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A microcosm system and an analytical protocol to assess PAH degradation and metabolite formation in soils

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Abstract During bioremediation of polycyclic aromatic hydrocarbon (PAH)-polluted soils accumulation of polar metabolites resulting from the biological activity may occur. Since these polar metabolites are potentially more toxic than the parental products, a better understanding of the processes involved in the production and fate of these oxidation products in soil is needed. In the present work we describe the design and set-up of a static soil microcosm system and an analytical methodology for detection of PAHs and their oxidation products in soils. When applied to a soil contaminated with phenanthrene, as a model PAH, and 1-hydroxy-2-naphthoic acid, diphenic acid, and phthalic acid as putative metabolites, the extraction and fractionation procedures resulted in recoveries of 93%, 89%, 100%, and 89%, respectively. The application of the standardized system to study the biodegradation of phenanthrene in an agricultural soil with and without inoculation of the high molecular weight PAH-degrading strain *Mycobacterium* sp. AP1, demonstrates its suitability for determining the environmental fate of PAHs in polluted soils and for evaluating the effect of bioremediative treatments. In inoculated microcosms 35% of the added phenanthrene was depleted, 19% being recovered as CO_2 and 3% as diphenic acid. The latter, together with other two unidentified metabolites, accumulated in soil.

Keywords Biodegradation · Polycyclic aromatic hydrocarbons · Soil microcosms · Phenanthrene · Bacterial PAH metabolites

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Introduction

Characterization of biodegradation processes in soils is a key issue in determining the fate of contaminants in polluted soils and should provide valuable information for the design, optimization and risk assessment of decontamination treatments. PAHs are pollutants of special concern due to their ubiquitous presence in soils contaminated with oil and coal derivatives, their persistence, and potential toxicity/carcinogenicity (Kanaly and Harayama 2000).

Biodegradation routes for 2- and 3-ring PAHs by soil bacteria that use them as a sole source of carbon and energy are well characterized. In general, the studied Gram-negative strains possess a complete set of enzymes for the total assimilation/mineralization of those compounds (Cerniglia 1992). Soil enrichment cultures with four-ring PAHs as a sole carbon source usually leads to the isolation of actinobacteria,

often belonging to the genus *Mycobacterium*. These *Mycobacterium* strains oxidize 3- and 4-ring PAHs using multibranched pathways that seem to include both, productive routes potentially leading to the complete assimilation/mineralization of the substrates, and non-productive routes that result in the accumulation of partially oxidated compounds in the medium, for which further degradation has not been demonstrated (i.e., ketones, quinones, and dicarboxylic acids) (Vila et al. 2001; López et al. 2006; Kim et al. 2005).

It is also known that, due to the relaxed specificity of some degradative enzymes (Selifonov et al. 1995), PAH-degrading bacterial strains may act on chemical analogues of their growth substrates producing cometabolic or partial degradation products that also accumulate in the medium. For example, the fluorene degrading strain *Bulkholderia cepacia* F297 was shown to grow on creosote-PAHs utilizing some of the components as a carbon source while transformed others to a number of polar aromatic compounds (Grifoll et al. 1995). These types of processes are expected to naturally occur in soils and may be stimulated by bioremediative strategies. In fact, a few studies have demonstrated the presence of some aromatic ketones and carboxylic aromatic acids in polluted soils and sediments (Fernández et al. 1992; Meyer et al. 1999).

There are a few available standardized methods for the detection of PAHs and their oxidation products in soils (Meyer and Steinhart 2000; Langbehn and Steinhart 1994); however, the complexity of the contaminant mixtures, the microbial communities, and the soil matrix make it enormously difficult to accurately describe the biodegradation processes (Alexander 1999). Soil microcosms provide a way to study microbial interactions with contaminants in a controlled and reproducible way, while retaining the complexity of the matrix and being representative of the processes occurring in the field. Laboratory microcosms allow the measuring of biodegradation as well as mineralization (CO_2 production) rates (Kästner and Mahro 1996) and can be used to study the effect of bioremediation treatments, including bioaugmentation (Wagner-Döbler et al. 1992; Reid et al. 2001; Cavalca et al. 2002). Radio labeled substrates are widely used in microcosms to quantify mineralization rates; however, this methodology has some disadvantages, such as the contamination of laboratory material and the analytical equipment (Schoef et al. 2005).

