, alternatively, by omitting the 16S rRNA gene sequences. The partial sequences of each gene for strain UBF-P1, and for the type strain of *C. gelatinilyticus* DSM 18289^T, were obtained by using the primers, amplification conditions, and sequencing approach described elsewhere [28]. The accession numbers for the newly sequenced partial genes are FN598910 to FN598917. For the phylogenetic reconstruction, the homologous genes were selected only from the collection of type strains of the *Alphaproteobacteria* for which a fully sequenced genome was available [20]. The homologous sequences were retrieved from the public repository MicrobesOnline [1], and each gene was independently aligned by using the program Clustal X 1.83 [24]. Hypervariable and unalignable positions of each alignment were removed by using the available online program Gblocks (http://molevol.cmima.csic.es/castresana/Gblocks_server.html) and the default conditions [2]. The resulting improved alignments were concatenated in a single dataset (Supplementary Table S1). The phylogenetic analyses were performed by using the RAxML algorithm version 7.0 with the GTRGAMMA model [22]. The bootstrap analyses were carried out by using 100 replicates. As shown in Fig. 1, the concatenated five partial sequences, using only highly conserved positions, reconstructed a very stable tree where most of the bootstrap values were close to 100%. Both strains UBF-P1 and *C. gelatinilyticus* DSM 18289^T affiliated together consistently. Nearly identical results were obtained when analysing the concatenate with the omission of the 16S rRNA gene sequence (Supplementary Figure S5). The identity of both strain-concatenates ranged from 87.8% to 85.6%, depending on whether or not the 16S rRNA gene sequence was included. The results of the isolation of strain UBF-P1 within the fully sequenced *Alphaproteobacteria* resulted in agreement with the observations made after the reconstruction of the 16S rRNA (Supplementary Figures S1–S4). In both cases, the phylogenetic divergences were in agreement with the classification of UBF-P1 as a new alphaproteobacterial genus.

According to the results (i.e. a 92% 16S rRNA gene sequence identity to the type strain of the type genus of the family, and the stable affiliation of the concatenates after MLSA) the new isolate seemed to fall into the recently proposed family *Cohaesibacteraceae* [8]. However, the 16S rRNA gene sequence of UBF-P1 did not share the signature positions 194 (G instead of T), 678 (T instead of A), 712 (A instead of T) given for the family [8], nor the 1244 (G instead of A) or 1293 (C instead of T) positions. The signatures found in this study more closely resembled the majority of the other families of the order. Consequently, these observations led to the reliability of signature positions for circumscribing families to be questioned.

Morphological and physiological tests for both strains studied were carried out as follows: Gram staining was performed as described by Reddy et al. [19]. Cellular morphology and the presence of flagella were observed using transmission electron microscopy (GEOL JEN-1010, operating at 80 kV). Images were

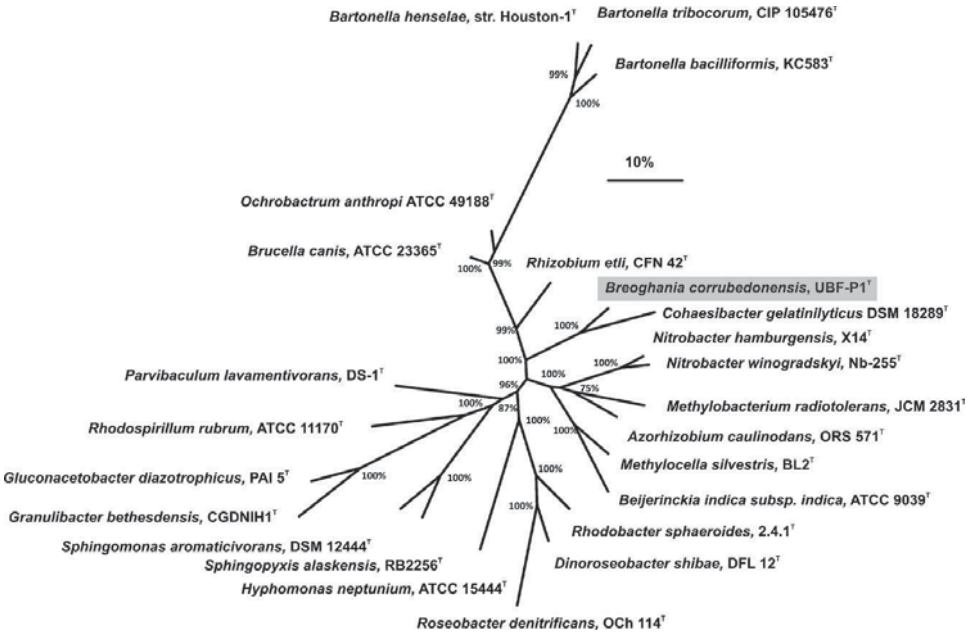

Fig. 1. MLSA phylogenetic reconstruction based on concatenated genes *atp*D, *pyr*G, *rpo*B, *fus*A, and 16S rRNA of strain UBF-P1^T, the type strain of *Cohaesibacter gelatinilyticus* DSM 18289^T, and the selection of type strains of the *Alphaproteobacteria* for which fully sequenced genomes are available in the public repositories. The tree was reconstructed by using the RAxML algorithm version 7.0 with the GTRGAMMA model [22]. The bootstrap analysis was carried out by using 100 replicates. The bar indicates 10% alignment divergence. The similarity of the concatenated sequences of UBF-P1^T and the type strain of *Cohaesibacter gelatinilyticus* DSM 18289^T is 87.8%.

taken with a Megaview camera III (Soft Imaging System SIS). Motility was determined by the hanging drop method [19]. Anaerobic growth was checked on LB 3% NaCl and in Marine Agar using the GasPak anaerobic system incubated both in natural light and the dark. The temperature range for growth was determined on the basis of both OD_{600} increment in liquid LB 3% NaCl (7 days incubation) and colony formation on LB 3% NaCl plates (20 days) incubated at 4, 10, 15, 21, 25, 30, 37, 40, 42 and 45 °C. The pH range (pH 4.5–10 at intervals of 0.5 units) for growth was determined in LB 3% NaCl liquid medium. To test salt tolerance, liquid LB containing various concentrations of NaCl [0, 0.5, 1, 3, 5, 7, 8.5, 10, 15 and 20% (w/v)] was used. Catalase and oxidase activities were determined according to the protocols of Smibert and Krieg [21]. Nitrate and nitrite reduction, the production of indole, arginine dihydrolase, urease, gelatinase, and the hydrolysis of aesculin were tested using the API 20NE kit (bioMérieux), according to the manufacturer's instructions, except that the colonies used as inocula were suspended in a 3% NaCl aqueous solution (w/v). Utilization of different organic compounds as carbon sources was tested in ASW supplemented with mineral nutrients and yeast extract (25 mg L^{-1}), containing each of the test compounds at 5 mM except for complex substrates (yeast extract and casaminoacids, 1 g L^{-1}), starch (0.1 g L^{-1}), hydrocarbons (0.5 g L^{-1}), or solvents (methanol and acetone, 50 mM). After 20 days incubation, growth was considered positive when the cell protein concentration in cultures (determined using a modification of the Lowry method [3]) was three fold that found for cultures without the test compound. All phenotypic assays (except the temperature range for growth) were carried out at 30 °C, which was the optimum temperature for growth of the strain. The tests for hydrolysis of DNA, starch and Tween 80 were performed according to Reddy et al. [19] by the Identification Services of DMSZ, Braunschweig, Germany.

The fatty acid methyl ester, polar lipid, respiratory quinone, and DNA base composition analyses were carried out at the DSMZ. Fatty acid methyl esters were obtained from whole cells grown on Marine Agar (Difco) at 25 °C for 3 days using the methods of Miller [15] and Kuykendall et al. [12], and were analysed by GC using the Sherlock Microbial Identification System (MIS) (MIDI, Microbial ID, Newark, DE). The analyses of polar lipids (2D-TLC) and respiratory quinones (TLC-HPLC) were performed by Dr. B.J. Tindall [26,27]. The DNA G+C content was determined according to Mesbah et al. [16], and Tamaoka and Komagata [23]. DNA was enzymically hydrolysed and dephosphorilated, and the resultant nucleosides were analysed by HPLC.

The results showed that strain UBF-P1 was a Gram-negative bacterium forming regular, irregular or bulbous rods, motile by one or two subpolar flagella, and approximately 0.6–0.7 μm wide and 2–3.5 μm long, that reproduced by asymmetric division (Fig. 2 and Supplementary Figure S6). The morphological, physiological and biochemical characteristics found for strain UBF-P1 are given in the corresponding genus and species descriptions. Given the phenotypic heterogeneity within each of the different families forming the order [8,13] it was not easy to differentiate strain UBF-P1 from the rest of the families on this basis. However, strain UBF-P1 could be differentiated from its closest relative genus, *Cohaesibacter*, by means of nitrite reduction, gelatine hydrolysis, ADH, ODC, and urease tests (Table 1). Other biochemical differences are shown in the supplementary material (Supplementary Table S2). It is also important to notice that our results for strain DSM 18289^T showed some differences compared to the original descrip-

Table 1
Distinctive characteristics of strain UBF-P1^T and *C. gelatinilyticus* DSM 18289^T. Unless indicated, the tests were simultaneously performed using both strains.

Characteristic	Bacterial strain	
	UBF-P1^T	*C. gelatinilyticus* CL-GR15^T
Cell shape	Regular, curved, bulbous rods	Irregular rods[a]
Cell size (mm)	0.6–0.7 × 2.0–3.5	0.2–0.4 × 1.0–3.0[a]
Division type	Binary fission or asymmetric division	Binary fission or asymmetric division[a]
Motility	+	+
Flagella	1 or 2, subpolar	Polar[a]
Growth temperature (°C)		
Optimal	30	25–30
Range	15–40	15–31
NaCl range for growth (%)		
Range	1–10%	2–5%
Growth pH		
Optimal	7.5	8.0
Range	5.0–8.5	6.0–9.0
Oxygen requirement	Aerobic	Facultatively anaerobic
Catalase	+	+
Oxidase	+	+
Nitrate reduction	+	+[b]
Nitrite reduction	–	+[b]
Gelatine hydrolysis	–	+
ADH test	+	–
Urease	+	–
G + C content (mol%)	63.9	52.8–53
Major quinone	Q-10	Q-10
Major fatty acid(s) (>10%)	$C_{18:1}\omega7c$ (75.3%)	$C_{18:1}\omega7c$ (58.8); Summed feature 3[c] (20.8)
Major polar lipids	Diphosphatidylglycerol (DPG) Phosphatidylglycerol (PG) Phosphatidylethanolamine (PE) Phosphatidylmonomethylethanolamine (PME) Phosphatidylcholine (PC)	Diphosphatidylglycerol (DPG) Phosphatidylglycerol (PG) Phosphatidylethanolamine (PE) Phosphatidylmonomethylethanolamine (PME) Phosphatidylcholine (PC)

[a] Data reported by Hwang and Cho [8].
[b] Our results differ from the previously reported data by Hwang and Cho [8].
[c] Feature 3 contains one or more of $C_{16:1}$ ω 7c and/or $C_{15:0}$ ISO 2-OH.

tion, and these are indicated in the same tables. The cellular fatty acid profile of UBF-P1 (Table 2) was characterized by $C_{18:1}\omega7c$ (75.3%), while other fatty acids found in smaller amounts were $C_{19:0}$ cycloω8c (6.4%) and $C_{16:0}$ (4.4%). This predominance of octadecenoic acids together with a cyclic $C_{19:0}$ fatty acid is typical of the *Rhizobiales* [4,9,11,17,25,32]. *Cohaesibacter* also had $C_{18:1}\omega7c$ as a major fatty acid, but, in addition, it showed considerable amounts of $C_{16:1}$ $\omega7c$ and/or $C_{15:0}$ iso 2-OH (20.8%) and $C_{20:1}$ $\omega7c$ (9.2%), while $C_{19:0}$ cycloω8c was detected in a very low concentration (0.6%). The polar lipid content (Table 1 and Supplementary Figure S7) was similar in both strains, including diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylmonomethylethanolamine (PME), and phosphatidylcholine (PC) as major components. The respiratory lipoquinone was Q-10, which is present in all the families of the order. The DNA G + C content of UBF-P1 was 63.9 mol%, which is more than 10% higher than that of *Cohaesibacter*, but falls within the variability of the members of the order [8].

Finally, a matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis was performed

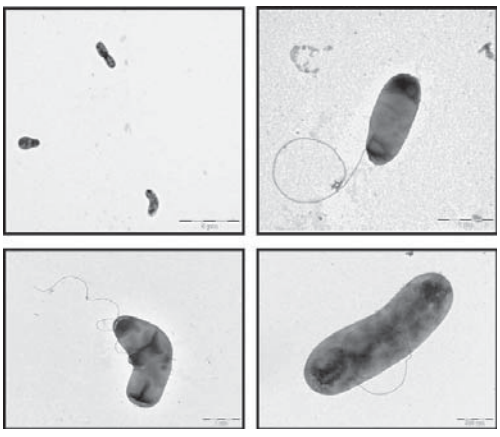

Fig. 2. Transmission electron micrographs of negatively stained cells of strain UBF-P1^T.

Table 2
Fatty acid composition of strain UBF-P1^T and *C. gelatinilyticus* DSM 18289^T. Values are percentages of total fatty acids. Cells were grown on Marine Agar at 25 °C. The analyses were simultaneously performed on both strains.

Fatty acid	Content (%)	
	UBF-P1^T	*C. gelatinilyticus* CL-GR15^T
Summed feature 2[a]	0.9	2.5
Summed feature 3[a]	2.5	20.8
$C_{16:0}$	4.4	0.7
$C_{17:1}$ ω6c	0.5	–
$C_{17:0}$	0.9	–
$C_{18:1}$ $\omega7c$	75.3	58.8
$C_{18:0}$	2.2	3.0
$C_{19:0}$ cyclo ω8c	6.4	0.6
$C_{19:0}$ 10 methyl	–	0.7
$C_{18:0}$ 3-OH	2.4	2.5
$C_{20:1}$ ω7c	–	9.2
$C_{20:1}$ ω9c	1.3	–
Other[b]	3.3	1.1

[a] Summed features contain one or more of the following: feature 2, $C_{14:0}$ 3-OH and/or $C_{16:1}$ ISO I; feature 3, $C_{16:1}$ $\omega7c$ and/or $C_{15:0}$ iso 2-OH.
[b] Sum of identified components that made up less than 0.5% or unknown (less than 2%).

on UBF-P1 and *C. gelatinilyticus* DSM 18289^T in order to evaluate the discrimination power of this technique. The cells were grown on LB solid medium until colonies of about 1 mm in diameter were visible. For mass spectral analyses, a small amount of cells (10^5 to 10^6 cells) was transferred to a FlexiMass stainless steel target with a sterile pipette tip. The cells were extracted on the target with 1 μL matrix solution consisting of a saturated solution of α-cyano-4-hydroxy-cinnamic acid in a mixture of acetonitrile:ethanol:water (1:1:1) acidified with 3% (v/v) trifluoroacetic acid. The suspension of cells in the matrix solution was allowed to evaporate at room temperature and crystal formation was observed visually. For each strain, mass spectra were prepared in duplicates and analysed on an AXIMA Confidence instrument (Shimadzu/Kratos, Manchester, UK) in linear positive ion extraction mode. Mass spectra were accumulated from 100 profiles each from five nitrogen laser pulse cycles, scanning the entire sample spot. Ions were accelerated with pulsed extraction at a voltage of 20 kV. Raw mass spectra were processed automatically for baseline correction and peak recognition. As shown in Supplementary Figure S8, both strains had a completely different profile for the major detectable macromolecules, and the profiles have been stored in the SARAMIS reference spectra database for future identification [http://qww.anagnostec.de; [10]].

All the phylogenetic, genetic and phenotypic data analysed in this study indicated that strain UBF-P1 clearly isolated within the order *Rhizobiales*, and affiliated with the family *Cohaesibacteraceae* [8]. However, the dissimilarities with the type strain of the closest genus *C. gelatinilyticus* DSM 18289^T, makes it plausible to circumscribe our isolate as a new genus and species. For this reason, we propose *Breoghania corrubedonensis* gen. nov. sp. nov., as a novel member of the order *Rhizobiales*. The strain UBF-P1^T has been deposited in three international culture collections with the numbers CECT 7622, LMG 25482, DSM 23382.

In an attempt to understand the role that strain UBF-P1^T may play in the degradation of pyrene by the enrichment culture from which it was isolated or in the degradation of fuel by the original marine microbial consortium UBF [30], several hydrocarbons and bacterial metabolites from polycyclic aromatic hydrocarbons were assayed as carbon sources. Strain UBF-1^T was not able to grow on the hydrocarbons hexadecane, pyrene or phenanthrene, the pyrene metabolites 4-phenanthrene carboxylic acid and phthalic acid, or the phenanthrene metabolite diphenic acid, typically produced during the degradation of these PAHs by soil bacteria [29]. However, growth was positive for protocatechuic acid, a common product formed from phthalate in the metabolic pathways for the degradation of a number of aromatic hydrocarbons by bacteria, including pyrene. These results, together with the capacity for utilizing several small organic acids, indicated that in the enrichment cultures with pyrene or fuel, strain UBF-P1^T did not participate in the initial attack of hydrocarbons, but grew on acidic compounds resulting from the degradation of pyrene or other hydrocarbons by different bacterial populations present in these microbial communities.

Description of *Breoghania* gen. nov

Breoghania (Bre.o.gha'ni.a. N.L. fem. n. *Breoghania*, named after *Breoghan*, according to Celtic mythology (*Leabhar Ghabhala*, XII century), the first Celtic king of *Gallaecia* (actual Galicia), founder of the city of *Brigantia* (probably A Coruña) that built a tower on the coast from where *Eire* (Ireland) could be seen.

Cells are Gram-negative, aerobic rods, motile by polar flagella. Cells divide by binary fission or asymmetric division. Oxidase and catalase positive. The G+C content of the DNA of the type strain is 63.9 mol%. The respiratory quinone is ubiquinone 10 (Q-10). The predominant cellular fatty acid is $C_{18:1}\omega7c$ (75.3%), and other fatty acids found in smaller amounts are $C_{19:0}$ cyclo$\omega8c$ and $C_{16:0}$. The major polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylmonomethylethanolamine, and phosphatidylcholine. On the basis of 16S rRNA gene sequences, UBF-P1^T affiliates with the Class *Alphaproteobacteria* and the type species is *B. corrubedonensis*.

Description of *Breoghania corrubedonensis* sp. nov.

Breoghania corrubedonensis (Co.ru.be.do.nen'sis N.L. fem. adj. corrubedonensis, of or belonging to Corrubedo, northwest Spain, isolated from the beach of Corrubedo, the location where the sand sample used to inoculate the enrichment cultures from which strain UBF-P1^T was isolated).

In addition to the characteristics reported for the genus, cells are regular, irregular or bulbous rods, motile by one or two subpolar flagella, and approximately 0.6–0.7 μm wide and 2–3.5 μm long. They reproduce by asymmetric division. On LB ASW, LB 3% NaCl, or Marine Agar, colonies are circular, entire, convex, mucoid, and creamy white in colour. After incubation for 5 days at optimal growth conditions, colonies are approximately 1 mm in diameter. The strain grows at temperatures in the range of 15–40 °C (optimum 30 °C) and at pH 5–8.5. Growth occurs with NaCl concentrations of 1–10%. Hydrolysis of gelatine or aesculin does not occur. Aminopeptidase, arginine dihydrolase, ornithine decarboxylase and urease activities are present. Nitrate is reduced to nitrite. Negative for nitrite reduction, indole production, and H_2S formation. β-Glucosidase and β-galactosidase are not produced. Starch, DNA or Tween 80 are not degraded. Acid is only weakly produced from L-arabinose. Growth occurs on glucose, arabinose, mannitol, D-sorbitol, adipate, gluconate (w), phenyl acetate, acetate, succinate, malate, pyruvate, casaminoacids (Difco), acetone, and Tween 20. No growth occurs on D-xylose, mannose, maltose, starch, caprate, *N*-acetyl-glucosamine, citrate, lactate, oxalacetate, propionate or methanol. The fatty acids are $C_{18:1}\omega7c$ (75.3%), $C_{19:0}$ cyclo $\omega8c$ (6.4%), $C_{16:0}$ (4.4%), $C_{16:1}$ $\omega7c$ and/or $C_{15:0}$ iso 2-OH (summed feature 3, 2.5%), $C_{18:0}$ 3-OH (2.4%), $C_{18:0}$ (2.2%), and $C_{20:1}$ $\omega9c$ (1.3%). Other minor (<1%) fatty acids are $C_{14:0}$ 3-OH or $C_{16:1}$ iso I (summed feature 2), $C_{17:1}$ $\omega8c$, $C_{17:1}$ $\omega6c$, and $C_{17:0}$. Major polar lipids are diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylmonomethylethanolamine (PME), and phosphatidylcholine (PC). The DNA content is 63.9 mol%. The discriminative traits of the species are listed in diagnostic Table 1 and Supplementary Material Table S2.

The type strain of the species UBF-P1^T has been deposited in three culture collections under the following numbers: CECT 7622, LMG 25482, and DSM 23382.

Emended description of the family *Cohaesibacteraceae* Hwang and Cho 2008

The description of the family was mainly based on the signature sequence and the taxonomic information of the two described members of the type species *C. gelatinilyticus* [8]. Given the differences between *C. gelatinilyticus* and *B. corrubedonensis* type strain sequences, the signature positions given as indicative of the family are no longer valid. For instance, the 16S rRNA gene sequence of *B. corrubedonensis* does not share the signature positions 194 (G instead of T), 678 (T instead of A), 712 (A instead of T) given for the family [8], nor the 1244 (G instead of A) or 1293 (C instead of T). The signatures found more closely resemble the majority of the other families of the order. In addition, reduction of nitrate is positive. The DNA composition ranges from 52 to 64 mol% G+C.

Emended description of *Cohaesibacter gelatinilyticus* Hwang and Cho 2008

The description is as proposed by Hwang and Cho [8] with the following modifications: nitrate and nitrite reduction are positive. It does not utilize citrate or *N*-acetyl-glucosamine. Oxidative acids from sugars are not produced. Aesculin is not hydrolysed. It does not utilize the hydrocarbons hexadecane, pyrene or phenanthrene, nor acetone or methanol for growth. The type strain is *C. gelatinilyticus* CL-GR15^T (=DSM 18289^T).

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.syapm.2010.06.005.

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SUPPLEMENTARY MATERIAL

Supplementary Table S1. Information on the sequence length of each partial gene of strain UBF-P1^T used and the position of each gene in the concatenated alignment after having been filtered with the program Gblocks [2]. The use of the program Gblocks removed 12% of the homologous positions in the alignment as they were highly variable.

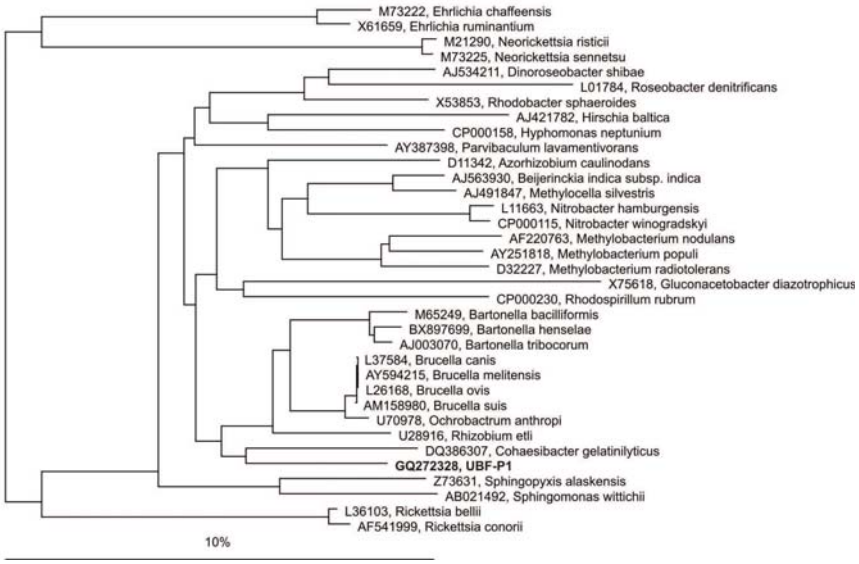
Gene	Length of the partial gene (nucleotides)	Initial and final position of each gene in the concatenated alignment (after Gblocks)	Accession Numbers
atpD	543	1 - 490	FN598910
pyrG	370	491 - 827	FN598913
rpoB	1230	828 - 1761	FN598917
fusA	761	1762 - 2506	FN598914
16S	1481	2507 - 3618	GQ272328
Total	4385	3618	

Supplementary Table S2. Additional information on the differential characteristics between strain UBF-P1^T and *Cohaesibacter gelatinilyticus* DSM 18289^T. Tests were simultaneously performed with both strains.

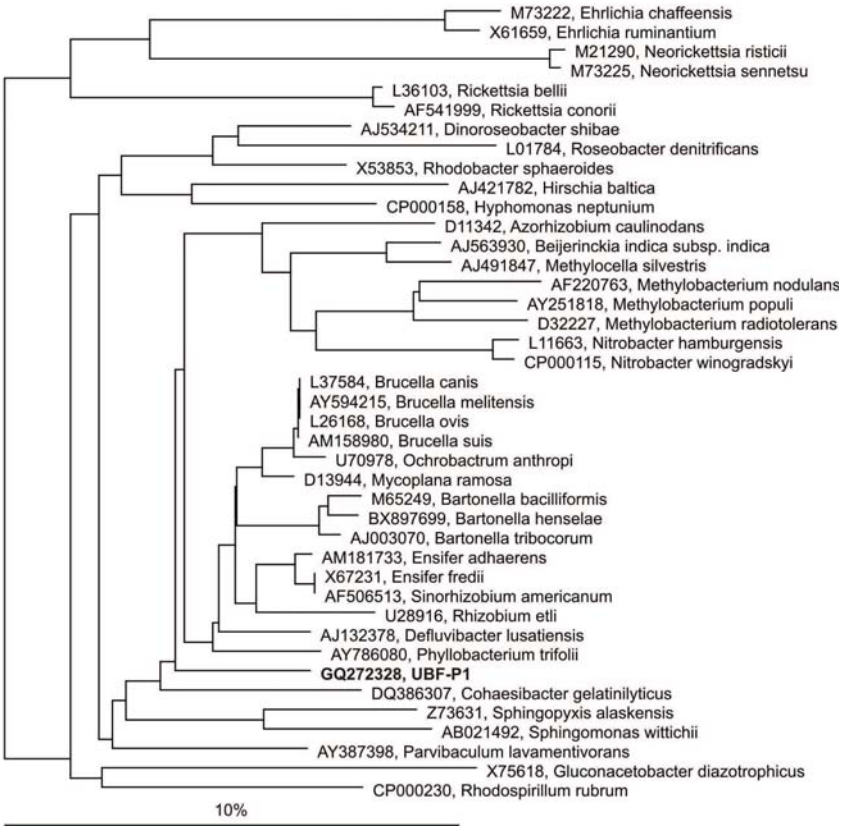
Characteristic	Bacterial strain	
	UBF-P1^T	*C. gelatinilyticus* DSM 18289^T
Colony coloration	Cream, circular, opaque, mucoid, 1 mm	Cream, circular, entire, convex, 2 mm
Aminopeptidase	+	nd
Lysis in 3% KOH	+	nd
LDC	+ (w)	-
ADH test	+	-
ODC	+	-
Indol	-	-
Voges-Proskauer	-	-
H_2S formation	-	-
TDA	-	-
β-galactosidase (ONPG and PNPG)	-	-
Utilization/assimilation of:		
Glucose	+	+
Citrate	-	-*
Arabinose	+	-
Mannose	-	+
Mannitol	+	+
Maltose	-	-
Malate	+	+
N-Acetyl-glucosamine	-	-*
Phenylacetate	+	-
Gluconate	+ (w)	+ (w)
Caprate	-	-
Adipate	+	-
D-xylose	-	-
Starch	-	-
D-cellobiose	-	-
D-sorbitol	+	+
Phthalic acid	-	-
Protocatechuic acid	+	-
Diphenic acid	-	-
4-Phenanthroic acid	-	nd
Acetate	+	+
Propionate	-	+
Succinate	+	+
Lactate	-	+
Oxalacetate	-	-
Pyruvate	+	+
Acetone	+	-
Methanol	-	-
Casaminoacids	+	+
Yeast extract	+	+
Hexadecane	-	-
PAHs (Pyrene or Phenanthrene)	-	nd
Tween 20	+	+
Tween 80	-	-
Oxidative acid from:		
D-glucose	-	-*
L-arabinose	+	-*
D-sucrose	-	-
L-rhamnose	-	-
D-mannose	-	-*
Inositol	-	-*
D-sorbitol	-	-*
D-melobiose	-	-
Amygdalin	-	-
Hydrolysis of:		
Aesculin	-	-*
Starch	-	-
DNA	-	-
Tween 80	-	-
Anaerobic growth in marine rich media	-	-

* Our results differ from the previously reported data by Hwang and Cho [8].
(w): weak, nd: Not determined.

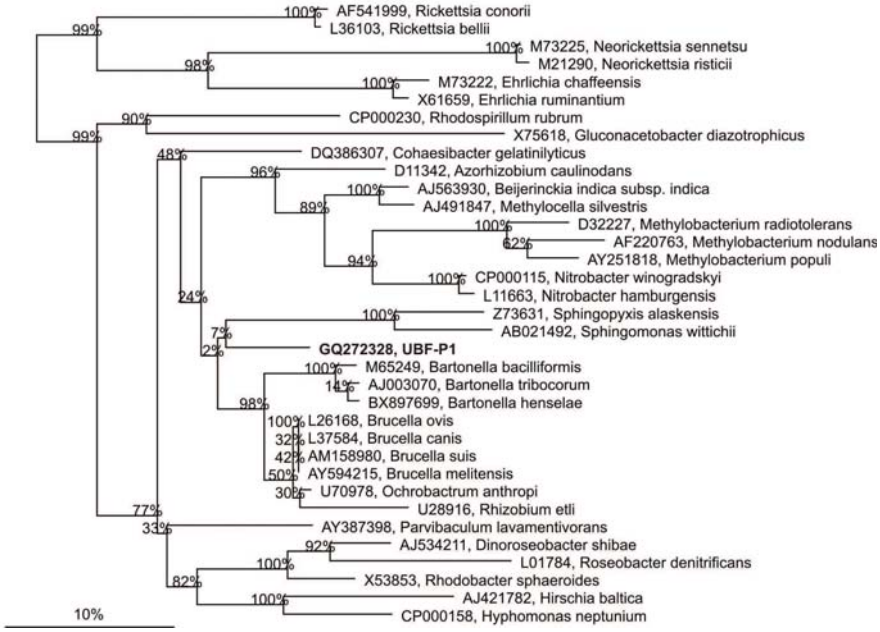
Supplementary Figure S1. Neighbour joining tree reconstruction based on the 16S rRNA gene sequences of all type strains of the *Alphaproteobacteria* for which a full genome sequence is available in the public repositories. In addition to the new isolate strain UBF-P1, the type strain of *Cohaesibacter gelatinilyticus* is included as the closest relative due to its sequence identity. The tree is based on the Jukes Cantor algorithm and by using the 40% conservation filter as produced in the LTP98 ARB database [14].



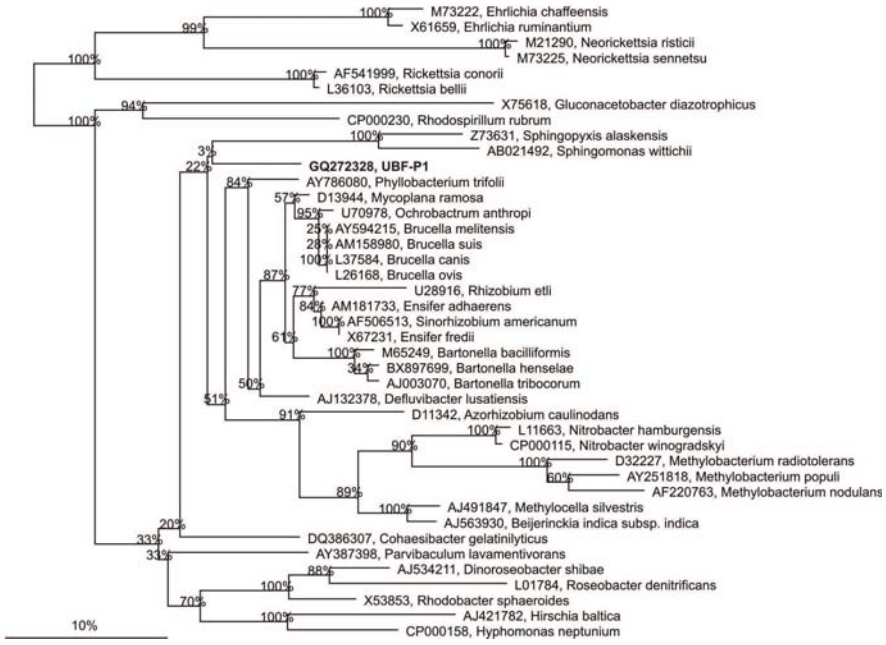
Supplementary Figure S2. Neighbour joining tree reconstruction based on the 16S rRNA gene sequences of all type strains of the *Alphaproteobacteria* for which a full genome sequence is available in the public repositories. In addition to the new isolate strain UBF-P1, the type strain of *Cohaesibacter gelatinilyticus* is included as the closest relative due to its sequence identity. In addition, the six additional close relatives *Phyllobacterium trifolii*, *Mycoplana ramosa*, *Ensifer adhaerens*, *Sinorhizobium americanum*, *Ensifer fredii*, and *Defluvibacter lusatiensis* are included as the nearest similar sequences. The tree is based on the Jukes Cantor algorithm and by using the 40% conservation filter as produced in the LTP98 ARB database [14].



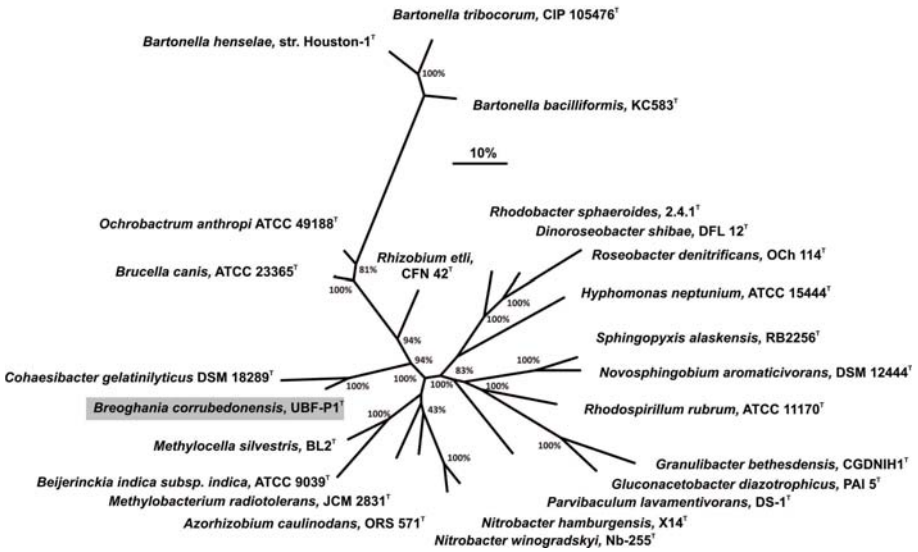
Supplementary Figure S3. RAxMl tree reconstruction based on the 16S rRNA gene sequences of all type strains of the *Alphaproteobacteria* for which a full genome sequence is available in the public repositories. In addition to the new isolate strain UBF-P1, the type strain of *Cohaesibacter gelatinilyticus* is included as the closest relative by its sequence identity. The tree is based on using the 40% conservation filter as produced in the LTP98 ARB database [14].



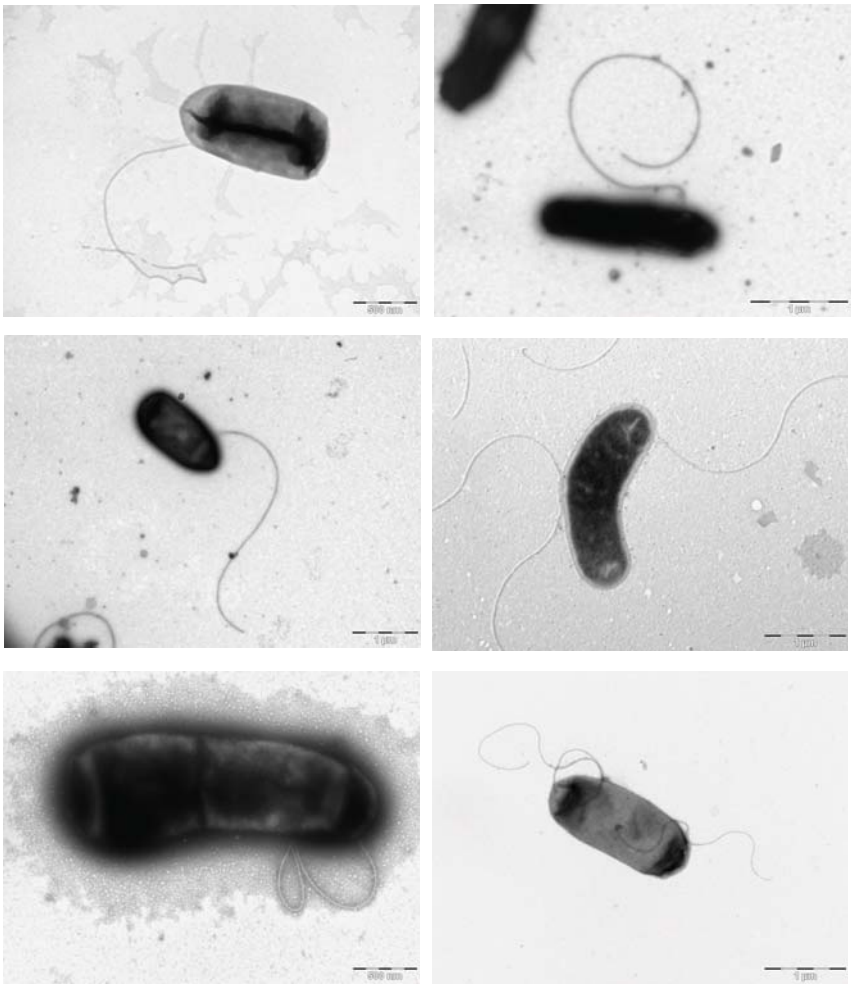
Supplementary Figure S4. RAxMl tree reconstruction based on the 16S rRNA gene sequences of all type strains of the *Alphaproteobacteria* for which a full genome sequence is available in the public repositories. In addition to the new isolate strain UBF-P1, the type strain of *Cohaesibacter gelatinilyticus* is included as the closest relative due to its sequence identity. In addition, the six additional close relatives *Phyllobacterium trifolii*, *Mycoplana ramosa*, *Ensifer adhaerens*, *Sinorhizobium americanum*, *Ensifer fredii*, and *Defluvibacter lusatiensis* were included as the nearest similar sequences. The tree is based on using the 40% conservation filter as produced in the LTP98 ARB database [14].

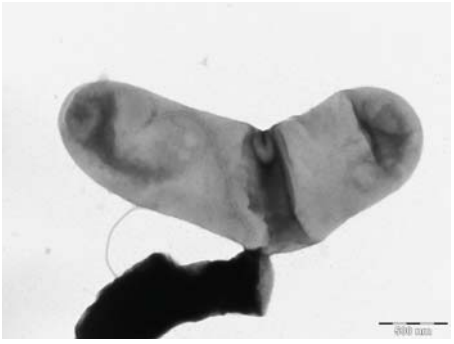
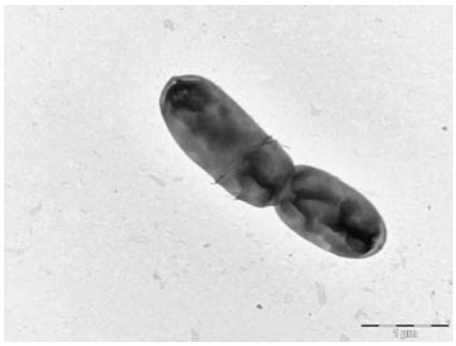
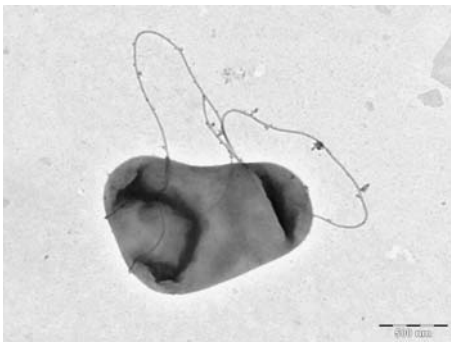
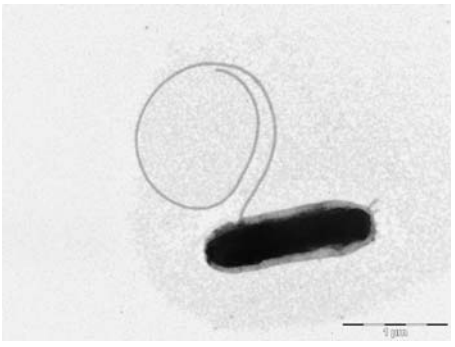
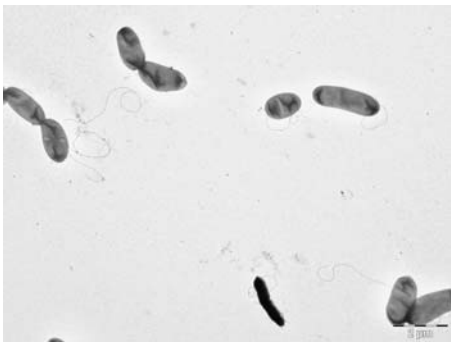
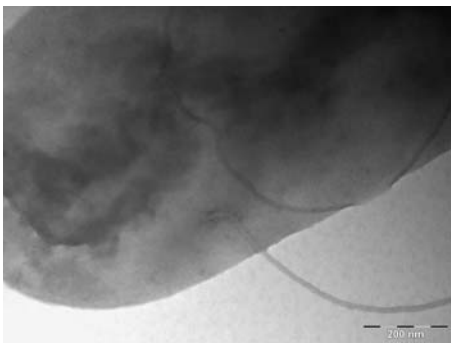
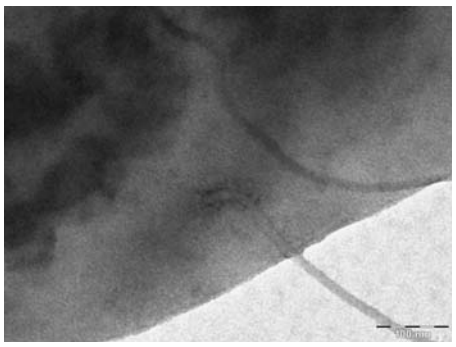


Supplementary Figure S5. MLSA phylogenetic reconstruction based on the concatenated genes *atp*D, *pyr*G, *rpo*B, and *fus*A of strain UBF-P1, the type strain of *Cohaesibacter gelatinilyticus* DSM 18289^T, and the selection of type strains of the *Alphaproteobacteria* for which a fully sequenced genome is available in the public repositories. The tree was reconstructed by using the RAxML algorithm version 7.0 with the GTRGAMMA model [22]. The bootstrap analysis was carried out by using 100 replicates. The bar indicates 10% alignment divergence. The similarity of the concatenated sequences of UBF-P1 and the type strain of *Cohaesibacter gelatinilyticus* DSM 18289^T is 85.6%.

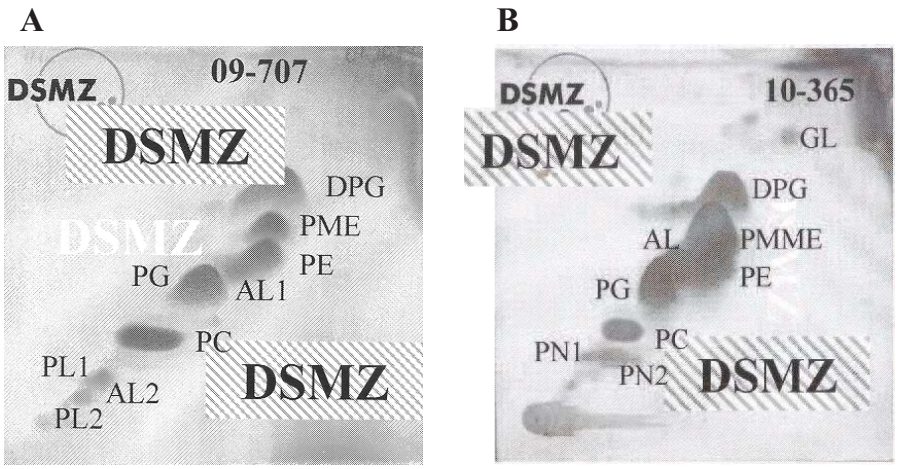


Supplementary Figure S6. Additional transmission electron micrographs obtained after negative staining of strain UBF-P1^{T}.