The objective of the present work was to develop and validate a microcosm system and the analytical procedures necessary to describe the biodegradation processes involved in the fate of PAHs in polluted soils, including kinetics of depletion of parent compound, mineralization rates, and potential accumulation of partially oxidated compounds. The system is applied to the study of degradation of phenanthrene as a model PAH in an agricultural soil, and to a soil inoculated with *Mycobacterium* sp. AP1, a strain isolated for its capacity to utilize pyrene, but that also degrades phenanthrene in liquid cultures accumulating several oxidation products (Vila et al. 2001).

Materials and methods

Soil characteristics

The soil used was a non-contaminated agricultural soil obtained from Andújar, Spain, with a pH value of 8.2, water holding capacity of 0.25 ml/g and an organic matter content of 2.6%. The soil was air dried, sieved through a 2 mm mesh screen and stored at 4°C until needed.

Microcosms system design and operation

The basic system initially designed consisted of a total of 24 microcosms, each one connected to an individual alkali trap by polytetrafluoroethylene (PTFE) tubing. Groups of six microcosms were connected to a common humid air distributor flask, and all the humid air distributors were connected to a glass air manifold, which was finally connected to the air pump (Air Admiral aquarium pump, Cole-Parmer, USA). Before entering the glass manifold distributor, the air produced by the pump was passed through a carbon dioxide trap (a 500 ml Pyrex bottle with a screw cap and an air diffuser submerged in 2 M NaOH), an activated carbon filter (a glass cylinder, filled with granulated activated carbon, 4–12 mesh, Aldrich, Milwaukee, WI), and a biological filter (cellulose acetate, 0.2 μm, 45 mm diameter, Schleicher & Schuell, Dassel, Germany). This way each

microcosm received CO_2-free sterile fresh air that, after being loaded with the CO_2 produced, was directed to the corresponding CO_2 trap (Fig. 1).

During the setup of the system several experiments were carried out using agricultural soil with glucose (0.1 and 1%) as an easy mineralizable substrate, in which different types of microcosm flasks, traps, lining materials, connections, and general settings were assayed. In these experiments each data point corresponded to the mean of the CO_2 production values obtained from at least triplicate microcosms with glucose. Triplicate microcosms without carbon source served as controls to measure the intrinsic respiration of soil, while triplicate microcosms with sodium azide (0.05 M) served as negative (abiotic) controls. Every group of six microcosms was air flushed (approximately 150 ml/min) for 10 min every 24 h. The rest of the time the system was kept closed. For each data point the corresponding microcosms and traps were removed from the system and the amount of CO_2 trapped was determined by titration.

In the final system used to test PAH degradation, the microcosms and CO_2 traps consisted of 100 ml-Pyrex® flasks with open screw caps equipped with silicon (top) and PTFE (bottom) liners. The humid air distributors consisted of similar flasks with 250-ml capacity. All the tubing conducting the air was PTFE (1 mm i.d.) and was fitted directly through the silicon and PTFE septa. The PTFE tubing was connected to other elements of the system (i.e., glass distributor manifold) by short sections of silicon tubing (2 or 4 mm i.d.) and each one was provided with a hosecock clamp. Similarly, the outlet PTFE tube of each CO_2 trap was connected to a small section of silicone tube with a clamp, which allowed keeping the system closed. Each microcosm contained 20 g of soil (dry weight), while the traps and humid air distributors contained 50 ml of 0.05 M NaOH, and 100 ml of distilled H_2O, respectively. Every group of six microcosms was flushed during 10 min every 48 h. The rest of the time the system was kept closed. Given that 100 ml of air contains about 890 micromoles of oxygen, and the obtained mineralization rates (maximum rates of CO_2 production were 40 micromoles/48 h), the amount of oxygen supplied largely exceeded the oxygen consumed. Therefore, phenanthrene mineralization was never limited by oxygen supply. At each indicated time the corresponding microcosms and traps were removed for analysis, the CO_2 trapped being determined by titration.