Supplementary Figure S7. Polar lipid analyses (2D-TLC) of strain UBF-P1^{T} (A) and C*ohaesibacter gelatinilyticus* DSM 18289^{T} (B), as obtained by the Identification Service of the DMSZ and Dr. B.J. Tindall, DSMZ, Braunchweig.



DPG Diphosphatidylglycerol

PME/PMME Phosphatidylmonomethylethanolamine

PE Phosphatidylethanolamine

PG Phosphatidylglycerol

PC Phosphatidylcholine

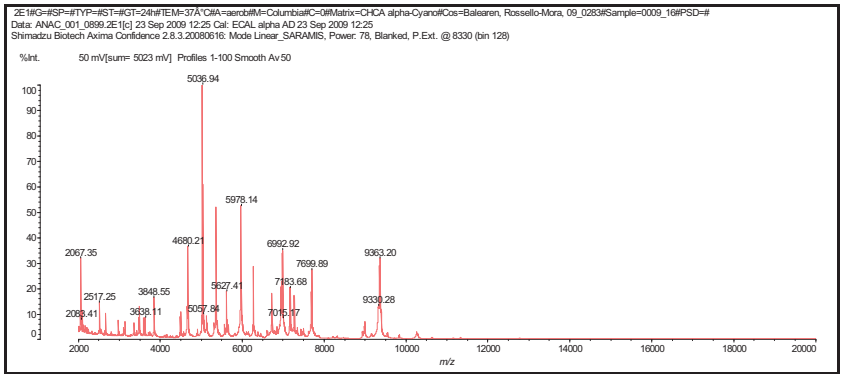
PL1-PL2 Phospholipids

AL, AL1-AL2 Aminolipids

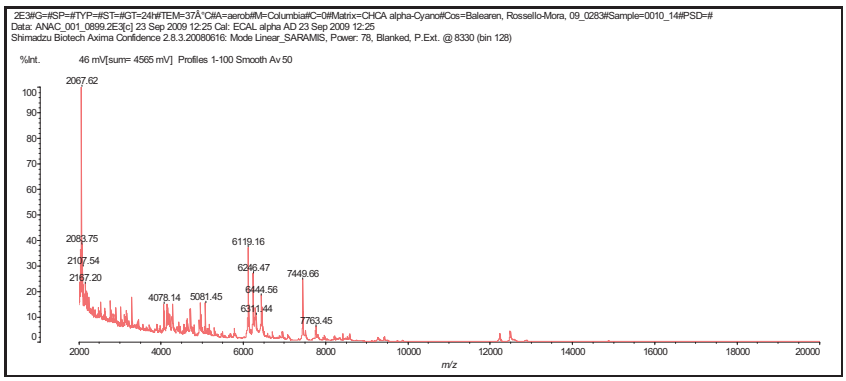
PN1-PN2 Aminophospholipids

GL Glycolipid

Supplementary Figure S8. Maldi-Tof profiles of total cell extracts of the strain UBF-P1^{T} (A) and *C. gelatinilyticus* DSM 18289^{T} (B).



A



B

Efecto de la Fertilización en la Interfase sobre la Biodegradación de Hidrocarburos Aromáticos Policíclicos Presentes en Fases Líquidas No Acuosas

El principal objetivo de este estudio fue usar un bioestimulante oleofílico (S-200) para identificar posibles limitaciones nutricionales de hidrocarburos aromáticos policíclicos (HAPs) en la interfase entre fases líquidas no acuosas (FLNA) y la fase acuosa. La biodegradación de HAPs presentes en fuel en FLNA fue lenta y de cinéticas de orden cero, indicando restricciones en la biodisponibilidad. El bioestimulante potenció la biodegradación, produciendo cinéticas logísticas (forma de S) y aumentos de 10 veces en las tasas de mineralización de fenantreno, fluoranteno y pireno. Los análisis químicos del residuo de fuel oil además evidenciaron una potenciación de la biodegradación de los alquil-HAPs y *n*-alcanos. El incremento no fue debido a un resultado en el aumento de la tasa de partición de los HAPs en la fase acuosa, ni tampoco fue causado por la compensación de cualquier déficit nutricional en el medio. Nosotros sugerimos que la biodegradación de los HAPs por bacterias adheridas a la FLNA puede estar limitada por la disponibilidad de nutrientes, debido al simultáneo consumo de los componentes de la FLNA, pudiendo ser esta limitación superada por el uso de fertilizantes en la interfase.

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Effect of Interface Fertilization on Biodegradation of Polycyclic Aromatic Hydrocarbons Present in Nonaqueous-Phase Liquids

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The main goal of this study was to use an oleophilic biostimulant (S-200) to target possible nutritional limitations for biodegradation of polycyclic aromatic hydrocarbons (PAHs) at the interface between nonaqueous-phase liquids (NAPLs) and the water phase. Biodegradation of PAHs present in fuel-containing NAPLs was slow and followed zero-order kinetics, indicating bioavailability restrictions. The biostimulant enhanced the biodegradation, producing logistic (S-shaped) kinetics and 10-fold increases in the rate of mineralization of phenanthrene, fluoranthene, and pyrene. Chemical analysis of residual fuel oil also evidenced an enhanced biodegradation of the alkyl-PAHs and *n*-alkanes. The enhancement was not the result of an increase in the rate of partitioning of PAHs into the aqueous phase, nor was it caused by the compensation of any nutritional deficiency in the medium. We suggest that biodegradation of PAH by bacteria attached to NAPLs can be limited by nutrient availability due to the simultaneous consumption of NAPL components, but this limitation can be overcome by interface fertilization.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are pollutants typically found as part of complex organic matrixes, for example, in the form of nonaqueous-phase liquids (NAPLs) such as creosote and coal tar. The biodegradation of NAPL-associated PAHs is typically slow (*1*−*3*), and consequently, the chemicals show, in this physical state, a high tendency to remain in the environment for long periods. Biodegradation of NAPL-associated PAHs may be severely limited by their slow kinetics of abiotic partitioning into the water phase. However, specific microbial mechanisms, such as attachment to the NAPL/water interface, biosurfactant production, and chemotaxis, may enhance this process (*4*−*6*). Nutritional constraints may also limit biodegradation of NAPL-associated chemicals when the major components in the NAPL mixtures are degraded simultaneously with the chemicals of concern, thus causing shortages in the N and P supply that can be more pronounced in the vicinity of the NAPL/water interface (*7*). Treatments, including addition of the limiting nutrients, often have a positive effect on biodegradation rates during laboratory-scale bioremediation of NAPL-polluted soils (*8*, *9*). However, in spite of the importance of biodegradation processes at the NAPL/water interface, little is known on how nutritional limitations operate with attached bacteria and how their activity can be promoted specifically to enhance biodegradation of NAPL constituents.

The main goal of this study was to use an oleophilic biostimulant to target possible nutritional deficiencies during PAH biodegradation at the NAPL/water interface. The enhancing effect of oleophilic biostimulants on biodegradation of fuel components during bioremediation of marine spills is a well-known phenomenon (*10*). The main feature of oleophilic biostimulants is that they associate with the oil phase, being primarily recommended for use on rocky shores or where wave action hinders the effectiveness of slow-release and water-soluble biostimulants. However, experimental studies reporting precise measurements of the effect of these additives on biodegradation rates at the fuel/water interface are very scarce (*11*). Laboratory estimations obtained from homogenized suspensions may not be able to discriminate the nutritional benefits of the biostimulants from those derived from fuel emulsion and its additional effects on specific surface area, bioavailability, and subsequently biodegradation rates. The scarcity of published information on the effect of oleophilic biostimulants on biodegradation by attached bacteria may also be due to inattention to this subject by manufacturing companies.

In our study, we employed a biphasic NAPL/water system, described previously (*6*), that maintains the integrity of the organic phase and hence a constant interfacial area. The biostimulant was added at a low concentration to the water phase and contributed negligible amounts to N and P sources already present in excess as inorganic salts. Biodegradation of PAHs initially present in fuel-containing NAPLs as well as in single-component NAPLs—heptamethylnonane (HMN), hexadecane (HD), and di-2-ethylhexyl phthalate (DEHP)—was evaluated by radiorespirometry and by measuring the residual concentrations of PAHs and the appearance of metabolites.

Materials and Methods

Chemicals. Heavy fuel oil, RMG 35 (ISO 8217), was obtained from the Technical Office of Accidental Marine Spills, University of Vigo, Spain (OTVM). This fuel has similar characteristics to the Prestige heavy fuel oil, with special resistance to degradation due to its high viscosity and composition of high molecular mass aliphatic and aromatic compounds. These heavy fuels are obtained as residues after the thermal distillation (visbreaking) of crude oils to obtain the lighter oil fractions. Fluoranthene, phenanthrene, pyrene, di-2-ethylhexyl phthalate (DEHP), *n*-hexadecane (HD), and 2,2,4,4,6,8,8-heptamethylnonane (HMN) were purchased from Sigma Chemical Co., Steinheim, Germany. [^{14}C]Phenanthrene (13.1 mCi·mmol^{-1}, radiochemical purity >98%), [^{14}C]fluoranthene (45 mCi·mmol^{-1}, radiochemical purity >98%), and [^{14}C]pyrene (58.7 mCi·mmol^{-1}, radiochemical purity >98%) also were obtained from Sigma. We used the nonionic alkyl poly(ethylene glycol) ether surfactant Brij 35, which was supplied by Sigma−Aldrich. Oleic acid (90%) was supplied by Aldrich and urea by Fluka. The 16-PAH standard solution used for gas chromatography−mass spectrometry (GC-MS) quantification (PAH-mix 9) was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Reference

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compounds for identification of PAH metabolites were obtained from Sigma−Aldrich. Diazomethane was generated by alkaline decomposition of Diazald (*N*-methyl-*N*-nitroso-*p*-toluenesulfonamide) by use of a Diazald kit with Clear-Seal joints from Sigma−Aldrich. Organic solvents were obtained from J. T. Baker, Deventer, The Netherlands.

The physicochemical constants of each NAPL used, relevant for this study and available in the literature (*12*) or provided by OTVM, are solubility in water, S_w (milligrams per liter); octanol−water partition coefficient, log K_{ow}; density, D (grams per milliliter); and viscosity, V (centiStokes, cSt). The values corresponding to each NAPL are as follows: DEHP (S_w, 2.24×10^{-5}; log K_{ow}, 7.45; D, 0.981; V, 81.4 at 20 °C); HD (S_w, 0.283×10^{-3}; log K_{ow}, 9.16; D, 0.773; V, 3.03 at 25 °C); HMN (S_w, 0.278×10^{-3}; log K_{ow}, 8.25; D, 0.793; V, 3.18 at 25 °C); and heavy fuel (D, 0.981; V, 380 at 50 °C). To reduce viscosity and allow reproducible results in biodegradation and partitioning experiments with a constant interfacial area, fluidized samples of fuel were obtained by mixing 1 g of fuel with 1 mL of HMN or DEHP. The resulting NAPL samples, referred to as fuel/HMN and fuel/DEHP, had viscosities at 25 °C of 13.99 ± 0.28 and 31.16 ± 0.14 cSt, respectively. These viscosity values were determined as the ratio of time for passage through a 10 mL pipette to time for passage of HMN (*13*).

Bacteria, Medium, and Cultivation. The bacterium used in this study, *Mycobacterium gilvum* VM552, originated from a PAH-polluted soil and is capable to use phenanthrene, fluoranthene, and pyrene as its sole source of carbon and energy. This bacterium can also grow with HD but not with HMN or DEHP. The bacterium was cultured with phenanthrene and prepared for mineralization experiments as previously described (*14*). The inorganic salts solution used in mineralization experiments (MM, pH 5.7) had the following composition: 900 mg·L^{-1} KH_2PO_4, 100 mg·L^{-1} K_2HPO_4, 80 mg·L^{-1} $CaCl_2$, 100 mg·L^{-1} NH_4NO_3, 100 mg·L^{-1} $MgSO_4 \cdot 7H_2O$, 2 μg·L^{-1} $Na_2B_4O_7 \cdot 10H_2O$, 2 μg·L^{-1} $MnSO_4 \cdot H_2O$, 2 μg·L^{-1} $CuSO_4 \cdot 5H_2O$, 1.4 μg·L^{-1} $Na_2MoO_4 \cdot 2H_2O$, 2 μg·L^{-1} $ZnSO_4 \cdot H_2O$, and 10 μg·L^{-1} $FeCl_3 \cdot 6H_2O$. Maximum growth rates (μ_{max}) for phenanthrene-pregrown *M. gilvum* VM552 in MM supplemented with either 1 g of solid phenanthrene or 1 mL of HD were derived from duplication periods (t_d) of optical density measurements at 600 nm (OD_{600}) according to $\mu_{max} = \ln 2 / t_d$.

Oleophilic Biostimulant. The biostimulant S-200 was kindly supplied by IEP Europe (Madrid, Spain). This additive is composed of urea (N source) and phosphoric esters (P source) in a mixture of saturated and unsaturated fatty acids (mainly oleic acid), butoxyethanol and glycol ether, as well as a base for carrying water (*15*). According to the analysis of a sample performed in our laboratory, the biostimulant (used in the experiments at 0.1 mL in 70 mL of MM) contributed 40 μg·mL^{-1} total organic carbon (TOC), 10 μg·mL^{-1} total N, and 0.4 μg·mL^{-1} total P to the composition of MM solution. This means increases of 0.4% and 0.003% in the concentrations of total N and P, respectively. The surface tension, determined at 25 °C with a TD1 Lauda ring tensiometer, of MM solution decreased from 62 to 34 mN·m^{-1} in the presence of the biostimulant. Its effect on NAPL viscosity was determined on a fuel/HMN sample amended with biostimulant (10:1 v/v), resulting in a viscosity value of 13.03 ± 0.7 cSt, only 7% lower than without biostimulant.

Mineralization Experiments. To measure the effect of the biostimulant on the mineralization of the radiolabeled PAHs present in NAPLs, we employed the constant-interfacial area method (*6*). These tests were performed in duplicate in sterile biometric flasks of 250 mL capacity (Bellco Glass), equipped with an open-ended glass tube (2 cm in diameter, 10 cm long, four slots in the base) that was placed vertically in each flask to contain the NAPL. The biphasic NAPL/water system used maintained the integrity of the NAPL, thus avoiding potential interferences resulting from emulsion of the NAPL caused by the surfactant component of the biostimulant. Duplicate 70 mL-portions of a bacterial suspension, containing 10^7 cells·mL^{-1} (OD_{600} 0.03) in MM, were added to the biometer flasks. Flasks containing the oleophilic biostimulant received 0.1 mL of the commercial preparation previously sterilized by dissolution in acetone. This organic solvent was evaporated completely (as evidenced by the return to the original volume) prior to use. In experiments with fuel, 1 mL of a fluidized fuel sample containing 80 000 dpm of ^{14}C-labeled phenanthrene, pyrene, or fluoranthene was added to the surface of the aqueous phase inside the tube. Experiments with single-component NAPLs were performed with 1 mL of HMN, DEHP, or HD containing ^{14}C-labeled phenanthrene and sufficient unlabeled substrate to give 1 mg·mL^{-1} NAPL. The flasks were sealed with Teflon-lined stoppers and incubated at 25 °C on a rotary shaker operating at 80 rpm. $^{14}CO_2$ production was measured as radioactivity appearing in the alkali trap (1 mL of 0.5 M NaOH) of the biometer flasks. The use of the same cell density in all mineralization experiments allowed comparisons among different treatments. No significant losses of $^{14}CO_2$ were expected during biodegradation experiments, given the Teflon-lined flask closures. Mass balances performed after mineralization experiments accounted for 90−105% of the initial radioactivity present in the system. Further details about the experimental procedures and method of calculation of mineralization rates can be found elsewhere (*6*). To determine the biodegradation percentages for alkanes, PAHs, and their alkyl derivatives and the potential accumulation of PAH metabolites in the assays with fuel-containing NAPLs, separate duplicate flasks were incubated under the same conditions but without the addition of ^{14}C-labeled compound. Uninoculated controls were also included to estimate abiotic loses. At the end of the incubation time (1500 h), both the NAPL and the water phase of cultures and controls were sampled, extracted, and analyzed by gas chromatography coupled to mass spectrometry (GC-MS).

Some mineralization experiments were performed in the presence of the nontoxic, nonionic surfactant Brij 35 (250 mg·L^{-1}). The critical micelle concentration of this surfactant, as determined at 25 °C with a TD1 Lauda ring tensiometer (Lauda, Germany), is 77 mg·L^{-1}. Other treatments included the addition of urea (14.3 mg·L^{-1}) and oleic acid (42.8 mg·L^{-1}), which were added to the water phase to reach the same nitrogen concentration and TOC, respectively, as those provided by the biostimulant.

Partitioning Experiments. These tests were conducted under conditions identical to those of mineralization experiments but in the absence of bacteria (*4, 6*). Measurements of partitioning of phenanthrene were carried out in 250 mL Erlenmeyer flasks containing 70 mL of an inorganic salts solution (pH 5.7) with or without biostimulant. The NAPL was added to the surface of the aqueous phase inside the glass cylinder. The flasks were sealed with Teflon-lined stoppers and maintained on a rotary shaker operating at 80 rpm. At certain time intervals, the aqueous phase outside the glass tube was sampled, and the concentration of phenanthrene in aqueous solution was measured by direct injection into a HPLC system.

In order to calculate the rate of mass transfer of phenanthrene into aqueous solution, a two-compartment model was fitted by nonlinear regression to partitioning data:

$$C = C_{eq}(1 - e^{-kt}) \quad (1)$$

In this equation, C is the concentration of phenanthrene in the aqueous phase, C_{eq} is the phenanthrene concentration in the aqueous phase at equilibrium, k is a mass-transfer

rate constant, and t is time. The maximum rates of partitioning or dissolution were calculated by multiplying C_{eq} by k.

Chemical Analysis of Residual Fuel Oil and PAH Metabolites. The residual fuel/HMN NAPLs were removed from control and culture flasks at the end of incubation to analyze the hydrocarbon composition. Each NAPL sample was dissolved in dichloromethane, dried over Na_2SO_4, and concentrated to 5 mL. A 0.5 mL aliquot of this solution was used for gravimetric analysis. The saturated and aromatic hydrocarbon fractions from another 0.5 mL aliquot were then obtained by column chromatography via U.S. Environmental Protection Agency (EPA) method 3611b and analyzed by GC-MS as indicated elsewhere (*16*). *o*-Terphenyl (Sigma−Aldrich) was used as internal standard. Alkane degradation percentages were determined by comparing the hopane-normalized areas from GC-MS reconstructed ion chromatograms (ion m/z 85) obtained for the saturated fraction of cultures, with those obtained for noninoculated controls. 17α(H),21β(H)-Hopane was used as the conservative internal biomarker and was detected by use of ion m/z 191. The 16 PAHs included in the U.S. EPA list of priority pollutants and alkyl derivatives were analyzed from reconstructed ion chromatograms of the aromatic fraction, obtained by using the corresponding molecular ions, and were quantified by utilizing standard calibration curves obtained for the nonsubstituted PAHs (*17*). All the analyses were performed on samples from separate duplicate flasks.

To analyze the hydrocarbons and metabolites present in the aqueous phase, 50 mL samples were removed from cultures and controls and solvent-extracted (20 mL of dichloromethane, five times) first in neutral conditions and then after acidification at pH 2.0. Neutral extracts were concentrated, dried, and directly analyzed by GC-MS. Acidic extracts were treated with diazomethane prior to analysis. When possible, oxidation products were identified by comparison of their MS spectra and GC retention time with those obtained for authentic commercial standards or for metabolites isolated and identified in previous biodegradation studies (*3, 16, 18*). When authentic products were not available, identification was suggested on the basis of data in databases (National Institute of Standards and Technology) or fragmentation patterns.

All results are given as means of duplicate measurements ± standard deviation (SD). Error bars in figures represent 1 SD. Statistical comparisons were performed with a Student t-test at $P = 0.05$.

Results

Influence of Oleophilic Biostimulant on Biodegradation of Fuel Components. The mineralization of phenanthrene, fluoranthene, and pyrene initially present in fuel/HMN is shown in Figure 1. The data, obtained simultaneously for the three plots, indicate that mineralization was different when the oleophilic biostimulant was present. The biostimulant did not affect the initial (first 100 h) phase of mineralization, but the subsequent increase in the rate of transformation did not occur if the medium had no biostimulant. In the absence of biostimulant, mineralization of the three chemicals was linear ($r > 0.98$) during the entire test period and occurred simultaneously at a rate of 0.10 ± 0.01 ng·mL^{-1}·h^{-1} for phenanthrene, 0.0035 ± 0.0003 ng·mL^{-1}·h^{-1} for pyrene, and 0.0006 ± 0.00005 ng·mL^{-1}·h^{-1} for fluoranthene. Mineralization curves in the presence of the biostimulant were S-shaped and evidenced the respiration of the chemicals at increased maximum rates of 1.12 ± 0.07 ng·mL^{-1}·h^{-1} (phenanthrene), 0.065 ± 0.002 ng·mL^{-1}·h^{-1} (pyrene), and 0.027 ± 0.01 ng·mL^{-1}·h^{-1} (fluoranthene).