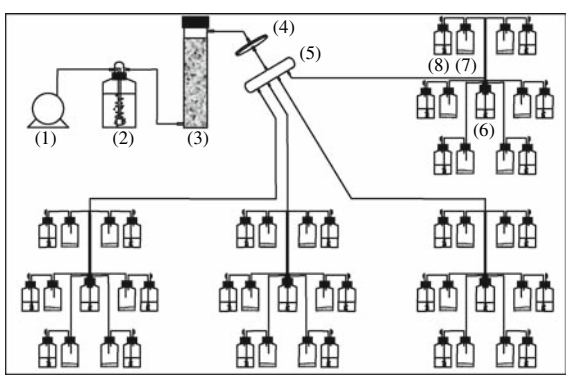

Fig. 1 Design and components of the basic microcosms system. (1) Air pump, (2) Initial CO_2 trap, (3) carbon-activated filter, (4) biological filter, (5) glass manifold distributor, (6) humidifier, (7) microcosm flask, (8) CO_2 microcosm trap

CO_2 production measurement

The quantity of total CO_2 trapped was determined by acid–base titration (Bartha and Pramer 1965). The carbonates of 20 ml aliquots of the contents of each trap were precipitated with 2 ml of a barium chloride ($BaCl_2 \cdot H_2O$) solution (1 M) and phenolphthalein was added as indicator. The mixture was titrated with standardized hydrochloric acid (HCl, 0.05 M).

Set-up and validation of an analytical protocol for detection of PAH-metabolites

The chemical fractionation procedures for neutral extracts were set up using a stock solution containing phenanthrene, 9-fluorenone, 1-hydroxy-2-naphthoic acid, and 2-naphthoic acid (4 mg/ml each) in dichloromethane; while the clean-up process for the acidic extract was assayed with a stock solution of phthalic acid and diphenic acid (4 mg/ml each).

The chemical extraction procedures were set up using 20 g of soil contaminated with an acetone solution containing phenanthrene (final concentration in soil 1.25 mg/g), 1-hydroxy-2-naphthoic acid, phthalic acid and diphenic acid (1 mg/g each). The soil was dried by combining it with anhydrous sodium sulphate (10 g). The mixture was then extracted by sonication with dichloromethane: acetone (2:1 v/v)

(5 × 40 ml). In order to improve recoveries for neutral and acidic metabolites during the set-up and optimization of the method we assayed adjusting the pH of the soil to different values by adding HCl (1 M).

The organic extracts were concentrated to near dryness in a rotary evaporator, dried under a gentle stream of dry nitrogen, and dissolved in 1 ml of the appropriate solvent. The chemical fractionation and clean-up of the extracts was performed on Baker-Bond® SPE-Mini glass columns (20 mm i.d., 8 ml capacity; J.T. Baker Inc., Deventer-Holland) fitted with BakerBond® PTFE frits, and set in a SPE-12G Baker® column system.

Dry neutral extracts were dissolved in 1 ml of hexane and transferred to a column packed with 2.5 g of silica gel (70–230 mesh, Merck, Darmstadt-Germany) previously activated at 120°C and deactivated with 5% water. Three fractions were eluted as follow: F1 (15 ml 20% dichloromethane in hexane) to collect PAHs, F2 (1 ml dichloromethane followed by 7 ml of methanol) to collect neutral metabolites, and F3 (15 ml of acidic methanol, 0.05N HCl) to collect possible acidic metabolites. Each fraction was dried and dissolved in methanol (10 ml for F1, and 1 ml for F2 and F3) for HPLC analysis. Later, 500 µl from each of those solutions were transferred to 100 µl of dichloromethane and derivatized with diazomethane for GC-MS analysis.