The GC-MS analyses of the aliphatic and aromatic fractions from the residual fuel/HMN NAPL from inoculated

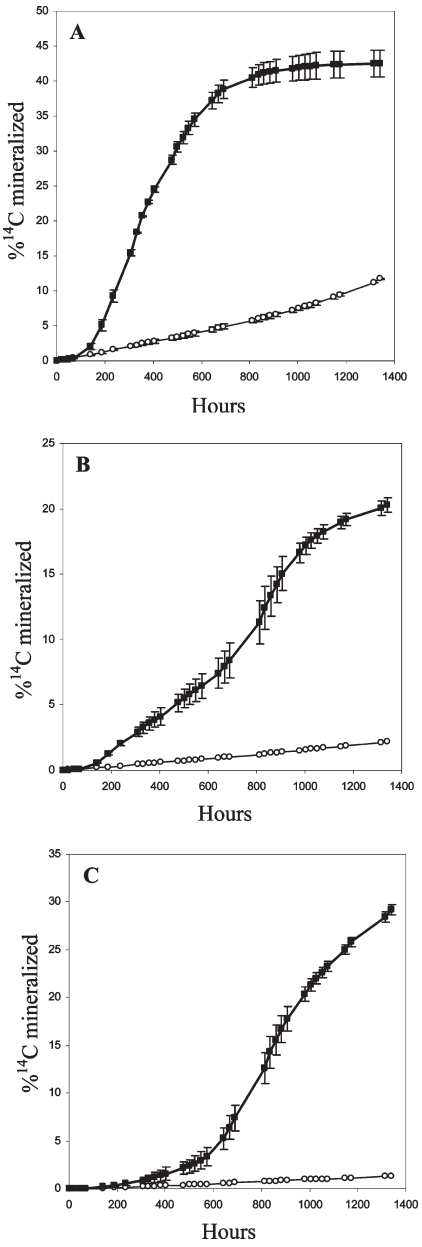

FIGURE 1. Effect of an oleophilic biostimulant (S-200) on mineralization of (A) phenanthrene, (B) pyrene, and (C) fluoranthene present in fuel/HMN by *Mycobacterium gilvum* VM552. Results in the three panels were obtained from experiments carried out simultaneously. Symbols represent percent ^{14}C mineralized without biostimulant (○) and with biostimulant (■). Error bars represent 1 standard deviation of duplicates.

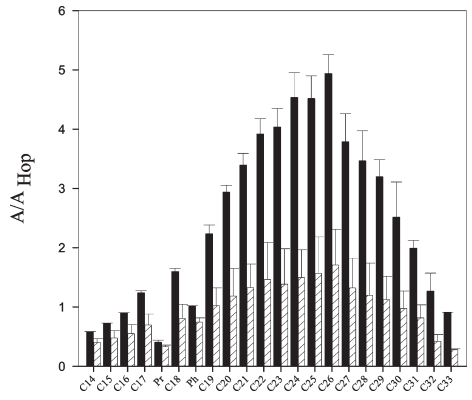

FIGURE 2. Hopane normalized areas (A/A_{HOP}) of the *n*-alkanes (*m*/*z* 85) detected by GC-MS analysis in the fuel/HMN residue of the abiotic controls (black bars) and cultures (striped bars) of *Mycobacterium gilvum* VM552, with the biostimulant. Compounds from C_{18} to C_{33} were significantly ($P < 0.05$) degraded. Cultures without biostimulant did not show significant alkane degradation as compared with controls. Pr, pristane; Ph, phytane, Cn, *n*-alkanes, n indicating the number of carbon atoms.

flasks and abiotic controls confirmed that the oleophilic biostimulant enhanced the biodegradation of all the families of hydrocarbons analyzed. As shown in Figure 2, the aliphatic fraction from abiotic controls exhibited a modal distribution of the *n*-alkanes from *n*-C_{14} to *n*-C_{35} with maxima at *n*-C_{24} and *n*-C_{26}, what is characteristic of the heavy fuel used. In inoculated flasks, the total alkanes (*m*/*z* 85) were significantly degraded (60.6% ± 12.8%, $P < 0.05$) only when the oleophilic stimulant was present. Similar results were found when the degradation percentages were calculated on the basis of C_{17}/pristane or C_{18}/phytane ratios (results not shown). Interestingly, high molecular weight saturated hydrocarbons (i.e., $C_{24}-C_{29}$) were more extensively degraded (64−67%) than smaller compounds with higher solubility in water ($C_{18}-C_{20}$, 49−59%; $C_{14}-C_{17}$, not significantly degraded). These results are remarkable, since previous studies on biodegradation of crude oil by bacteria have led to the general assumption that short-chain liquid alkanes are generally biodegraded faster than the long-chain compounds (*19*).

Biodegradation of PAHs and alkyl derivatives reached about 22% in the absence of S-200, while in the presence of the biostimulant this percentage increased to 60%. Only fluorene (43% ± 4.7%) and phenanthrene (38% ± 15%) were significantly degraded in the absence of biostimulant, whereas the cultures with the biostimulant presented a more extensive removal of those PAHs (84% ± 6.4% for fluorene and 99% ± 0.3% for phenanthrene), in addition to an important degradation of anthracene (40% ± 12.9%), fluoranthene (68% ± 3.4%), and pyrene (43% ± 4.7%) (Figure S1, Supporting Information). These results are in agreement with those obtained in the mineralization tests with ^{14}C-labeled compounds (Figure 1), when it is taken into account that the determinations based on the production of $^{14}CO_2$ are minimum estimates of the biodegradation (and do not include the incorporation into the biomass and metabolite production). As expected, methylated PAHs, more abundant than their nonalkylated counterparts, were attacked in a lesser extent, showing only a light but significant degradation in the presence of the biostimulant. Indeed, the corresponding fragmentograms (Figure S2, Supporting Information) showed only a selective degradation of mono- and dimethylnaphthalenes and monomethylphenanthrenes, following the patterns described in the literature (*16*, *19*).

The GC-MS analysis of the neutral and acidic extracts from the aqueous phase with and without biostimulant presented similar profiles of metabolite accumulation. However, the concentration in the flasks with biostimulant was about 10-fold higher (values went from about a tenth to several micrograms per milliliter, i.e., 120 ng·mL^{-1} for 1-indanone, 140 ng·mL^{-1} for diphenic acid, 690 ng·mL^{-1} for 2-carboxycinnamic acid, or 3.6 μg·mL^{-1} for 4-methylphthalic acid; Figure 3 and Tables S1 and S2, Supporting Information). Neutral metabolites corresponded to alkyl oxidized or phenolic naphthalenes, while the acidic compounds were mainly dicarboxylic acids, typically accumulated during the degradation of PAHs and their methyl derivatives by *Mycobacterium* strains. Specifically, phtalic acid is a common intermediate in the degradation of naphthalene, phenanthrene, fluoranthene, and pyrene, while carboxycinnamic and diphenic acids are naphthalene and phenanthrene metabolites, respectively (*3*, *18*). The presence of metabolites with methyl groups indicates similar biodegradation pathways for the corresponding alkyl PAH (*3*, *16*).

Role of Partitioning from NAPLs on Biodegradation of Phenanthrene. The PAH mass transfer from the NAPL to water was measured to determine whether the observed enhancement caused by the biostimulant could be explained by an increase in partitioning. However, partitioning of phenanthrene, chosen as a representative PAH, from fuel-containing NAPLs was not substantially modified by the presence of the biostimulant (Figure S3, Supporting Information). The measured rates of partitioning and equilibrium concentrations in the aqueous phase are shown in Table 1. The rates for the abiotic process are compared with the maximum mineralization rates. In the absence of biostimulant, the rate of mineralization of phenanthrene initially present in fuel/HMN was not significantly different from the measured partitioning rate. This confirms the consistency between HPLC and ^{14}C measurements in the experimental system used. However, when the biostimulant was present, the maximum mineralization rate was higher than the measured rate of partitioning.

A similar situation was observed when the surfactant Brij 35 was added to increase partitioning of phenanthrene from fuel/HMN into the aqueous phase (Table 1). Under these conditions, the oleophilic biostimulant had, again, no significant effect on partitioning rates, but mineralization of phenanthrene was enhanced in its presence, yielding an S-shaped plot (Figure S4, Supporting Information) and occurring at higher maximum rates than those predicted by abiotic partitioning. In the absence of biostimulant, mineralization was also linear but occurred at significantly lower rates than those of partitioning (Table 1), suggesting that partitioning exceeded the catabolic potential of microorganisms. The stimulatory effect of biostimulant on mineralization of phenanthrene was also observed when HMN was changed by DEHP as the fluidizing medium in the NAPL mixture (Table 1). Mineralization rate was, in the presence of the biostimulant, lower than that observed with the HMN mixture but still double the predictions of partitioning rate.

Mineralization and partitioning experiments were also performed with phenanthrene dissolved in single-component NAPLs, with the aim of discriminating possible effects caused on the enhancement by the biodegradability of the NAPL. Therefore, we determined the mineralization of phenanthrene initially dissolved in a NAPL that could be used as carbon and energy source (HD) and in two NAPLs that were not degraded by the bacterial strain used (HMN and DEHP). Under these conditions, partitioning rates and equilibrium concentrations measured in the absence of bacteria were increased with HMN and DEHP but not with HD (Table 2).

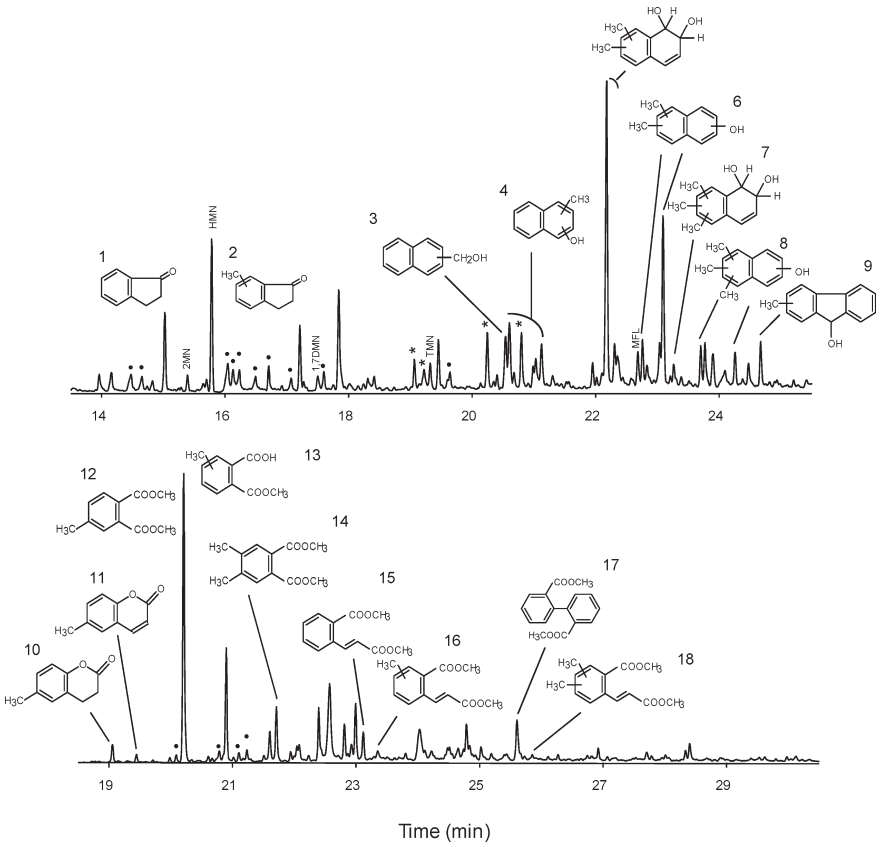

FIGURE 3. Typical GC-MS chromatograms of the neutral (top) and acid (bottom) extracts from the aqueous phases of the *Mycobacterium gilvum* VM552 culture in the presence of biostimulant indicating the identified peaks (Table S1, Supporting Information). Acidic metabolites were identified as the corresponding methyl esters. (•) Monoaromatic acids; (*) aromatic hydroxy acid lactones; MN, methylnaphthalene; HMN, heptamethylnonane; DMN, dimethylnaphthalene; TMN, trimethylnaphthalene; MFL, methylfluorene. For identification of compounds 1−18, see Tables S1 and S2, Supporting Information.

The exact reason for these differences remains unknown, but it may be related to the higher log K_{ow} value of HD as compared with HMN and DEHP. Nevertheless, irrespective of the NAPL used, the curves of phenanthrene mineralization were S-shaped (Figure S5, Supporting Information). With HD, two different phases could be observed after the onset of mineralization. The enhancement by the oleophilic biostimulant was evident only during the initial phase of mineralization (up to 234 h), driving mineralization to its maximum value. The subsequent rate (up to 404 h) was not statistically different with and without biostimulant. As shown by a lag period of 150 h and a doubling time of 71.7 h (μ_{max} = 0.009 h^{-1}) in separate growth experiments with HD, it is possible that proliferation on HD occurred during the phase of maximum phenanthrene mineralization. When phenanthrene was supplied as crystals and under the same growth conditions, *M. gilvum* showed no lag phase and a doubling time of 22.7 h (μ_{max} = 0.03 h^{-1}). The exponential growth observed in these growth experiments excluded any trace-nutrient limitation under the experimental conditions used. Mineralization was also enhanced by the biostimulant with HMN and DEHP, but the differences in rates were maintained during the whole phase of maximum mineralization (Table 2). In all cases, mineralization rates were higher than partitioning rates.

Effect of Shaking Conditions and Nutrients on Biodegradation. The possible effects of shaking and individual fertilizer components on biodegradation were also investigated. Mineralization of phenanthrene present in fuel/HMN was measured in shaken flasks where the NAPL was freely suspended in the water and, therefore, the NAPL/water interface was not kept constant. Although partitioning rates were not measured under these conditions, according to previous research (*12*), they were expected to increase as a result of an increased mixing and greater interfacial area. The promoting effect of the oleophilic biostimulant was less evident under these conditions (Figure S6A, Supporting Information). Indeed, mineralization in the absence of biostimulant was still linear (r = 0.98) and occurred at a rate of 0.31 ± 0.04 ng·mL^{-1}·h^{-1}, which was significantly higher (P < 0.05) than that detected under constant interfacial area, 0.11 ± 0.01 ng·mL^{-1}·h^{-1} (Table 1). The addition of the biostimulant also induced a shift in mineralization to an S-shaped curve (Figure S6A, Supporting Information) and

TABLE 1. Effect of Oleophilic Biostimulant S-200 on Partitioning and Mineralization of Phenanthrene Initially Present in Fuel-Containing Nonaqueous-Phase Liquids[a]

	partitioning		mineralization		
S-200	C_{eq} (ng·mL^{-1})	rate (ng·mL^{-1}·h^{-1})	rate (ng·mL^{-1}·h^{-1})	extent (%)	time[b] (days)
		Fuel/2,2,4,4,6,8,8-Heptamethylnonane (HMN)			
−	1.62 ± 0.006 a	0.18 ± 0.002 Aa	0.11 ± 0.012 Aa	12.64 ± 0.16 a	58
+	1.86 ± 0.047 a	0.13 ± 0.007 Aa	1.13 ± 0.07 Bb	42.5 ± 1.96 b	58
−[c]	26.60 ± 9.41[d] a	0.70 ± 0.18[e] Aa	0.14 ± 0.001 Ba	8.12 ± 0.54 a	35
+[c]	33.83 ± 17.69[d] a	0.77 ± 0.31[e] Aa	1.67 ± 0.091 Bb	32.57 ± 0.94 b	35
		Fuel/Di-2-ethylhexyl Phthalate (DEHP)			
−	2.30 ± 0.34 a	0.30 ± 0.12 Aa	0.07 ± 0.002 Ba	2.96 ± 1.02 a	35
+	3.82 ± 0.38[d] a	0.17 ± 0.04[e] Aa	0.29 ± 0.18 Ba	5.54 ± 2.89 a	35

[a] Reported values are means ± 1 standard deviation. Values in a row followed by the same capital letter are not significantly different ($P = 0.05$). For each NAPL, values in a column followed by the same lower-case letter are not significantly different ($P = 0.05$). Statistical analysis of partitioning data was performed separately among treatments with and without Brij 35. [b] Time period in which mineralization was measured. [c] Treatment with Brij 35. [d] Last experimental value because no equilibrium was achieved within the experimental period. [e] Values were calculated by linear regression with 10 time points (first 24 h).

TABLE 2. Effect of Oleophilic Biostimulant S-200 on Partitioning and Mineralization of Phenanthrene Initially Present in Single-Component Nonaqueous-Phase Liquids[a]

	partitioning		mineralization		
S-200	C_{eq} (ng·mL^{-1})	rate (ng·mL^{-1}·h^{-1})	rate (ng·mL^{-1}·h^{-1})	extent (%)	time[b] (days)
		n-Hexadecane (HD)			
−	43.84 ± 19.50 a	1.73 ± 0.19 Aa	4.10 ± 0.30[c] Ba 10.00 ± 4.00[c] Bb	24.48 ± 0.56 a	24
+	49.21 ± 4.39 a	2.00 ± 0.64 Aa	10.52 ± 1.57 Bb	24.78 ± 1.57 a	24
		2,2,4,4,6,8,8-Heptamethylnonane (HMN)			
−	42.88 ± 8.53[d] a	2.43 ± 0.16[e] Aa	38.28 ± 9.78 Ba	48.96 ± 0.88 a	12
+	64.74 ± 5.84 b	5.64 ± 0.95 Ab	62.93 ± 2.03 Bb	45.40 ± 2.37 a	12
		Di-2-ethylhexyl Phthalate (DEHP)			
−	12.59 ± 3.63 a	0.57 ± 0.003 Aa	52.48 ± 0.89 Ba	43.43 ± 2.36 a	12
+	25.01 ± 0.22[d] b	2.02 ± 1.03[e] Ab	62.25 ± 6.18 Bb	42.19 ± 5.46 a	12

[a] Reported values are means ± 1 were standard deviation. Values in a row followed by the same capital letter are not significantly different ($P = 0.05$). For each NAPL, values in a column followed by the same lower-case letter are not significantly different ($P = 0.05$). [b] Time period in which mineralization was measured. [c] Rates were calculated by linear regression to five time points ($r > 0.99$) during the first phase (136−234 h) and the second phase (307−404 h) of mineralization results in Figure S5A (Supporting Information). [d] Last experimental value because no equilibrium was achieved within the experimental period. [e] Values were calculated by linear regression with 10 time points (first 24 h).

an increase in mineralization rate (0.66 ± 0.04 ng·mL^{-1}·h^{-1}), but this rate was significantly lower ($P < 0.05$) than that observed under constant interfacial area (1.13 ± 0.07 ng·mL^{-1}·h^{-1}).

Mineralization of phenanthrene was not enhanced under constant interfacial area when urea was added to give a N concentration matching that of the cultures with biostimulant, resulting in a mineralization rate of 0.15 ± 0.07 ng·mL^{-1}·h^{-1} (Figure S6B, Supporting Information). However, urea promoted biodegradation significantly in the presence of the surfactant Brij 35, resulting in an S-shaped curve and a maximum mineralization rate of 0.83 ± 0.02 ng·mL^{-1}·h^{-1}. The mineralization rate in the presence of the surfactant alone was 0.14 ± 0.01 ng·mL^{-1}·h^{-1} and not significantly different ($P<0.05$) than the control (0.11 ± 0.01 ng·mL^{-1}·h^{-1}, Table 1). Similarly, no enhancement was observed in treatments where oleic acid was added (42.8 mg·L^{-1}, resulting in the same TOC as biostimulant-containing MM solutions) or with a MM solution supplemented with 8.7-fold the concentration of the inorganic N source [0.876 g·L^{-1} $(NH_4)NO_3$], resulting in linear mineralization rates of 0.09 ± 0.01 ng·mL^{-1}·h^{-1} and 0.07 ± 0.005 ng·mL^{-1}·h^{-1}, respectively (data not shown).

Discussion

The occurrence of linear kinetics of mineralization of fuel-associated PAHs is consistent with bioavailability-limited biodegradation. Furthermore, measurements of partitioning rate of phenanthrene, used as representative PAH, yielded, in the absence of biostimulant, values that were not significantly different than mineralization rates. Therefore, we can conclude that the mass transfer into the aqueous phase was the main limiting step for biodegradation of PAH. However, the enhancement in biodegradation observed in the presence of the oleophilic biostimulant was not the result of an increase in partitioning rate, as revealed by independent estimations in the absence of bacteria (Table 1). The enhancement was not caused by the compensation of a general nutritional deficiency in the medium, since the amounts of additional N and P contributed by the low concentrations of biostimulant used were negligible (0.4%

and 0.003%, respectively), in comparison to those already present in excess in the mineral salts solution used. The oleophilic nature of the biostimulant further suggests that the enhancement was caused by a localized enrichment in nutrients (N and P) of the NAPL/water interface that promoted the growth of attached bacteria, thus resulting in higher rates of PAH biodegradation than predicted by abiotic partitioning. Additional support for interface fertilization being the cause for the biodegradation enhancement is given by (i) the degradation of the *n*-alkanes only in the presence of biostimulant and the higher extent of degradation of the long-chain components ($C_{21}-C_{29}$), with a high affinity for the NAPL (*9*); (ii) the increased (10-fold) production of metabolites from known productive pathways (methyl phthalates) in the presence of the fertilizer; and (iii) the dependence of the biodegradation-enhancing effect of urea on the presence of a surfactant. In the latter case, the surfactant very likely partitioned into the NAPL and would have eventually facilitated, through interactions with its hydrophilic moiety, the association of the nitrogen source with the NAPL/water interface.

The observed shift in biodegradation kinetics of fuel-associated PAH from zero-order to logistic ("S-shaped") caused by the biostimulant (Figure 1) is also consistent with the promotion of bacterial growth at the NAPL/water interface. Logistic kinetics has been commonly observed in studies of biodegradation of organic chemicals dissolved in single-component NAPLs, and this kinetics is attributed to attached bacteria (*4, 6*). In our study, phenanthrene initially dissolved in HD, HMN, or DEHP was also mineralized following logistic kinetics and reached maximum rates exceeding partitioning predictions (Table 2), which confirms those previous results. The effect of the oleophilic biostimulant was observed only at the initial stages of mineralization of phenanthrene dissolved in a biodegradable, single-component NAPL (HD) and was more important with nonbiodegradable NAPLs (HMN and DEHP), possibly as a result of the enhancement in partitioning rate (Table 2). Therefore, our observations indicate that fuel-containing NAPLs reacted very differently to the studied single-component NAPLs in relation to interface fertilization.