Dry acidic extracts were dissolved in methanol and cleaned-up in mini glass columns packed with BakerBond® Octadecyl (C18) 40 µm Prep LC Packing (J.T. Baker Inc., Deventer-Holland), using 20 ml of acidic methanol (0.05 N HCl) as eluent. The cleaned extracts were concentrated to 1 ml of methanol and analysed by HPLC and GC-MS as indicated for the neutral extracts.

Phenanthrene biodegradation in soil microcosms inoculated with *Mycobacterium* sp. AP1

Mycobacterium sp. AP1, isolated from a sand sample from a crude oil-contaminated beach in Tarragona (Spain), is able to utilize pyrene, fluoranthene and phenanthrene as sole sources of carbon and energy (Vila et al. 2001). Pure cultures of *Mycobacterium* sp. AP1 are routinely maintained by subculturing in mineral salts agar medium (mineral salts medium [$KH_2PO_4 \cdot 3H_2O$, 4.25 g; $NaH_2PO_4 \cdot H_2O$, 1.0 g; NH_4Cl, 2.0 g, $MgSO_4 \cdot 7H_2O$, 0.2 g; $FeSO_4 \cdot 7H_2O$, 0.012 g; $MnSO_4 \cdot H_2O$, 0.003 g; $ZnSO_4 \cdot 7H_2O$, 0.003 g; $CoCl_2 \cdot 6H_2O$, 0.001 g; $C_6H_7NO_6Na_2$, 0.123 g per liter] solidified with 15 g/l of agar) with pyrene (0.2 g/l) as a carbon source. Cells used to inoculate the soil microcosms were harvested from cultures grown for 7 days in modified Luria Bertrani agar (Bactopeptone [Difco, Detroit, MI] 10 g, glucose 5 g, yeast extract 2.5 g, NaCl 2 g, agar 15 g, per liter of mineral salts medium) at 30°C, and were resuspended in phosphate buffer (3.2×10^6 cell/ml; pH 7.2).

Soil microcosms were prepared with 20 g of agricultural soil (dry weight) that was then contaminated by adding 25 mg of phenanthrene in acetone solution (25 mg/ml). The solvent was allowed to evaporate for 4 h and the humidity was adjusted to 80% of the water holding capacity with sterile mineral medium. The pH of soil was not adjusted and no additional nutrients were used. Four different series of microcosms were set up: *Soil with no additions*, to determine the intrinsic respiration due to the utilization of the organic mater in the soil by soil indigenous microorganisms; *soil with phenanthrene*, to determine the possible degradation of phenanthrene by the indigenous microorganisms; *soil with phenanthrene inoculated with Mycobacterium sp. AP1* (1.6×10^5 cells/g), to determine the effect of the inoculum in phenanthrene biodegradation and metabolite accumulation; and *soil with phenanthrene and sodium azide* (NaN_3 0.05%), as abiotic and phenanthrene volatilization control.

The system was incubated at room temperature for 60 days. During this time, microcosms were air flushed for 10 min every 2 days at a constant flow. This allowed for evolved CO_2 to be trapped by the air bubbling into the alkali traps. Triplicates for each different series of microcosms were removed at 0, 15, 30 and 60 days and analyzed to determine residual phenanthrene concentration and presence of metabolites.

Chemical analyses

Reverse-phase HPLC was performed with a Hewlett-Packard model 1050 chromatograph equipped with an HP-1040 M diode array UV-visible detector set at 254 nm. Separation was achieved on a Chromospher

C_{18} (Chrompack) (25 cm 4.6 mm, 5-μm particle size) column, applying a linear gradient of methanol [10–95% (v/v) in 20 min] in acidified water (0.6% H_3PO_4). Flow was maintained at 1 ml/min.