The results may be explained by postulating the existence of nutritional limitations at the NAPL/water interface caused to bacteria by the fuel components. These limitations restrained the growth of bacteria attached to fuel-containing NAPLs unless interface fertilization was accomplished, thus causing logistic kinetics and partitioning-uncoupled mineralization. The precise cause for these nutritional constraints is unknown, but it may involve the simultaneous biodegradation of substrates present in fuel (PAHs, their alkyl derivatives, and alkanes) by attached bacteria. The efficient utilization of these multiple carbon sources may have caused a higher demand for N and P than biodegradation occurring in single-component NAPLs, where only one or two carbon sources were consumed at a time. According to this mechanism, the higher nutrient demand of bacteria attached to fuel-containing NAPLs may have been fulfilled by interface fertilization but not by diffusion from the bulk aqueous phase of the inorganic N and P sources already present in the mineral solution. Alternately to supplying nutrients at the interface, biostimulant components (fatty acids and surfactants) could have enhanced biodegradation by decreasing the NAPL viscosity and therefore diffusion of PAH in the NAPL side, thus facilitating the growth of PAH-degrading bacteria at the NAPL/water interface. However, several lines of experimental evidence indicate that this alternative explanation of the results is unlikely: (1) the absence of any effect on partitioning rates by the biostimulant [an increase would have been expected in case of a decreased NAPL viscosity (*20*)], (2) the inability of fatty acids (oleic acid) to promote biodegradation at comparable TOC, and (3) the limited effect of biostimulant on direct measurements of NAPL viscosity.

During degradation of complex PAH mixtures, a variable portion of depleted parent compounds is transformed to partially oxidized metabolites that may accumulate in the medium as a result of cometabolic or incomplete degradation processes (*3, 16*). Here, the enhancement of biodegradation by the oleophilic fertilizer was accompanied by higher production and accumulation of neutral and acidic metabolites in the water phase, some of which had not been identified previously in biodegradation studies with oil mixtures. This higher production of partially oxidized PAHs, some of which are intermediate metabolites resulting from carbon furnishing bacterial reactions (i.e., phthalic acids), is also consistent with enhanced bacterial growth in the presence of the biostimulant. In addition, it demonstrated that the enhanced biodegradation produced by the biostimulant may be accompanied by polar compounds that partition into the aqueous phase, being more mobile and bioavailable than the parent compounds.

In summary, the data show that interface fertilization was an effective mechanism to enhance biodegradation of NAPL-dissolved PAH when present in complex mixtures such as fuel. The study provides, by evidencing experimentally the enhancement of bacterial metabolism at the surface of fuel mixtures, new insights into the causes of persistence of NAPL-associated chemicals and the mechanism by which oleophilic biostimulants promote the biodegradation of petroleum hydrocarbons. It has also implications for bioremediation of subsurface-NAPL sources of pollutants, which is often limited by the slow kinetics of partitioning into the water phase. Determining the effects of fertilization on growth rates of bacteria attached to the NAPL/water interface is essential in order to devise practical biostimulation strategies, and this will be the subject of future investigations.

Acknowledgments

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Supporting Information Available

Two tables and six figures showing quantification of metabolites and residual PAHs; fragmentograms of alkyl-PAHs; effect of biostimulant on partitioning and mineralization of phenanthrene; and effect of shaking conditions and nutrients on mineralization of phenanthrene in NAPLs. This information is available free of charge via the Internet at http://pubs.acs.org/.

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Supporting Information

Effect of Interface Fertilization on Biodegradation of Polycyclic Aromatic Hydrocarbons Present in Nonaqueous-Phase Liquids

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TABLE S1. GC R_t and electron impact mass spectral properties of identified metabolites detected in the neutral extracts from cultures of *Mycobacterium VM552* with fuel, in the presence and the absence of the oleophilic biostimulant (S-200).

Num	R_t	Ion Fragments *m/z* (%)	Identification
1	15.0	132 (M^+,100), 104 (M^+-CO, 86.1), 89 (M^+-CH_3-CO,4.2), 78 (30.9), 51 (17.1)	1-Indanone[1]
2	17.2	146 (M^+, 94.4), 131 (M^+-CH_3, 4.5),117 (M^+-CH_3-CH_2, 100),103 (M^+-CH_3-CH_2-CH_2, 10.2),91 (22.1), 77 (9.1),63 (8.9), 51 (8.2)	Methyl 1-Indanone[2]
3	20.5	158 (M^+, 56.2), 141 (M^+-OH, 20.7), 129 (M^+-CHO, 100), 115 (17.2), 102 (4.1), 98 (1.0)	Naphthalene methanol[2]
4	20.6	158 (M^+,100), 141 (M^+-OH, 31.1),129 (M^+-CHO, 93.2),115 (M^+-CHO-CH_2, 79.7),105 (79.7),91 (50),77 (36.5)	Methyl naphthol[2]
	20.9	158 (M^+,87.8), 141 (M^+-OH, 28.4),129 (M^+-CHO,100),115 (M^+-CHO-CH_2, 55.4),104 (18.9),91 (23.0),77 (21.6)	
	21.1	158 (M^+,100), 141 (M^+-OH, 4.1),129 (M^+-CHO, 32.4),115 (M^+-CHO-CH_2, 19.0),102 (4.1),89 (2.7)	
5	22.2	190 (M^+, 5.4), 172 (M^+-H_2O, 100), 157 (M^+-H_2O-CH_3, 35.2), 143 (M^+-H_2O-CH_3-CH_2, 32.6), 129 (M^+-H_2O-CH_3-CH_2-CH_2, 39.2), 115 (M^+-H_2O-CH_3-CH_2-CH_2-CH_2, 26.1), 104 (2.9)	1,2-dihydroxy 1,2-dihydro dimethyl naphtahlene[3]
	22.3	190 (M^+, 6.6), 172 (M^+-H_2O, 100), 157 (M^+-H_2O-CH_3, 35.0), 143 (M^+-H_2O-CH_3-CH_2, 30.2), 129 (M^+-H_2O-CH_3-CH_2-CH_2, 41.4), 115 (M^+-H_2O-CH_3-CH_2-CH_2-CH_2, 25.5), 104 (4.1)	
	22.3	190 (M^+, 19.6), 172 (M^+-H_2O, 100), 157 (M^+-H_2O-CH_3, 15.7), 143 (M^+-H_2O-CH_3-CH_2, 52.9), 129 (M^+-H_2O-CH_3-CH_2-CH_2, 26.5), 115 (M^+-H_2O-CH_3-CH_2-CH_2-CH_2, 24.5), 105 (6.9),91 (10.8), 77 (10.8)	
6	22.8	172, (M^+,100), 157 (M^+-CH_3, 20.6), 143 (M^+-CH_3-CH_2, 9.8), 128 (M^+-CH_3-CH_2-CH_3, 26.8), 115 (M^+-CH_3-CH_2-CH_3-CH_3, 12.74), 102 (M^+-CH_3-CH_2-CH_3-CH_3-CH, 2.0), 86 (M^+-CH_3-CH_2-CH_3-CH_3-CH-O, 4.5)	Dimethyl naphtol[2]
	22.8	172 (M^+, 100), 157 (M^+-CH_3,39.1), 141 (M^+-CH_3-O, 12.6), 128 (M^+-CH_3-O-CH, 22.5), 115 (M^+-CH_3-O-CH-CH, 12.7), 103 (1.8), 91 (5.0)	
	23.0	172 (M^+, 100), 157 (M^+-CH_3, 18.4), 143 (M^+-CH_3-CH_2, 8.3), 128 (M^+-CH_3-CH_2-CH_3, 25.4), 115 (M^+-CH_3-CH_2-CH_3-CH_3, 15.0), 102 (M^+-CH_3-CH_2-CH_3-CH_3-CH, 2.8), 86 (M^+-CH_3-CH_2-CH_3-CH_3-CH-O, 4.7)	
7	23.3	204 (M^+, 7.3), 186 (M^+-H2O, 51.1),171 (M^+-H_2O-CH_3, 100),158 (M^+-H_2O-CH_3-CH, 15.8),143 (M^+-H_2O-CH_3-CH-CH_3, 13.3),128 (M^+-H_2O-CH_3-CH-CH_3, 23.9),115(17.1)	1,2-dihydroxy 1,2-dihydro trimethyl naphthalene[3]
	24.1	204 (M^+4,9),186 (M^+-H_2O, 100),171 (M^+-H_2O-CH_3 57.3),157 (M^+-H_2O-CH_3-CH_2, 7.8),141 (53.4),128 (20.4),115 (33.0)	
8	23.7	186 (M^+, 100),168 (M^+-H_2O, 66.7),157 (70.2),143 (55.5),128 (50.8),115 (54.3)	Trimethylnaphtol[3]
	24.2	186 (M^+, 46.3), 171 (M^+-CH_3, 100),153 (M^+-CH_3-H2O, 3.1), 141 (8.7),128 (M^+12.8),115 (9.7),102 (1.8)	
9	24.7	196 (M^+,48.0),181(M^+-CH_3, 100),165 (M^+-CH_3-O, 31.4),152 (M^+-CH_3-O-CH, 25.9)	Methyl fluorenol[3]

Identification was based on analysis of authentic standards[1], fragmentation pattern and a match higher than 90%with the NIST library[2], or was inferred according to the fragmentation pattern and literature data[3].

TABLE S2. GC R_t and electron impact mass spectral properties of identified metabolites detected in the acidic extracts from cultures of *Mycobacterium VM552* with fuel, in the presence and the absence of the oleophilic biostimulant (S-200). Compounds were identified as methyl esters.

Num	R_t	*m/z* of ion fragments (%)	Identification [a]
10	19.1	162 (M^+,100), 147 (M^+-CH_3, 3.9), 134 (M^+-CO, 57.7), 119 (31.6), 105 (12.6), 91 (62.9)	2H-1-Benzopyran-2-one, 3,4-dihydro-6-methyl [2]
11	19.5	160 (M^+,100), 132 (M^+-CO, 69.4), 131 (M^+-CO-H, 71.0), 104 (21.3), 77(18.0)	2H-1-Benzopyran-2-one, 6-methyl[2]
12	20.2	208 (M^+,13.7), 177 (M^+-CH_3, 100), 149 (M^+-$COOCH_3$, 5.7), 118 (2.8), 91 (11.5)	4-methyl-phthalic acid DME [1]
13	20.9	194 (M^+, 31.1), 162 (M^+-OCH_3-H, 100), 134 (M^+-OCH_3-H-CO, 77.7), 105 (12.6), 91 (60.2)	Methyl phthalic ME[3]
14	21.7	222 (M^+, 13.5), 191 (M^+-OCH_3, 100), 163 (M^+-COOCH3, 4.9), 148 (1.5), 132 (1.9), 120 (1.2), 105 (5.8), 91 (2.7), 77 (4.9)	4,5-dimethyl phthalic acid, DME[2]
15	23.1	220 (M^+, 3.6), 189 (M^+-OCH_3, 10.6), 173 (14.5), 161 (M^+-$COOCH_3$, 100), 145 (7.6), 130 (6.7), 118 (11.0), 115 (6.17), 102 (7.8), 91 (5.7)	2-Carboxycinnamic acid DME[1]
16	23.4	234 (M^+, 6.8), 220 (M^+-CH_3, 4.9), 203 (M^+-OCH_3, 3.9), 189 (M^+-CH_3-OCH_3, 10.7), 175 (M^+-$COOCH_3$, 100), 161 (M^+-CH_2-$COOCH_3$, 32.0), 145 (30.6), 131 (14.6), 118 (8.7), 115 (16.5), 105 (6.8), 91 (16.5)	Methyl carboxycinnamic acid DME[3]
17	25.6	270 (M^+, 2.7), 239 (M^+-OCH_3, 3.8), 211 (M^+-$COOCH_3$, 100), 196 (M^+-$COOCH_3$-CH_3, 13.4), 180 (M^+-COOCH-OCH_3, 13.3), 168 (6.5), 152 (12.4), 139 (10.1)	Diphenic acid DME[1]
18	26.1	248 (M^+, 7.7), 234 (M^+-CH_3, 10.2), 217 (M^+-OCH_3, 20.7), 189 (M^+-$COOCH_3$, 100), 174 (M^+-CH3-$COOCH_3$, 26.0), 161 (M^+-CH-$COOCH_3$, 7.0), 145 (26.6), 131 (13.1), 115 (42.5), 105 (9.4), 91 (22.5)	Dimethyl carboxycinnamic acid DME[3]

Identification is based on analysis of authentic standards[1], fragmentation pattern and a match with the NIST library higher than 90%, or is inferred according to fragmentation pattern of and literature data[3].

[a]ME, methyl ester, DME dimethyl ester.

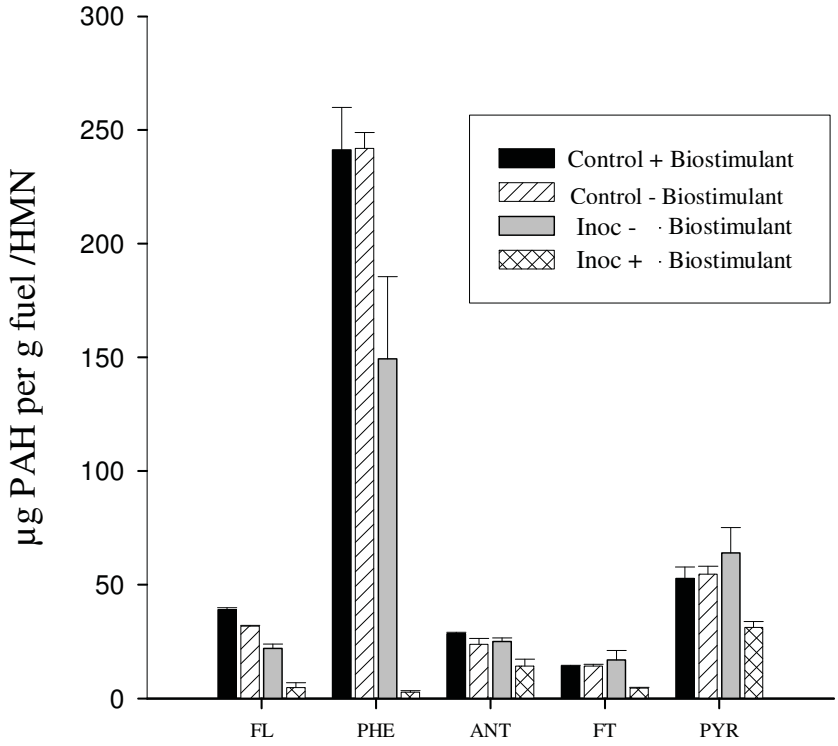


FIGURE S1. Concentrations of PAHs recovered from the NAPL phase from controls and inoculated flasks, both without and with biostimulant. Only PAHs with significative degradation ($p<0.05$) are shown. The bars show the ratio between the peak area and the area hopane for each type of sample. FL, fluorene; PHE, phenanthrene; ANT, Anthracene; FLT, fluoranthene; PYR, pyrene.

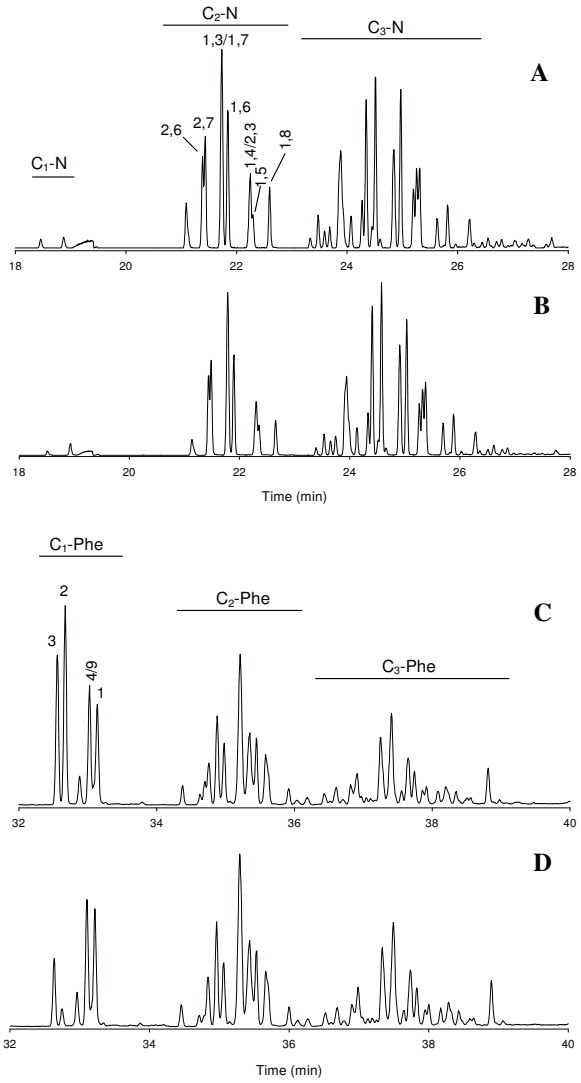


FIGURE S2. GC-MS fragmentograms showing the selective degradation of the alkyl naphthalene (A and B) and alkyl phenanthrene (C and D) families in flasks inoculated with *Mycobacterium* sp VM552 (B and D) in the presence of biostimulant, as compared with non-inoculated controls (A and C).

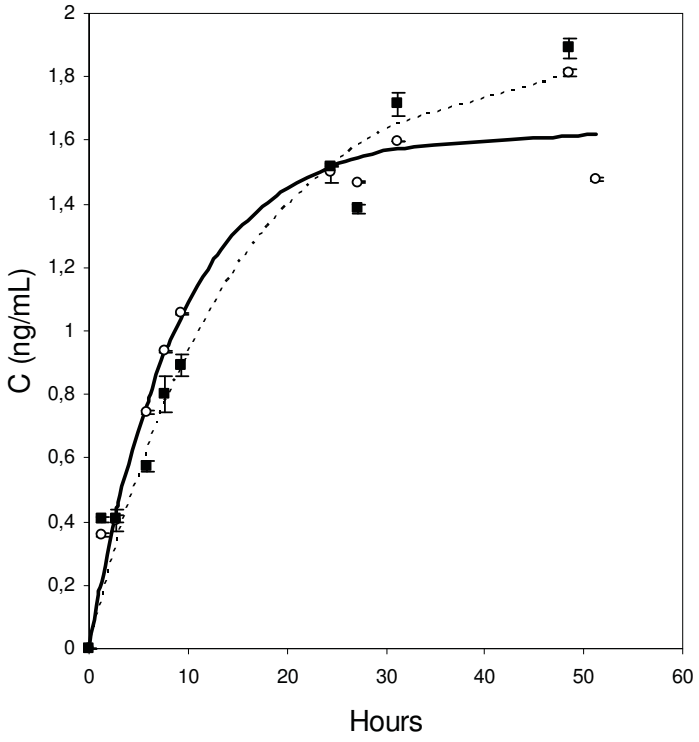


FIGURE S3. Partitioning of phenanthrene from fuel/HMN in the presence and absence of the oleophilic biostimulant S-200. Symbols represent phenanthrene concentrations in the aqueous phase without biostimulant (o) and with biostimulant (■). Solid and dashed lines represent, respectively, fits of experimental data without and with biostimulant to eq 1. Error bars represent one standard deviation of duplicates.

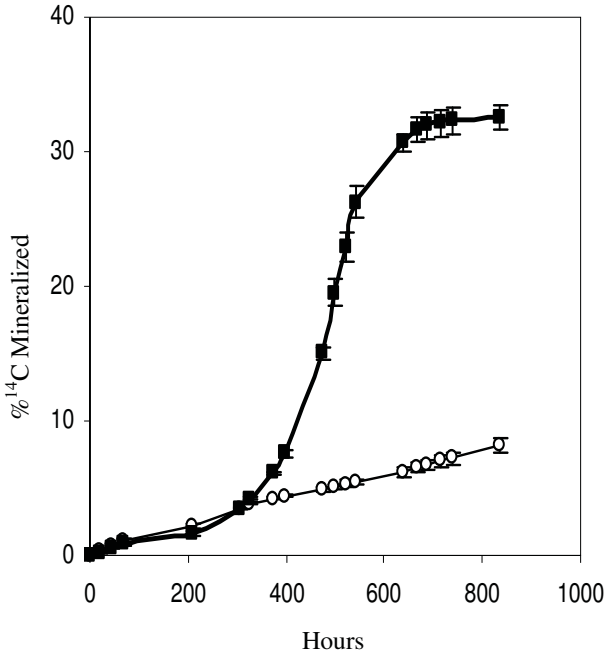
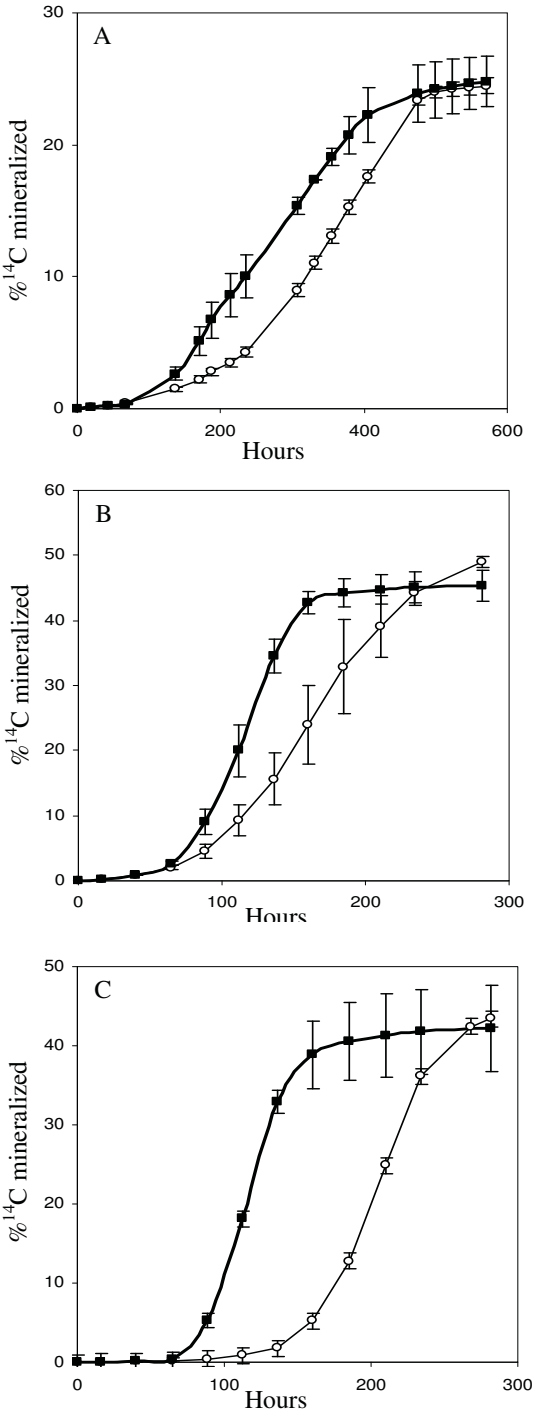


FIGURE S4. Effect of an oleophilic biostimulant (S-200) on mineralization of phenanthrene present in fuel /HMN by *Mycobacterium gilvum* VM552 in the presence of the surfactant Brij 35. Symbols represent % ^{14}C-phenanthrene mineralized without biostimulant (o) and with biostimulant (■). Error bars represent one standard deviation of duplicates.