GC-MS analyses were conducted using a Hewlett-Packard 5890 series II with a 5989 mass selective detector. Compounds were separated on an HP-5 capillary column [30 m × 0.25 mm (internal diameter)] with 0.25 μm film thickness, and helium as the carrier gas. The column temperature was maintained at 50°C for 1 min, and then raised to 310°C at a rate of 10°C/min. The mass spectrometer was operated at 70 eV of electron ionization energy. Injector and analyzer temperatures were set at 290 and 315°C, respectively. Concentrations of residual phenanthrene and oxidation products were determined from the corresponding peak areas from triplicate samples by using standard calibration curves for each chemical.

Results and discussion

Design and operation of the microcosms system

The design of the microcosms system was based on that described by Bartha and Pramer (1965), where a known amount of the test chemical is added to a sample of soil in a biometer flask; then the evolved CO_2 is collected in an alkali trap and analyzed by titration. The percent of theoretical CO_2 evolution can be then calculated to determine the degree of mineralization of the test compound. The use of external traps where the gas bubbles pass through the alkaline solution allows for a greatly increased mass transfer rate respect to internal traps, where the mass transfer of CO_2 into the alkaline solution only occurs at the static-gas liquid interface (Haderlein et al. 1999).

After a number of experiments with agricultural soil and glucose as a carbon source the system shown in Fig. 1 and generally described in *Materials and methods* was set up. A great improvement in sensitivity was found when the volume of the traps was increased from 2 to 50 ml, decreasing simultaneously the concentration of NaOH from 1 M to 0.05 M (results not shown). The final basic system established included four humidifier flasks fed by one glass manifold air distributor. This system could be easily duplicated depending on the number of treatments, controls and replicates needed in each experiment.

In typical experiments with glucose using this system the coefficient of variability within both, triplicate microcosms connected to the same humidifier, and triplicates connected to different humidifiers, was less than 10%. Regarding CO_2 evolution, the system showed good discrimination between soil microcosms amended with 1% glucose [values increasing linearly from 0 to an average value of 0.7 mmol (11% mineralization) in 12 days], those with 0.1% of glucose (0–0.15 mmol, 23% mineralization), and the intrinsic respiration and abiotic controls (0 to 0.05–0.06 mmol, respectively).

Set-up and validation of an analytical protocol for detection of PAH-metabolites in soil microcosms

Soil extracts may contain a complex mixture of organic compounds that can hamper the detection of the test pollutants and their metabolites. Also, a high residual concentration of the parent compound or of a given metabolite may conceal less concentrated oxidation products. Our previous works in the study of bacterial metabolic routes for degradation of PAHs, both as single components (Grifoll et al. 1994, Vila et al. 2001, López et al. 2006) or in mixtures (Grifoll et al. 1995) showed that aromatic carboxylic acids are often accumulated as a result of partial degradation (Vila et al. 2001, Grifoll et al. 1995), cometabolism (Vila et al. 2001, Grifoll et al. 1995) or lateral non-productive routes that coexist with the main degradative routes (Vila et al. 2001, López et al. 2005, 2006). For example, the strain used in this study, *Mycobacterium* sp. AP1 grows on pyrene, fluoranthene, and phenanthrene accumulating 2,2′-dihydroxy-6,6′-biphenyl dicarboxylic acid, *Z*-9-carboxymethylenefluorene-1-carboxylic acid, and diphenic acid as a result of apparently non-productive alternative routes that involve *ortho*-cleavage in the K-region of those compounds. According to this, the soil extraction protocol should include the separation between neutral and acidic extracts, and further chemical fractionation and/or *clean-up* procedures.