FIGURE S5. Effect of an oleophilic biostimulant (S-200) on mineralization of phenanthrene initially present in hexadecane (A), heptamethylnonane (B) and di-2-ethylhexyl phthalate (C) by *Mycobacterium gilvum* VM552. Symbols represent $\%^{14}C$ mineralized without biostimulant (o) and with biostimulant (■). Error bars represent one standard deviation of duplicates.



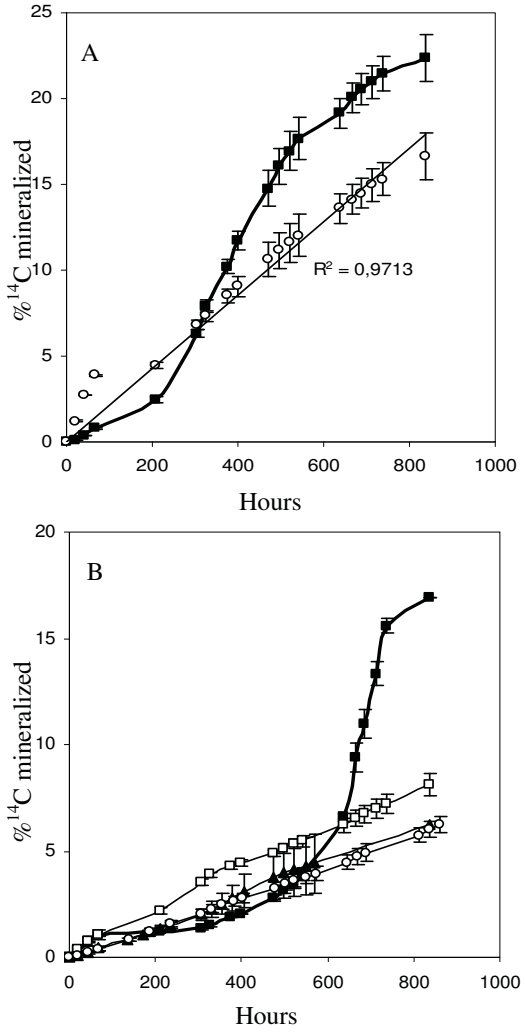


FIGURE S6. A, Effect of an oleophilic biostimulant (S-200) on mineralization of phenanthrene present in fuel/HMN freely suspended in the water. Symbols represent $\%^{14}C$ mineralized without biostimulant (o) and with biostimulant (■). The solid line represents linear fitting to data without biostimulant. B, Mineralization of phenanthrene initially present in fuel/HMN under constant interfacial area in the control with no addition (o), with urea and Brij 35 (■), with Brij 35 (□) and with urea (▲). In both panels, error bars represent one standard deviation of duplicates.

Influencia de la rizosfera de girasol en la bioaccesibilidad y biodegradación de HAPs en suelos

La reducida bioaccesibilidad de los microorganismos del suelo es probablemente el factor más limitante en la biorremediación de hidrocarburos policíclicos aromáticos (HAPs) en suelos contaminados. Nosotros hemos utilizado macetas que contienen suelo plantado con girasol para determinar la influencia de la rizosfera en la habilidad de la microbiota en reducir los niveles de HAPs. La concentración total de HAPs disminuyó un 93% en 90 días cuando el suelo contaminado fue cultivado con girasol, representando un aumento del 16% comparado con el suelo contaminado sin plantas. Este gran aumento en la degradación de HAPs fue consistente con el efecto positivo de la rizosfera en el crecimiento selectivo de poblaciones degradadoras de HAPs. Los análisis moleculares mostraron que el aumento en el número de degradadores fue acompañado con un dramático cambio en la estructura de las comunidades bacterianas del suelo, favoreciendo grupos con una bien conocida capacidad degradadora de HAPs como *Sphingomonas* (α-*Proteobacteria*), *Commamonas* y *Oxalobacteria* (β-*Proteobacteria*) y los filos recientemente descritos *Acidobacteria* y *Gemmatimonadetes*. Nosotros hemos realizado además experimentos de mineralización sobre suelo contaminado con creosota en presencia y ausencia de exudados de la raíz de girasol poder evaluar la capacidad que tienen estos exudados como bioestimulantes en la degradación de HAPs. Mediante la realización de experimentos en invernadero y de mineralización, hemos separado el impacto químico de los exudados de la raíz de cualquier fenómeno de superficie, indicando que los exudados de la raíz del girasol tienen el potencial de aumentar la degradación de xenobióticos mediante el crecimiento de microorganismos del suelo. Hemos caracterizado los exudados de girasol obtenidos *in vitro* para determinar el carbono orgánico total (COT) y su composición química. Nuestros resultados indican que la rizosfera promueve la degradación de HAPs mediante el aumento de la bioaccesibilidad de los contaminantes y el aumento del número y diversidad de degradadores de HAPS. Nosotros proponemos que la bioestimulación que proporcionan las plantas está basada en la composición química de los exudados.

Influence of the sunflower rhizosphere on the bioaccessibility and biodegradation of PAHs in soil

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Abstract

Reduced bioaccessibility to soil microorganisms is probably the most limiting factor in the bioremediation of PAH-polluted soils. We used sunflowers planted in pots containing soil to determine the influence of the rhizosphere on the ability of soil microbiota to reduce PAH levels. The concentration of total PAHs decreased by 93% in 90 days when the contaminated soil was cultivated with sunflowers, representing an improvement of 16% compared to contaminated soil without plants. This greater extent of PAH degradation was consistent with the positive effect of the rhizosphere in selectively stimulating the growth of PAH-degrading populations. Molecular analysis revealed that the increase in the number of degraders was accompanied by a dramatic shift in the structure of the bacterial soil community favoring groups with a well-known PAH-degrading capacity, such as *Sphingomonas* (α-*Proteobacteria*), *Commamonas* and *Oxalobacteria* (β-*Proteobacteria*), and *Xhanthomonas* (γ-*Proteobacteria*). Other groups that were promoted for which degrading activity has not been reported included *Methylophyllus* (β-*Proteobacteria*) and the recently described phyla *Acidobacteria* and *Gemmatimonadetes*. We also conducted mineralization experiments on creosote-polluted soil in the presence and absence of sunflower root exudates to advance our understanding of the ability of these exudates to serve as bio-stimulants in the degradation of PAHs. By conducting greenhouse and mineralization experiments, we separated the chemical impact of the root exudates from any root surface phenomena, indicating that sunflower root exudates have the potential to increase the degradation of xenobiotics due to the growth of soil microorganisms. We characterized the sunflower exudates in vitro to determine the total organic carbon (TOC) and composition of PAHs. Our results indicate that the rhizosphere promotes the degradation of PAHs by increasing the bioaccessibility of the pollutants and the number and diversity of PAH degraders. We propose that the biostimulation exerted by the plants is based on the chemical composition of the exudates.

Keywords: rhizosphere, microbial community structure, bioremediation, PAHs, sunflower root exudates, biodegradation

1.Introduction

Bioremediation techniques are routinely applied to recover soils polluted by polycyclic aromatic hydrocarbons (PAHs). These techniques are based on the well-established capability of soil microorganisms to degrade PAHs through growth-linked or co-metabolic reactions (Kanaly and Harayama, 2010). However, a major limiting factor in the bioremediation of PAH-polluted soils is the reduced bioaccessibility that is often exhibited by these pollutants, which results in difficulty in predicting whether an acceptable end-point decontamination level can be achieved. Bioacessibility can be defined as the concentration of a pollutant that is potentially biodegradable over time in the absence of limitations to biodegradation other than restricted phase exchanges.

Microorganisms can potentially overcome bioaccessibility restrictions through a variety of mechanisms, including biosurfactant production, attachment and chemotaxis (Garcia-Junco et al., 2003; Velasco-Casal et al., 2008; Tejeda-Agredano et al., 2011b). Bioaccessibility can also be increased in the soil externally, for example, by adding surfactants (Bueno-Montes et al., 2011) or applying electrokinetic techniques (Niqui-Arroyo and Ortega-Calvo, 2010).

Rhizoremediation, i.e., the use of ecosystem services provided by the plant rhizosphere to decontaminate polluted soils, has recently gained attention in relation to organic pollutants, such as PAHs. Translocation of dissolved contaminants in the rhizosphere and the microbial utilization of root exudates as co-substrates in the biodegradation of PAHs have been proposed as mechanisms through which plants contribute to the elimination of PAHs (El-Shatnawi and Makhadmeh, 2001); (Siciliano et al., 2003); (Newman and Reynolds, 2004). The sunflower (*Helianthus annuus*, L) has been used as a pilot system in phytoremediation assays for PAHs (Kummerova et al., 2001) and antibiotics (Gujarathi et al., 2005). The sunflower rhizosphere removes a greater quantity of fluorene, anthracene and pyrene from contaminated soil than the rhizospheres of other plant species, such as wheat, oat and maize, and exhibits a better response to seed germination and root elongation in the presence of these PAHs (Maliszewska-Kordybach and Smreczak, 2000). Olson et al. (2007) reported the sunflower as the best plant among 11 dicotyledonous species to use in assays of PAH bioavailability. Further advantages of focusing on the sunflower as a model plant for use in PAH rhizoremediation studies are related to the importance of this species as an edible oil producer. The ability to investigate the root exudation process and the role of the exudates under natural conditions has been hampered by a number of significant quantification problems, due to interference by microbial metabolites and components of the soil (Grayston et al., 1996). These problems can be overcome through the development of appropriate *in vitro* techniques to obtain root exudates that allow analysis of the products secreted by the plant roots.

The research approach applied in the present study was to generate soils polluted with aged PAHs at concentrations that would be realistic for polluted soils that had undergone extensive bioremediation, and we used these samples to test the hypothesis that the germination and development of sunflower plants would enhance the bioaccessibility and biodegradation of PAHs in the soil. We used both culture-dependent and culture-independent (i.e., based on DNA) techniques to determine the effects of planting on the dissipation of the chemicals from the soil under greenhouse conditions and on the structure of the soil microbial communities. We also developed a method to produce sunflower root exudates, which were chemically characterized and tested for possible effects on biodegradation by soil microorganisms through a dual radiorespirometry/residue analysis method that allowed precise estimation of compound bioaccessibility.

2. Materials and Methods

2.1. Soil

Two soils were used in this study: a creosote-polluted clay soil and an agricultural soil. The polluted soil (calcaric fluvisol) constituted the source of aged contaminants as well as PAH-degrading microorganisms for our greenhouse and laboratory experiments. This soil was provided by EMGRISA (Madrid, Spain) from a wood-treating facility in southern Spain that had a record of creosote pollution exceeding 100 years. The agricultural, non-polluted soil was a loamy sand soil from Coria del Río, Seville, Spain (Typic Xerochrepts). A PAH-containing soil mixture was obtained from these soils in two steps. First, the agricultural soil was mixed (67:33 w/w) with washed sand (Aquarama), and subsequently autoclaved. Next, this mixture (referred to as uncontaminated soil) was homogenized with polluted soil (1:1 w/w) in a cement mixer for seven days (9 hours per day), with regular changes in the direction of rotation. This homogenization

period was necessary to allow reproducible results to be obtained. The mixture was then dried for 18 hours at 30°C, ground and sieved (2 mm mesh). The resulting material was used in all experiments as a source of polluted soil with the following composition: pH 8.1; 15.9% $CaCO_3$; 0.9 % total organic carbon (TOC); 0.055% organic nitrogen (Kjeldahl); 7 $mgkg^{-1}$ available phosphorus; 461 $mgkg^{-1}$ potassium; particle size distribution 46.6% coarse-grained sand, 4.3% fine-grained sand, 15.8% silt, and 33.2% clay; and 21.75 $mgkg^{-1}$ of total PAHs (as the sum of 6 PAHs: fluorene, phenanthrene, anthracene, fluoranthene, pyrene and chrysene; Table 1). The resulting profile of PAH concentrations was consistent with soils that have undergone extensive bioremediation (Niqui-Arroyo and Ortega-Calvo, 2010; Bueno-Montes et al., 2011).

2.2. Greenhouse Experiments

2.2.1 Experimental Design

For this study, we used sunflower (*Helianthus annuus L.* cv. *PR 63A90*) seeds from the University of California that were certified for agronomic crop production. The greenhouse experimental design consisted of 5 pots with 2 kg of soil per treatment. The treatments included uncontaminated soil planted with seeds (as a positive control for plant growth) and contaminated soil with or without seeds. Five seeds were used per planted pot. The experiment was carried out in a greenhouse at 23±1 ºC and 20% field capacity. After 45 and 90 days, soil samples were collected for measurements of residual PAH contents and microbiological determinations. Soil samples (20 g) were carefully extracted from the rhizosphere zone with the aid of a glass tube. Care was taken to avoid damaging the plants. Samples for the PAH analyses were stored at -20ºC, and samples for the microbiological analyses were stored at 4ºC. At the end of the experimental period, the percentage of germination was evaluated for each treatment, and the fresh and dry weights of stems and roots were determined separately. Dried stems and roots were generated by incubating the separated plant materials in a desiccation oven (70 ºC) for 72 hours.

2.2.2 PAH Analysis

Triplicate soil samples (1 g of soil per sample) were dried completely using anhydrous sodium sulfate to grind the mixture in a mortar and pestle. Samples were extracted in a Soxhlet with 100 mL dichloromethane for 8 h. Once the extract was obtained, the organic solvent was evaporated in a vacuum to nearly complete dryness, and the residue was dissolved in 5 mL dichloromethane and cleaned by passing through a Sep-Pak Fluorisil cartridge. The purified extracts were evaporated with N_2, and the residues were dissolved in 2 mL of acetonitrile. Finally, the samples were filtered through a nylon syringe filter (0.45 µm, 13 mm Ø, Teknokroma, Barcelona, Spain). Quantification of PAHs was performed using a Waters HPLC system (2690 separations module, 474 scanning fluorescence detector, Nova-Pak C_{18} Waters PAH column, 5 µm particle size and 4.6 x 250 mm, 1 mL/min flow and mobile phase with an acetonitrile-water gradient). The column was installed in a thermostatic oven maintained at 30ºC.

2.2.3 Autochthonous microbiota

2.2.3.1 Enumeration of heterotrophic and hydrocarbon-degrading microbial populations

Bacterial counts from soil samples were performed using the miniaturized most probable number (MPN) method in 96-well microtiter plates with 8 replicate wells per dilution (Wrenn and Venosa, 1996). Total heterotrophs were counted in diluted (1:10) Luria-Bertani medium; low

molecular weight (LMW) PAH-degraders were counted in mineral medium (Grifoll et al., 1995) containing a mixture of phenanthrene (0.5 gL^{-1}), fluorene, anthracene, and dibenzothiophene (each at a final concentration of 0.05 gL^{-1}); and high molecular weight (HMW) PAH-degraders were counted in mineral medium containing pyrene at a final concentration of 0.5 gL^{-1}. Hydrocarbon was added to the plates dissolved in pentane, and medium was added after solvent evaporation. MPN plates were incubated at room temperature (25ºC±2ºC) for 30 days. Positive wells were detected based on turbidity (heterotrophs) and observable coloration (brownish/yellow) for PAH degraders.

2.2.3.2 DNA extraction and PCR amplification of eubacterial 16S rRNA genes

Total DNA from soil and rhizosphere samples was extracted using a Power Soil DNA isolation kit (Mobio, Carlsbad, USA). Eubacterial 16S rRNA gene fragments were amplified from the extracted total DNA through PCR using pureTaq™Ready-To-Go™ PCR bead tubes (GE healthcare, United Kingdom) in a final volume of 25 µL containing 1 µl of DNA extract as the template and 25 pmol of each primer (Sigma-Aldrich, Steinheim, Germany). To obtain clone libraries, we used the primers 27f and 1492r (Weisburg et al., 1991), and for the denaturing gradient gel electrophoresis (DGGE) fingerprinting analysis, we used GC40-63f and 518r (El Fantroussi et al., 1999). After 10 min of initial denaturation at 94°C, 30 cycles of amplification were carried out, each consisting of 30 sec of denaturation at 94°C, 30 sec of annealing at 56°C and 1 min (DGGE) or 2 min (clone libraries) of primer extension at 72°C followed by a final primer extension step of 10 min at 72°C. All of the PCR amplifications were performed in an Eppendorf Mastercycler.

2.2.3.3 DGGE analysis

The 16S rRNA PCR amplification products were purified using the Wizard®SV Gel and PCR Clean-Up system (Promega, Madison, USA) and quantified in a NanoDrop® Spectrophotomer ND-1000 prior to DGGE analysis. Identical amounts of PCR products were loaded in 6% polyacrylamide gels with denaturing gradients ranging from 45% to 70% (100% denaturant contains 7 M urea and 40% formamide). Electrophoresis was performed at a constant voltage of 100 V for 16 h in 1x TAE buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, pH 7.4) at 60°C in a DGGE-2001 System (CBS Scientific, Del Mar, CA, USA) machine. The gels were stained for 30 min with 1x SYBR Gold nucleic acid gel stain (Molecular Probes, Eugene, OR, USA) and photographed under UV light using a Bio-Rad molecular imager FX Pro Plus multi-imaging system (Bio-Rad Laboratories, Hercules, CA, USA) in the DNA stain gel mode for SybrGold at medium sample intensity. DGGE bands were processed using Quantity-one version 4.5.1 image analysis software (Bio-Rad Laboratories) and corrected manually.

2.2.3.4 Construction, sequencing and phylogenetic analysis of 16S rRNA gene clone libraries

Amplified 16S rRNA gene fragments were purified as described above and were cloned using the pGEM®-T Easy Vector System (Promega, Madison, USA). Transformants were selected through PCR amplification using vector PCR primers. The PCR mixture contained 1.25 U of *Taq* DNA polymerase (Biotools B&M Labs, Madrid, Spain), 25 pmol of each primer (Sigma-Aldrich, Steinheim, Germany), 5 nmol of each dNTP (Fermentas, Hanover, MD) and 1x PCR buffer (Biotools B&M Labs) in a total volume of 25 µl. The obtained PCR products were purified, and inserts were sequenced using the ABI Prism Bigdye Terminator cycle-sequencing reaction kit (version 3.1) with the amplification primers 27f and 1492r and the internal primers 357f and 1087r (Lane, 1991). The sequencing reactions were performed using an ABI prism 3700 Applied

Biosystems automated sequencer at Scientific-Technical Services of the University of Barcelona. DNA sequencing runs were assembled using BioEdit Software (Hall, 1999). Sequences were aligned using the BioEdit software package (Hall, 1999) and manually adjusted. The resulting DNA sequence was examined and compared with BLAST alignment tool comparison software (Altschul et al., 1990) and the classifier tool of the Ribosomal Database Project II at http://rdp.cme.msu.edu/ (Maidak et al., 2000). The 16S rRNA gene sequences obtained for the bacterial clones were deposited in the GenBank database.

2.3. Experiments with exudates

2.3.1. In vitro production

In vitro production of sunflower root exudates was performed by placing 50 seeds in an inorganic salt solution (MM, pH 5.7) described elsewhere (Tejeda-Agredano et al., 2011). To avoid the introduction of alternative sources of organic carbon in the biodegradation experiments, the solution did not contain sucrose, vitamins or plant growth regulators. The medium was prepared using ultrapure water (MILLIPORE). We transferred 500 mL of MM to glass jars (1000 ml capacity, 28 x 11.5 cm) previously sterilized for 20 min. (121 ºC, 1 atm. of pressure). Inside these glass jars, we installed a square piece of stainless steel wire cloth (0.98 mm light and 0.40 mm in diameter), held in place by four stainless steel wires extending from the sides of each jar. The length of these wires is calculated such that that the seeds on the mesh were in contact with the surface of the MM without sinking into the solution to avoid producing anoxia. The jars were closed firmly with a pressure system using a glass lid.

To sterilize the seeds, a batch of 50 seeds was surface-sterilized in 250 mL of absolute ethanol for 3 minutes in sterilized Erlenmeyer flasks at 550 rpm. The ethanol was subsequently removed, and 250 mL of a solution of 57% sodium hypochlorite (14% active chlorine) was added for 25 min. Finally, the hypochlorite was eliminated, and the seeds were rinsed 3 times with sterilized distilled water for 5 min each time, working in a laminar flow biosafety cabinet. Next, the sterilized seeds were distributed on square cloth mesh. The size of the mesh allowed root growth to occur and kept the seeds in place. The jar was closed, sealed with Parafilm and placed in a culture room at 25 ± 1ºC, 65.24 $\mu Em^{-2}s^{-1}$ and with an 18-hour photoperiod for 30 days. After this period, the growth medium with the exudates was collected under sterile conditions and centrifuged for 3 h at 31,000 x g to obtain a solution that included the organic matter present in the sediment pore water (Haftka et al., 2008). These samples were stored at -20ºC until further use in the mineralization experiment. It is of particular note that the seeds were situated on the medium surface and never submerged such that only the developed roots were responsible for exudate production.

In vitro exudate extraction was repeated 6 times, and at the end of each repetition, we quantified the number of plants, determined the fresh and dry weight of the roots and assessed the relative growth rate (RGR) of whole plants calculated according to the equation $RGR = (\ln Bf - \ln Bi)\ D^{-1}$ (Merckx et al., 1987), where Bf is the final dry biomass; Bi is the initial dry biomass (average of 5 seedlings dried 3 days after germination of seeds); and D is the number of days of the experiment. The plants acquired at the beginning and end of the greenhouse experiment were dried by placing the plant material in the desiccation oven at 70 ºC for 72 hours.

2.3.2 Chemical analyses of sunflower root exudates

Total organic (TOC) and inorganic carbon estimations were carried out at IRNAS-CSIC based on measurements performed in a TOC Analyzer (TOC, model TOC-V CPH, Shimadzu, Japan) using anon-purgeable organic carbon (NPOC) analysis. The analyses of amino acids,

organic acids and sugar were carried out at Scientific-Technical Services of the University of Barcelona. Prior to analysis, the exudate sample was concentrated by freeze drying. The amino acid content was analyzed through cationic exchange chromatography (Amino acids analyzer, Biochrom 30, Biochrom, UK) and post-column derivatization with ninhydrin. The chromatograph was equipped with a polysterene-divinylbenzene sulphonate column (200x4 mm) with a 5-µm film thickness. Elution was carried out using lithium citrate buffer with a pH and ionic strength according to the manufacturer's instructions (Spackman et al., 1958).

Low-molecular-weight organic acids were analyzed using a Water Alliance 2695 chromatograph coupled to a PE SCIEX API 365 triple quadruple mass spectrometer. The column was an Aminex HPX-87H (300x7.8 mm) column (Bio-Rad, CA). The oven temperature was held at 40°C. The sample (100 µl) was injected with a flow rate of 0.8 $mLmin^{-1}$ of water acidified with acetic acid (0.1%) and subjected to a post-column addition of methanol acidified in the same manner. The analyses were perform using a Turbo Ion spray ionization source in negative polarity with the following parameters: capillary voltage −3500 V, nebulizer gas (N_2) 10 (arbitrary units), curtain gas (N_2) 12 (arbitrary units), declustering potential -60 V, focusing potential −200 V, entrance potential 10 V. The drying gas (N_2) was heated to 350°C and introduced at a flow-rate of 7000 ml/min. The results were analyzed in both Full Scan (40-400 Da) and SIM (selected ion monitoring Modes.

The sugar content was analyzed in a Waters Alliance 2695 chromatograph equipped with Aminex HPX-87P (300 x 7.8 mm) and Aminex HPX-87C (300 x 7.8 mm) columns (BioRad, CA) connected to a refraction index detector (Waters 2414) at a temperature of 37°C. The solvent system consisted of purified water at a flow rate of 0.6 mL min^{-1}. The oven temperature was held at 85°C.

Aromatic carboxylic acids and fatty acids were detected using GC-MS analysis based on methylated derivatives. After acidification with 1 M HCl (pH 2), 50 mL of the exudates was extracted with ethyl acetate (5 x 20 mL), and the extracts were concentrated under vacuum to 1 mL and derivatized *via* treatment with ethereal diazomethane. Analyses were performed on a Hewlett Packard HP5890 Series II gas chromatograph coupled to an HP 5989 mass spectrometer using a DB5 (J&W Scientific, Folsom, CA) capillary column (30 x 0.25 mm i.d.) with a 0.25-µm film thickness. The column temperature was held at 50°C for 1 min and increased to 310°C at 10°C min^{-1}, and this final temperature was maintained for 10 min. The mass spectrometer was operated at a 70 eV electron ionization energy. The injector and analyzer temperatures were set at 290°C and 315°C, respectively. The samples (1 µL) were injected in splitless mode using helium as the carrier gas at a flow rate of 1.1 $mLmin^{-1}$. When possible, products were identified and quantified through comparison of their MS spectra and GC retention times with those obtained for authentic commercial standards. When authentic products were not available, identification was suggested on the basis of data in databases (National Institute of Standards and Technology).

2.3.3. Bioaccessibility experiments with exudates

The bioaccessibility estimations relied on the determination of residual concentrations of native PAHs when ^{14}C-tracer biodegradation decreased in radiorespirometry assays performed in parallel. (Posada-Baquero et al., 2008; Niqui-Arroyo and Ortega-Calvo, 2010; Bueno-Montes et al., 2011; Posada-Baquero and Ortega-Calvo, 2011). To measure pyrene mineralization by indigenous bacteria in the presence or absence of sunflower root exudates, 1 g of soil was suspended in 70 mL of MM or sunflower root exudates. The suspensions were placed in 250 mL Erlenmeyer flasks under sterile conditions, and each treatment was performed in duplicate. Each of the flasks contained 30000 dpm of radiolabeled pyrene (58.7 $mCi \cdot mmol^{-1}$, radiochemical purity >98%) in 1 mL of MM. The flasks were sealed with Teflon-lined stoppers and were maintained at 25ºC on a rotary shaker operating at 80 rpm. The production of $^{14}CO_2$ was

measured as the radioactivity appearing in an alkali trap. The trap consisted of a 5 ml vial suspended from the Teflon-lined stopper; the vial contained 1 ml of NaOH (0.5 M). Periodically, the solution was removed from the trap and replaced with fresh alkali. The NaOH solution was mixed with 5 ml of a liquid scintillation cocktail (Ready Safe; Beckman Instruments), and the mixture was maintained in darkness for approximately 8 h to allow dissipation of chemiluminescence. Radioactivity was then measured with a liquid scintillation counter (model LS5000TD; Beckman Instruments).

To determine the biodegradation of the native PAHs present in the soil, separate duplicate flasks were incubated under the same conditions, but without addition of the ^{14}C-labeled compound. At the end of the incubation period (250 h), extraction and analysis of the PAHs present in the soil mixture suspension were conducted with a Soxhlet apparatus and then by HPLC (residual contents in aqueous phase are under the detection limit) by the same method as described above (Point 2.2.2). Analysis of microbial communities from cultures with and without exudates was performed as described previously in sections 2.2.3.2. and 2.2.3.3.

2.4 Statistical methods

Analysis of variance (ANOVA) and Tukey honest significant differences (HDS) were used to assess the significance of means, and Student's t-test was used to determine the significance of percentages. These statistical analyses were performed using SPSS v. 19 software. Differences obtained at the $p \leq 0.05$ level were considered to be significant.

3. Results

3.1 Greenhouse experiment

3.1.1 Plant response

All of the sunflower seeds germinated in both contaminated and uncontaminated soils within 15 days of the beginning of the experiment. However, after 90 days, the average stem height (67 cm) and dry weight of whole plants (6.51 g) were significantly higher ($p \leq 0.05$) in plants grown in contaminated soil than in those developing in uncontaminated soil (57.9 cm and 4.46 g, respectively). These differences may be related to the autoclaving procedure used for the uncontaminated soil. Additionally, the activity of microorganisms introduced into the soil mixture with the creosote-polluted soil may have been beneficial for the plants due to mobilizing soil nutrients. Therefore, the good development of plants in contaminated soil is an indirect indicator of the origin of the microbial populations developed during the greenhouse experiment.

3.1.2 Dissipation of PAHs in pots with polluted soil

Measurement of residual PAH concentrations showed the promoting effect of planting *H. annuus* on the dissipation of these chemicals from soil (Table 1). The concentrations of anthracene, fluoranthene, pyrene and crysene in planted soils decreased significantly below the levels detected in the unplanted controls after 45 and 90 d. A positive effect of planting on the dissipation of fluorene was only observed after 45 d, and its concentration remained below the detection limit in both planted and unplanted soils after 90 days. The presence of sunflower plants had no significant effect on the dissipation of phenanthrene in any of the sampling periods. The increased dissipation was reflected in the significantly lower ($P \leq 0.05$) concentration of total PAHs in planted pots compared to the unplanted controls, which resulted in a 60% additional decrease in the total PAH content in both sampling periods. With the exception of fluorene, extending the experimental period to 90 days did not result in a significantly lower

residual concentration of any of the PAHs in the soils. The chemical analysis of major soil characteristics (e.g., pH, texture) did not reveal significant differences after planting with sunflowers, with the only difference being found in the content of total organic carbon, which increased in the planted soils from 0.9 to 2.1% after 90 days.

Table 1
Effect of planting with sunflowers on residual PAH contents (mg kg^{-1}) in soil under greenhouse conditions after 45 and 90 days.

PAHs	C_0	45 days		90 days	
		Control	Planted	Control	Planted
Fluorene	1.23 ± 0.08A	0.18 ± 0.03B	0.04 ± 0.01C	< 0.00C	< 0.00C
Phenanthrene	3.98 ± 0.29A	0.77 ± 0.06B	0.39 ± 0.03B	0.54 ± 0.05B	0.12 ± 0.05B
Anthracene	8.14 ± 0.39A	2.14 ± 0.45B	0.67 ± 0.14C	1.63 ± 0.37B	0.36 ± 0.13C
Fluoranthene	5.10 ± 0.07A	1.51 ± 0.53B	0.46 ± 0.01C	1.47 ± 0.14B	0.37 ± 0.11C
Pyrene	2.04 ± 0.05A	0.39 ± 0.13B	0.15 ± 0.001C	0.34 ± 0.03B	0.14 ± 0.05C
Chrysene	1.26 ± 0.05A	1.16 ± 0.08A	0.68 ± 0.12B	1.02 ± 0.07A	0.51 ± 0.16B
Total PAHs	**21.75 ± 0.9A**	**6.15 ± 1.26B**	**2.39 ± 0.30C**	**4.99 ± 0.66B**	**1.50 ± 0.5C**

C_o, initial concentration of PAHs in the soil. The values shown are the mean ± standard deviation of triplicates. Values in a row followed by the same capital letter are not significantly different ($P \leq 0.05$).

3.1.3 Analysis of the autochthonous microbiota and its population dynamics

Microbial counts indicated that the soil used in this study was highly enriched in PAH degraders (Fig. 1). The heterotrophic microbial populations increased more than two orders of magnitude between days 0 and 45 under all the conditions. The treatments with plants did not seem to produce an additional enhancement of the growth of the heterotrophs in comparison to the untreated soil. These populations decreased slightly between days 45 and 90, except in the treatment with plants, where they remained at similar levels. This finding could be explained by general depletion of the available carbon sources, which would be compensated for by the rhizosphere in the plant treatment. The LMW PAH-degrading populations also increased in size by approximately two orders of magnitude between 0 and 45 days under all conditions but decreased thereafter in the control soil, while remaining approximately constant in the plant treatment. Interestingly, HMW PAH degraders experienced a substantial increase by 45 d, especially in the plant treatment, and remained at high levels until the end of the experiment. Because at 90 days, the ratio between the LMW PAH degraders and total heterotrophic populations was substantially higher in the treated than in the untreated soil, it could be concluded that in addition to stimulating the general growth of the heterotrophic populations (including that of PAH degraders), the rhizosphere treatment had an additional selective effect of enhancing the growth of the HMW PAH-degrading populations. These results were consistent with those obtained in the PAH analysis and confirm the results obtained by other authors (Miya and Firestone, 2000; Parrish et al., 2005). In addition, these results show that an increase in microbial growth can be obtained by supplementing soil with carbon sources and nutrients (present in exudates) and by improving the biodegradation of PAHs, possibly by increasing their bioaccessibility.

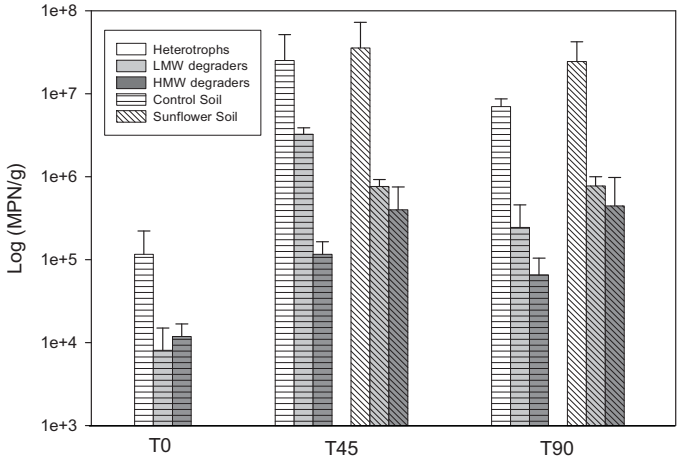

Fig. 1. Counts of heterotrophic and PAH-degrading microbial populations in the soil under the different treatments applied in the greenhouse experiment. MPN, most probable number. LMW, low-molecular-weight PAHs. HMW, high-molecular-weight PAHs.

It is known that the microbial communities in the rhizosphere can be considerably different than those in nearby soil that grow without the direct influence of roots. As a first step in understanding whether the increase in PAH degradation observed in the treated soil containing plants could be related to specific changes in the microbial community structure, we used DGGE and clone library analysis. The DGGE fingerprints obtained during the incubation period from replicate samples for each treatment showed very similar banding profiles (Fig. 2), indicating strong homogeneity within the pots for each condition. In general, the DGGE analysis revealed an initially diverse microbial community, with specific populations increasing in relative abundance throughout the incubation period in both the non-treated and the rhizosphere soil. A number of the bands obtained coincided in the two treatments, but their relative intensities differed, indicating that the shift in community structure induced by the rhizosphere was different than that induced by the simple potting and watering of the polluted soil.

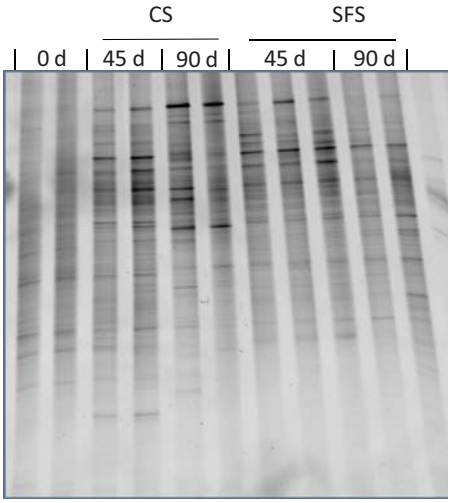

Fig. 2. DGGE profile of PCR-amplified 16S rRNA gene fragments from independent replicate samples from control soil (CS) and rhizosphere sunflower soil (SFS) samples after 0, 45 and 90 days. Each lane was loaded with an identical amount of DNA.

To gain insight into which microbial groups were selectively promoted by the rhizosphere in comparison to the non-treated soil, corresponding 16S rRNA gene libraries were obtained from samples taken at 90 days, and a total of 84 clones were analyzed. Table 2 shows the relative abundance and phylogenetic affiliation of each of the eubacterial populations detected, while Fig. 3 summarizes the importance of the different bacterial phyla in the non-treated and sunflower rhizosphere soils. Approximately two-thirds (60 and 68%) of the bacteria detected under both conditions belonged to the α-, β-, and γ-*Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, and *Chloroflexi* phyla. However, with the exception of the *Actinobacteria*, the relative abundances of these phyla and their compositions varied substantially with the treatment applied, confirming that the plants caused a dramatic shift in the community structure. The rhizosphere promoted the appearance of new populations within the three *Proteobacteria* subphyla, including representatives with a well-known capacity to degrade PAHs (Kanaly and Harayama, 2010). Within the α-*Proteobacteria,* the increase of the *Sphingomonas* group was interesting because this group included numerous members isolated from plant root systems and members with a versatile degrading capability allowing them to attack 2-, 3- and 4-PAHs (Fernandez-Luqueno et al., 2011). There was also a noticeable increase in the β-*Proteobacteria* (from 9% to 27%), as the rhizosphere promoted the appearance of members of the *Commamonas* group showing high similarity matches to members in the database isolated from PAH-contaminated soil or xenobiotic degraders (i.e., *Variovorax)*. In addition, the rhizophere promoted the appearance of members of the *Oxalobacteriaceae*, a recently described but uncharacterized family with root colonizing members (Green et al., 2007) that are closely related to the *Burkholderia*, which include important soil PAH degraders of both simple compounds and creosote mixtures (Grifoll et al., 1995). The increase observed in members of the *Methylophillus* group was interesting because in a recent study, we identified a methylotrophic bacterial species as one of the most abundant components of a heavy fuel-degrading consortium (Vila et al., 2010). Methylotrophic bacteria are more widely distributed than previously thought, but their

roles in natural habitats remain unknown (Lidstrom, 2006; Chistoserdova et al., 2009). The *Xanthomonas* group within the γ-*Proteobacteria* was also favored by the rhizosphere, with several of the detected representatives of this group corresponding to bacteria previously detected in polluted sites and identified as PAH degraders. For example, a *Pseudoxhantomonas* strain was recently described as being able to degrade the 4-ring PAH chrysene (Nayak et al., 2011). The reduction in the abundance of Bacteroidetes in the rhizosphere soil could be a direct consequence of the presence of nutrients from the exudates because this phylum has often been associated with non-nutrient environments (Vinas et al., 2005).

In the non-planted soil, 40% of the detected microorganisms belonged to seven phylogenetic groups not detected in the sunflower planted soil. Interestingly, among these microbes, we found members of the Candidate divisions OD1, OP11, TM7 and WS6, which are lineages of prokaryotic organisms for which there are no reported cultivated representatives but which present sufficiently well-represented environmental sequences to conclude that they represent major bacterial groups (Hugenholtz et al., 1998; Chouari et al., 2005). In addition to these sequences, the sequences retrieved from non-treated soil revealed a relatively high abundance of *Firmicutes*, while the *Planctomycetes* and *Deinococcus* groups were represented with lower proportions. In contrast, the sunflower rhizosphere soil promoted the presence of four phylotypes (*Acidobacteria, Gemmatimonadetes,* δ-*Proteobacteria* and *Cyanobacteria*) that were not detected in the non-treated soil. The most abundant, the *Acidobacteria* (14.6%) and *Gemmatimonadete*s (7.3%), constitute recently described new phyla (Ludwig et al., 1997; Zhang et al., 2003) and are broadly distributed in soils but poorly represented in cultures, which makes it difficult to ascertain their role in nature. The Acidobacteria have been observed previously in planted soil (Sipila et al., 2008; Yrjala et al., 2010), are usually found in non-polluted environments, and generally decrease in the presence of pollutants. Therefore, their higher abundance here after 90 days of treatment may be explained by both the rhizosphere effect and the high degree of removal of PAHs attained in this condition.

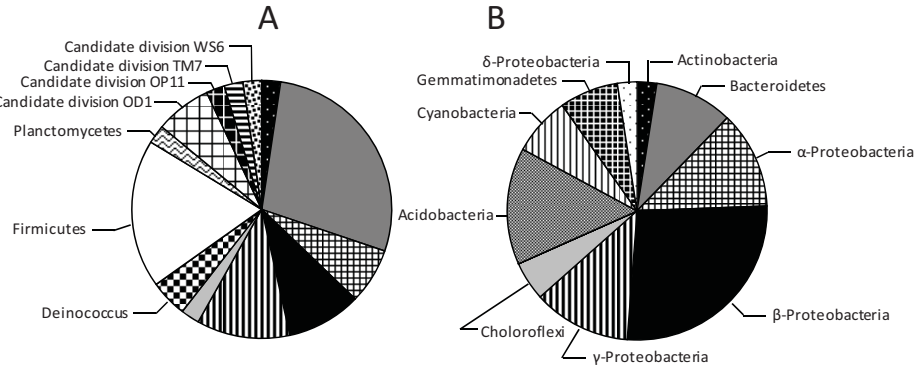

Fig. 3. Relative abundance of eubacterial phylogenetic groups identified in control soil (A) and sunflower rhizosphere soil (B) samples after 90 days of incubation in the greenhouse experiment.

Table 2. Sequence analysis of the 16S rRNA gene clone libraries from the PAH-polluted control soil (without plants) and sunflower rhizosphere soil.

Clone	Frequency (%)[a]		Fragment length (bp)	Sim %	Closest relative in GenBank database [b] (accession no)	Phylogenetic group
	CS	SFS				
CS1		2.4	1364	99	Uncultured *Acidobacteria* bacterium clone HEG_08_216 (HQ597545)	*Acidobacteriaceae (Acidobacteria)*
CS2		2.4	1373	98	Uncultured bacterium clone 60C1 (EU676416)	*Acidobacteriaceae (Acidobacteria)*
CS3		2.4	1422	99	Uncultured *Acidobacteria* bacterium clone SEG_08_603 (HQ729829)	*Acidobacteriaceae (Acidobacteria)*
CS4		2.4	1404	97	Uncultured *Acidobacteria* bacterium clone HG-J02120 (JN409027)	*Acidobacteriaceae (Acidobacteria)*
CS5		2.4	1421	99	Uncultured bacterium clone p6h2ok (FJ478980)	*Acidobacteriaceae (Acidobacteria*
CS6		2.4	1386	97	*Acidobacteria* bacterium IGE-018 (GU187039)	*Acidobacteriaceae (Acidobacteria)*
CS7	2.3		1466	99	Uncultured bacterium clone p27d24ok (FJ478675)	*(Actinobacteria)*
CS8		2.4	1384	99	*Lentzea waywayandensis* strain 173629 (EU570362)	*Pseudonocardiaceae (Actinobacteria)*
CS9	4.7		1444	98	Uncultured bacterium clone 125 (FM209343)	*Cytophagaceae (Bacteroidetes)*
CS10	7.0		1453	99	Uncultured bacterium clone p8c07ok (FJ479495)	*Chitinophagaceae (Bacteroidetes)*
CS 11	2.3		1412	98	Uncultured bacterium isolate 1112864242247 (HQ120332)	*Ohtaekwangia (Bacteroidetes)*
CS 12	2.3		1314	98	Uncultured *Bacteroidetes* bacterium clone HG-J01164 (JN408934)	*Ohtaekwangia (Bacteroidetes)*
CS 13	2.3		1443	95	Uncultured bacterium clone TX2_4C19 (JN178178)	*Rhodothermaceae (Bacteroidetes)*
CS 14	2.3		1329	92	Uncultured bacterium clone p27c17okm (FJ479173)	*Ohtaekwangia (Bacteroidetes)*
CS 15	4.7	4.9	1445	98	Uncultured *Bacteroidetes* bacterium clone g31 (EU979040)	*Ohtaekwangia (Bacteroidetes)*
CS 16	2.3	2.4	1393	99	Uncultured bacterium clone 224T (EU676412)	*Chitinophagaceae (Bacteroidetes)*
CS 17		2.4	1394	98	Uncultured soil bacterium clone UA2 (DQ298006)	*Chitinophagaceae (Bacteroidetes)*
CS 18	2.3		1475	99	Uncultured *Anaerolinae* bacterium clone AMAG11 (AM935836)	*(Choloroflexi)*
CS 19		2.4	1353	99	Uncultured bacterium clone S-Rwb_62 (DQ017911)	*(Choloroflexi)*
CS 20		2.4	1353	99	Uncultured bacterium clone H3-26 (JF703479)	*(Choloroflexi)*
CS 21		4.9	1373	99	*Phormidium autumnale* CCALA 143 (FN813344)	*(Cyanobacteria)*
CS 22		2.4	1324	99	Uncultured diatom clone H-101 (HM565019)	*Bacillariophyta (Cyanobacteria)*
CS 23	4.7		1455	96	Uncultured bacterium isolate 1112864242286 (HQ120393)	*Trueperaceae (Deinococcus)*
CS 24	2.3		1423	99	*Bacillus* sp. M71_D96 (FM992837)	*Bacillaceae (Firmicutes)*
CS 25	9.3		1440	99	*Bacillus* sp. R-36493 (FR682744)	*Bacillaceae (Firmicutes)*
CS 26	2.3		1486	98	*Virgibacillus carmonensis* (T) LMG 20964 (NR_025481)	*Bacillaceae (Firmicutes)*
CS 27	4.7		1438	99	*Bacillus* sp. BF149 (AM934692)	*Bacillaceae 2 (Firmicutes)*
CS 28		4.9	1399	96	Uncultured bacterium clone 15-4-139 (JN609373)	*Gemmatimonadaceae(Gemmatimonadetes)*
CS 29		2.4	1429	98	Uncultured bacterium clone TX5A_120 (FJ152828)	*(Gemmatimonadetes)*
CS 30	2.3		1368	94	Uncultured bacterium clone B6 (FJ660498)	*Planctomycetaceae (Planctomycetes)*