The application of a modification of the neutral extract fractionation protocol described by Meyer et al. (1999), to the stock solution provided recoveries of 98% for 9-fluorenone, 100% for 2-naphthoic

acid, 99% for 2-hydroxy-1-naphthoic acid, and 98% for phenanthrene. The acidic components were included because according to our experience they can be extracted in neutral conditions. The acidic extract *clean-up* procedure gave recoveries of 97% for phthalic acid and 99% for diphenic acid.

Once the neutral extract fractionation and acidic extract *clean-up* methods had been validated, we used a sample of soil contaminated with phenanthrene and representative metabolites to set up a sonication protocol for the obtention of the soil organic extracts. Ultrasound extraction has been widely accepted for the recovery of organic pollutants from soil and sediment samples (Grifoll et al. 1990; Guerin 1999; Song et al. 2002). A number of extraction conditions (varying the number of extractions, solvent volume, and pH), followed by the validated fractionation or *clean up* methods, were assayed. Phenanthrene and 1-hydroxy-2-naphthoic acid showed best recoveries (93% and 89%) with a first extraction at pH 7.2 followed by a second extraction in acidic conditions (pH 2.0). Phthalic and diphenic acids were only recovered in acidic extracts (89% and 100%). The recovery of all the compounds was maximum after five subsequent extractions at each pH. According to this results all the microcosms in the following experiments were extracted five times first at pH 7.2, then at pH 2.0.

Degradation of phenanthrene and metabolite accumulation in soil microcosms

The standardized microcosm system and analytical protocol were applied to study the biodegradation of phenanthrene as a model PAH in soil samples. Besides validating the system for the study of the biodegradation of PAHs in soils, these experiments examine the effect of inoculation of a specific mycobacterial strain in the biologically mediated fate of phenanthrene. As in previous experiments, azide-inhibited replicates were included as abiotic controls.

The validated system and operation conditions showed good discrimination between the kinetics of CO_2 production in abiotic controls, intrinsic respiration controls (microcosms without phenanthrene), and microcosms with phenanthrene (Fig. 2B shows the results from a typical experiment). The results confirm that most of the CO_2 produced in the latter

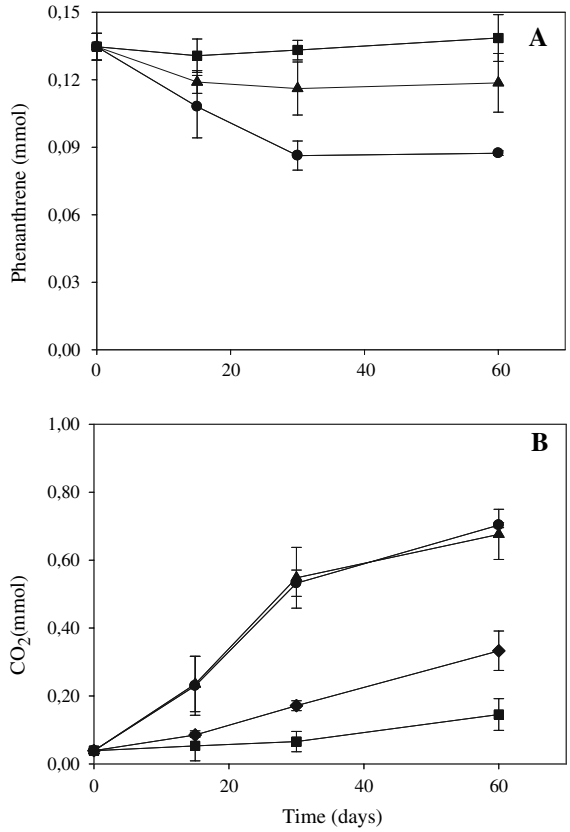

Fig. 2 Kinetics of phenanthrene depletion (**A**) and accumulative CO_2 evolution (**B**) in microcosms with phenanthrene contaminated soil (▲), microcosms with phenantrene contaminated soil inoculated with *Mycobacterium* sp. AP1 (●), soil microcosms with sodium azide (abiotic controls) (■), and non-contaminated soil microcosms (intrinsic respiration controls) (◆). Each represented value is the average of the values obtained for three replicates (±standard deviation)