CS 31	2.3		1285	99	*Chelatococcus asaccharovorans* CP141b (AJ871433)	*Methylobacteriaceae (α)*
CS 32	2.3		1276	99	Uncultured bacterium clone HDB_SIOP800 (HM186473)	*Bradyrhizobiaceae (α)*
CS 33	2.3	2.4	1410	97	Uncultured soil bacterium clone F6-154 (EF688392)	*Sphingomonadaceae (α)*
CS 34		2.4	1342	99	*Rhizobium* sp. AC93c (JF970343)	*Rhizobiaceae (α)*
CS 35		2.4	1355	97	Uncultured bacterium clone FCPS478 (EF516121)	*Rhizobiales (α)*
CS 36		2.4	1353	99	Uncultured alpha proteobacterium clone QZ-J4 (JF776915)	*Sphingomonadaceae (α)*
CS 37		2.4	1355	99	*Altererythrobacter* sp. JM27 (GU166344)	*Sphingomonadales (α)*
CS 38	2.3		1425	98	Uncultured bacterium clone SNR65 (AB608675)	*Burkholderiales (β)*
CS 39	4.7	7.3	1418	99	Uncultured bacterium clone HC18-11B13 (JF417848)	*Commamonadaceae (β)*
CS 40	2.3	4.9	1410	99	*Naxibacter suwonensis* (T) 5414S-25 (FJ969487)	*Oxalobacteraceae (β)*
CS 41		2.4	1389	99	*Variovorax* sp. RA8 (AB513921)	*Commamonadaceae (β)*
CS 42		2.4	1396	99	Uncultured beta proteobacterium clone E2006TS6.19 (GU983311)	*(β)*
CS 43		2.4	1394	99	Uncultured beta proteobacterium clone C173 (JF833705)	*Methylophilaceae (β)*
CS 44		2.4	1400	99	Uncultured beta proteobacterium clone C173 (JF833705)	*Methylophilaceae (β)*
CS 45		2.4	1391	99	Uncultured beta proteobacterium clone G2-50 (JF703344)	*(β)*
CS 46		2.4	1391	99	Uncultured ammonia-oxidizing bacterium clone FQ-13C-HF-1 (HQ678202)	*Nitrosomonadaceae (β)*
CS 47	2.3		1434	97	Uncultured bacterium clone 0-99 (GU444064)	*Xanthomonadaceae (γ)*
CS 48	9.3		1458	99	*Pseudomonas* sp. JQR2-5 (DQ124297)	*Pseudomonadaceae (γ)*
CS 49		2.4	1412	99	*Lysobacter niabensis* (AB682414)	*Xanthomonadaceae (γ)*
CS 50		2.4	1391	99	Uncultured bacterium clone Kas172B (EF203204)	*Sinobacteraceae (γ)*
CS 51		2.4	1409	99	*Pseudoxanthomonas* sp. XC21-2 (JN247803)	*Xanthomonadaceae (γ)*
CS 52		2.4	1410	99	Uncultured *Lysobacter* sp. clone T302B2 (HM438520)	*Xanthomonadaceae (γ)*
CS 53		2.4	1399	99	Uncultured bacterium clone BR121 (HQ190468)	*Pseudomonadaceae (γ)*
CS 54		2.4	1419	97	Uncultured bacterium clone RH1020 (AB511013)	*(δ)*
CS 55	7.0		1415	86	Uncultured candidate division OD1 bacterium clone AKYH1067 (AY922093)	Candidate division OD1
CS 56	2.3		1396	94	Uncultured bacterium clone B03-05G (FJ542974)	Candidate división OP11
CS 57	2.3		1368	97	Uncultured bacterium clone N1903_34 (EU104291)	Candidate división TM7
CS 58	2.3		1250	93	Uncultured bacterium clone FF_-aag84c04 (EU469637)	Candidate division WS6

[a] Frequencies in clone libraries obtained from CS (control soil) and SFS (sunflower soil). [b]In sequences showing an identical match to uncultured and to isolated strains, only the latter are listed. Sequences with more than 94% identity are grouped. α, β, γ, δ correspond to *alpha-*, *beta-*, *gamma-* and *deltaproteobacteria*, respectively.

3.2. In vitro production of exudates

3.2.1 Production

After 30 days, the average of germination rate was 55.33%, with the growth per day in terms of weight being 74 mg. The average fresh and dry weights of roots were 12.04 and 0.965 g, respectively, and the levels of TOC produced by the exudates were between 54.4 and 339 mgL^{-1}, with an average of 129.73 mgL^{-1}. A direct significant linear correlation (R=0.9125) ($p \leq 0.05$) was established between the RGR (74 mg per day) and fresh weight (12.04 g). There was also a direct linear correlation found between TOC (129.73 mgL^{-1}) with RGR (R=0.7293) and TOC with fresh weight (R=0.6366), although these correlations were not statistically significant.

3.2.2. Exudate Composition

Table 3 shows the compounds identified in the sunflower root exudates using different analytical techniques, including carbohydrates, amino acids, fatty acids, aromatic acids and certain secondary metabolites. As major carbohydrates, we identified fructose (2.44 ppm) and galactose (1.16 ppm); however, the chromatogram also showed a major unidentified peak that would have interfered with the detection of glucose if it had been present. Previous studies addressing exudate composition in tomato, sweet pepper, cucumber and Barmultra grass showed that fructose was one of most dominant sugars (Kuiper et al., 2002; Kamilova et al., 2006). Galactose is also present in root exudates, providing a favorable environment for the growth of microorganisms (Bertin et al., 2003), and has been detected in the root exudates of different species of Eucalyptus (Grayston et al., 1996). Amino acids were detected in a wide range of concentrations, among which asparagine (0.593 ppm) and glutamine (0.301 ppm) were the most abundant, while methionine, tryptophan, proline, glutamic acid and valine were not detected. Phosphoethanolamine was also detected at a relatively high concentration (0.571 ppm) and has been reported to be abundant in the cell membrane (Ofosubudu et al., 1990). The main fatty acids present were palmitic and estearic acids, whereas others, including the most abundant component of sunflower oil, linoleic acid, were detected at lower concentrations and could not be quantified. Several aromatic acids were identified, the most abundant of which were phthalic and protocatechuic acids. This result is of particular note, given that these compounds are typical intermediates in the metabolism of PAHs by bacteria (Kanaly and Harayama, 2010). The HPLC-MS analysis of organic acids revealed several products. The most intense signal corresponded to a compound with a mass compatible with gluconic acid. Other products were tentatively identified as caffeic, isocitric, butiric, pyruvic, propionic, fumaric, malic, and malonic acids, all of which are typically found in root exudates (Bertin et al., 2003). Abietic acid and the sesquiterpene tomentosin were identified as the methyl derivatives of organic acids in the GC-MS analysis, and in addition to having structures analogous to some PAHs, they exhibit different functions in the plant-microbe interaction.

In vitro, the sunflower root exudates showed a surface tension close to the surface tension of the mineralization medium (MM), showing either an absence or a low concentration of surfactants that could improve accessibility (Garcia-Junco et al., 2001).

Table 3. Organic compounds identified in the sunflower root exudates obtained in vitro.

Class of Compounds	Single compounds	Concentration (ppm)
Sugars[1]	Galactose	1.16
	Fructose	2.44
Amino acids[1]	Phenyl serine	0.423
	Taurine	0.083
	Phosphoethanolamine	0.571
	Aspartic acid	0.035
	Threonine	0.034
	Serine	0.069
	Asparagine	0.593
	Glutamine	0.301
	Glycine	0.016
	Alanine	0.017
	Cysteine	0.003
	Isoleucine	0.025
	Leucine	0.026
	Tyrosine	0.029
	Phenylalanine	0.012
	Ornithine	0.008
	Lysine	0.024
	Histidine	0.074
	Arginine	0.078
Fatty acids[3]	Azelaic acid[2]	nq
	Myristic acid[2]	nq
	Palmitic acid[1]	0.0353
	Linoleic acid[2]	nq
	Stearic acid[1]	0.0425
Aromatic organic acids[3]	Phtalic acid[1]	0.04358
	Paraben[2]	nq
	Protochatechuic[1]	0.00388
	Gallic acid[2]	nq
	5-Acetylsalicylic acid[2]	nq
	Abietic acid[2]	nq
	Hydroxydehydroabietic acid[2]	nq
Terpenoids[3]	Tomentosin[2]	Nq

*Identification was based on analysis of authentic standards[1] or on a match higher than 90% with the NIST library[2]. Identified as their methylated derivates[3] (diazomethane); nq= not quantified.

3.2.3. Effects of exudates on the bioaccessibility of PAHs and on soil microbial populations

Bioaccessibility experiments showed that the maximum rate of pyrene mineralization was enhanced twofold by the presence of exudates (from 0.024 ± 0.002 ng ml^{-1} h^{-1} to 0.052 ± 0.008 ng ml^{-1} h^{-1}, Figure 4). The maximum extent of pyrene C mineralization was also enhanced (from 29 ± 1.01% to 40 ± 1.41%), and the acclimation phase for pyrene mineralization was shortened from 75 h to 30 h. Interestingly, the results showed that the concentrations of total PAHs decreased to significantly lower values in the presence of exudates (Table 4), thereby demonstrating the positive influence of exudates on biodegradation for native chemicals. Furthermore, the residual contents of total PAHs, both with and without exudates, were not significantly different than those reached in the corresponding treatments in greenhouse experiments after 90 days ($P \leq 0.05$). Therefore, we can conclude that the degradation-promoting

effect of sunflower plants on the dissipation of PAHs from soil that occurred in the greenhouse experiment could be reproduced through laboratory incubation of the soil with shaking and the addition of root exudates.

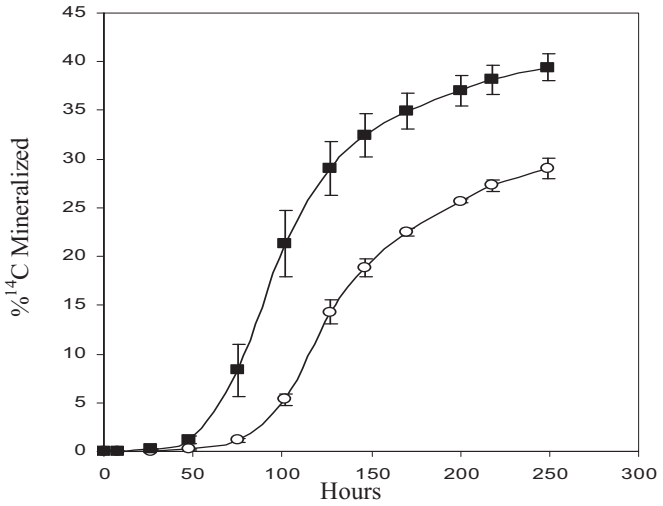

Fig. 4. Mineralization of pyrene in soil suspensions in the absence (o) and presence (■) of sunflower root exudates. Error bars represent the standard deviation of duplicates.

3.2.4 Changes in the structure of the soil bacterial community

Soil suspensions were sampled at the end of the experimental period (10 d) to determine the evolution of autochthonous microbiota using DGGE (Fig. 5). The DGGE profiles from cultures in the mineral medium with or without exudates indicated an increase in the number of microorganisms during the 10 days of the experiment in both conditions. In the absence of exudates, duplicate cultures showed similar banding profiles with slight differences in the relative intensity of each band. The banding profile changed as a result of exposure to exudates, which indicates that the enhanced PAH degradation was accompanied by the growth of specific microbial populations.

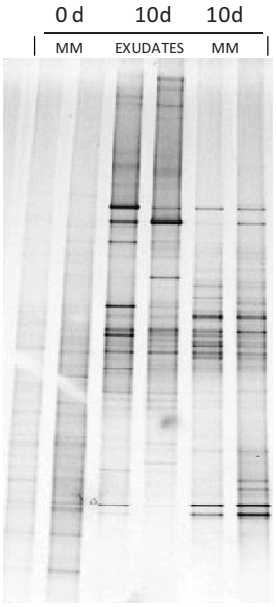

Fig. 5. DGGE profile of PCR-amplified 16S rRNA gene fragments in samples from soil suspensions in the bioaccessibility experiment presented in Figure 4 at the beginning (0 d) and at the end of the experimental period (10 d).

4. Discussion

Our data indicate that the development of sunflower plants enhanced the bioaccessibility of PAHs in the soil. The slowly degrading compounds remaining in the soil at the end of greenhouse and bioaccessibility assays probably exhibited slow desorption, which usually limits biodegradation of these compounds by microorganisms (Gomez-Lahoz and Ortega-Calvo, 2005; Bueno-Montes et al., 2011). This restriction on biodegradation would explain the absence of further decreases in the PAH concentrations in unplanted soils from 45 d to 90 d in the greenhouse experiment and the good agreement between the residual PAH concentrations in the greenhouse and bioaccessibility assays. The bioaccessibility experiments were designed to test the disappearance of the chemicals under laboratory conditions. These assays specifically addressed biodegradation using an excess of nutrients, radiorespirometry determinations with ^{14}C-pyrene and analysis of residual concentrations of native PAHs. This method had been applied previously to determine the efficiency of bioremediation approaches designed to increase the bioaccessibility of aged PAHs (Niqui-Arroyo and Ortega-Calvo, 2010; Bueno-Montes et al., 2011) and to determine the recalcitrant nature of background PAH soil pollution (Posada-Baquero and Ortega-Calvo, 2011). Despite the inherent difficulties in performing bioaccessibility estimations related to the specific the time period and/or target organisms considered (Alexander, 2000; Semple et al., 2004; Reichenberg and Mayer, 2006), this approach was very useful in the present study for reproducing the greenhouse results in the presence of root exudates produced *in vitro*, which indicates that the exudates played an important role in the effectiveness of the plants in promoting the bioaccessibility of PAHs.

The TOC content observed in the sunflower root extracts in this study, 129.73 mg L^{-1}, was representative of TOC values reported in other studies on the promoting effects of root extracts on PAH-degrading microorganisms. For example, Rentz et al. (2005) reported TOC concentrations of 84.2, 175.0 and 51.7 mgL^{-1} from root extracts of hybrid willow (*Salix alba x matsudana*), kou (*Cordia subcordata*) and milo (*Thespesia populnea*), respectively, whereas Miya and Firestone (2001) reported a TOC concentration of 54 mg L^{-1} for slender oat root exudates. In the present study, it is possible that the organic carbon in the exudates enhanced the bioaccessibility of PAHs through a mechanism related to the carbon's capacity to mobilize PAHs that are initially absorbed in the soil. Indeed, addition of DOM to contaminated soils results in enhanced biodegradation of PAHs, probably as a result of enhanced desorption (Haderlein et al., 2001; Bengtsson and Zerhouni, 2003; Bogan and Sullivan, 2003). DOM-mediated enhancement of biodegradation can also be caused by direct access to DOM-sorbed PAHs due to the physical association of bacteria and DOM (Ortega-Calvo and Saiz-Jimenez, 1998) and an increased diffusive flux toward bacterial cells (Haftka et al., 2008; Smith et al., 2009). The latter mechanism would be analogous to that described for the enhanced uptake of metals by plants in the presence of labile metal complexes, which is caused by an increased diffusional flux through unstirred boundary layers around roots (Degryse et al., 2006). The occurrence of DOM-mediated enhancement of bioaccessibility through root exudation would also explain the greater extent of biodegradation observed under greenhouse conditions, despite the significant increase of total organic carbon in the planted soils.

The chemical characterization of exudates also identified specific substances with the potential to directly enhance bioaccessibility. These substances include chemicals that are able to induce chemotaxis, which constitutes a relevant mobilization mechanism for motile microorganisms in the soil (Ortega-Calvo et al., 2003). For example, sugars such as fructose have a positive chemotactic effect on soil microorganisms (Heinrich and Hess, 1985). Amino acids, such as glutamine, aspartic acid and isoleucine, which were also found in this study as components of sunflower root exudates, are powerful chemoattractants for *Rhizobium* and *Bradyrhizobium japonicum* (Pandya et al., 1999). Zheng and Sinclair (1996) indicated that alanine, asparagine, glutamine, serine, and threonine in soybean root exudates may serve as chemoattractants to *Bacillus megaterium* strain B153-2-2. Finally, we detected fatty acids, such as palmitic acid and stearic acid, which are plant components with a known potential to enhance the bioaccessibility of PAHs in soil by acting as surfactants (Yi and Crowley, 2007). Vegetable oils have also been widely used as natural surfactants (Pannu et al., 2004; Gong et al., 2010), resulting in the dissolution of PAHs and consequently, in the enhancement of biodegradation. Therefore, the presence of these compounds may explain the greater decrease in PAHs observed in the sunflower soil treatments. Furthermore, it is also possible that the preferential growth of rhizosphere microorganisms observed on the exudate components at specific sites inside soil aggregates may have caused colony growth in the vicinity of pollutant sources and may have modified the structure of the soil aggregates to promote bioaccessibility through the excretion of extracellular polymeric substances and biosurfactants.

Therefore, the results obtained associated with root exudates indicated a role for promoting the bioaccessibility of PAHs. However, the present study may not allow complete discrimination between the effects on bioaccessibility from the enhanced biodegradation activity of microorganisms caused by the chemical components of exudates. The evolution of the heterotrophic bacterial population in the soil during the greenhouse experiment indicates that homogenization, aeration and watering had a general activation effect on this population, but planting sunflowers had a further positive impact due to maintaining their viability (Fig. 1). The chemical analysis of exudates reflected the presence of organic compounds in the root exudates with the potential to cause this effect. For example, fructose and galactose are known to provide a favorable environment for the growth of rhizosphere microorganisms (Grayston et al., 1996; Bertin et al., 2003); amino acids are a source of easily degradable N compounds, inducing

protease synthesis (Garcia-Gil et al., 2004); and ornithine and taurine are considered to be non-protein amino acids showing a protective function against stress to cell membranes (Hao et al., 2004; Kalamaki et al., 2009). Furthermore, the presence of plants had also a profound impact on the relative abundance of specific groups of bacteria in the soil, thereby increasing their biodiversity. The proportion of gram-negative bacteria increased in planted soils compared with unplanted controls, which is in agreement with previous observations (Anderson and Coats, 1995). For example, we observed better development of β-*Proteobacteria* in planted soils, which can be explained by the capability of this group of bacteria to readily assimilate the C present in sugars and residues of plant origin (Fierer and Jackson, 2006; Bernard et al., 2007).

Interestingly, certain aromatic organic acids were detected in the root exudates, such as phthalic and protocatecuic acids, that are intermediate metabolites in the degradation of PAHs by different bacterial groups, such as *Mycobacterium* for anthracene, pyrene, and fluoranthene degradation (Vila et al., 2001; van Herwijnen et al., 2003a; Lopez et al., 2005; Lopez et al., 2008) or *Sphingomonas* for phenanthrene, fluoranthene, anthracene and dibenzothiophene degradation (Bastiaens et al., 2001; van Herwijnen et al., 2003b). These secondary plant metabolites may stimulate PAH degradation by rhizosphere microorganisms and broaden the spectrum of their activity by inducing and promoting the development of organic pollutant-degrading enzymes (Singer et al., 2003) or acting as cosubstrates in cometabolic reactions. Indeed, the population of high-molecular-weight (HMW) PAH degraders increased in number in the planted soils compared with the unplanted controls, demonstrating the selective influence of the sunflower rhizosphere on these populations. These results agree with those from Parrish et al. (2005), who observed that after 12 months of plant development, the PAH degrader population was multiplied 100-fold in comparison with unplanted soil. Corgie et al. (2004) also found that the number of HMW PAH degraders decreased inversely with the distance from roots. Consistent with this selective effect on the PAH-degrading populations, there was a demonstrated increase in the relative abundance of bacterial groups with a know PAH-degrading capability or that were previously detected as key components in PAH-degrading microbial consortia, including *Sphingomonas* (within the α-*Proteobacteria*), *Comamonas*, *Oxalobacteria* and *Methylophillus* (β-*Proteobacteria*), and *Xanthomonas* (Kanaly and Harayama, 2010). Although the relative abundance of the *Actinobacteria* group does not change in the presence of sunflowers, it is known that this a group characterized by its ability to degrade recalcitrant organic compounds. Other microbes that are able to degrade recalcitrant organic compounds include *Actinomycetes,* which are able to compete with fungi for lignin degradation (de Boer et al., 2005), and *Mycobacteria*, which can degrade a variety of PAHs either as individual compounds (Vila et al., 2001; Lopez et al., 2005) or within fuel (Vila et al., 2010) and creosote mixtures (Lopez et al., 2008), particularly at sites where there is a low level of nutrients (Uyttebroek et al., 2006). Other bacterial phyla favored by the rhizosphere, including *Acidobacteria* and the *Gemmatimonadetes*, are recently described groups with few culturable representatives, and more research is needed to understand their potential role in polluted soils (Ludwig et al., 1997; Zhang et al., 2003).

The results presented in this report suggest that sunflower plants could be effective in promoting the bioaccessibility of PAHs in contaminated soils that have previously undergone extensive bioremediation but still contain unacceptable PAH levels, due to bioaccessibility restrictions. Considering the advantages of this plant species in relation to its agronomic interest and potential as a biofuel producer, this strategy seems to represent a promising alternative for increasing bioaccessibility in a sustainable and low-risk manner. Our results demonstrate that the rhizosphere caused a substantial shift in the structure of the autochthonous microbial populations in the soil that selectively favored the development of PAH degraders. Most of the literature discussed herein involves recent work on the effect of the rhizosphere on selected microbial PAH-degrading populations in artificially PAH-spiked soils. This study is the first to analyze the effect of the rhizosphere on autochthonous bacterial community structure from

environmental PAH-polluted soil. The exact contribution of the direct effects of the sunflower exudates and the effects related to the ecology of soil microorganisms will be the subject of future research.

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