is due to the mineralization of phenanthrene by either strain AP1 or the soil indigenous microorganisms. The milimoles of CO_2 detected in the traps from the abiotic controls remained approximately constant throughout the first 30 days of incubation to increase slightly at 60 days. This indicates that no physico-chemical oxidation occurred, that the sodium azide addition effectively inhibited the substrate mineralization keeping the residual fractions of phenanthrene constant over the incubation time (Fig. 2), and that the system presents a good air-tightness. The low variation observed between triplicates also indicates a well functioning of the all components of the system.

The analytical protocol proved to be efficient in the determination of the degradation of phenanthrene in microcosms (depletion of parent compound, Fig. 2A) and the detection of the expected accumulated metabolites (Table 1 and Fig. 3). The recovery of phenanthrene in abiotic controls was of 93–99% (23.3–24.6 mg) with slight variation throughout the experiment. According to those values, in this system the potential substrate volatility losses seem to be negligible. The expected acidic phenanthrene metabolites were produced and detected in microcosms inoculated with strain AP1 (see below), at concentration values in the milligram range (1.2 mg for diphenic acid) with acceptable variation between replicates (less than 10%). These results validate the system for the study of the biodegradation of PAHs.

The highest percentages of phenanthrene degradation were found for the microcosms inoculated with strain AP1 (Fig. 2A). The phenanthrene degradation rate proceeded linearly for the first 30 days, wherein 35.2% of the substrate had disappeared. No further degradation was observed by the end of the experiment (60 days). A similar kinetics was obtained with non-inoculated microcosm, although the percentages of phenanthrene depletion barely reached 11.6%. Initial high rates and subsequent attenuation during the biodegradation of PAHs has been generally observed in both, microcosm experiments and bioremediation of soils, being mainly attributed to a reduced bioavailability of the residual fraction due to sorption to organic matter (Cavalca et al. 2002; Bouwer and Zehnder 1993; Schwartz and Scow 1999).

Static solid state microcosm simulate soil conditions more accurately than shaked soil slurry microcosms, increasing the sorption process of the pollutant to soil pores and decreasing the contact with the microbial cell degraders into the micro-environment. The sorption processes together with the low mobility of *Mycobacterium* would explain the relative low levels of degradation observed in the inoculated microcosms in relation to those observed in liquid cultures with phenanthrene as sole carbon source (90%, non published results). In fact, the low mobility of mycobacteria may be a critical aspect in PAH degradation due to the difficulty of contact between the compound and microorganisms.

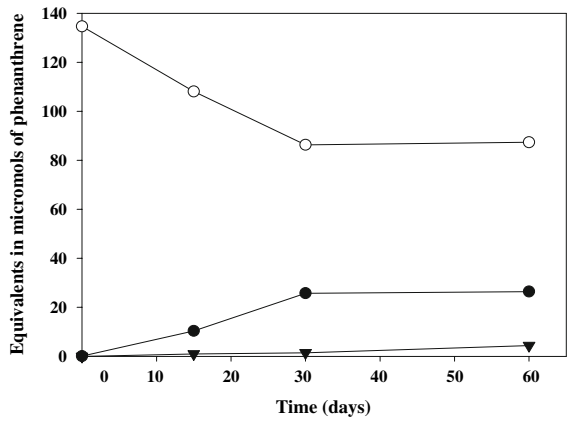

Fig. 3 Evolution of the concentration of phenanthrene equivalents recovered as residual phenantrene (○), CO_2 (●), and diphenic acid (▼) from microcosms inoculated with strain *Mycobacterium* AP1 at different times of incubation

Paralleling the degradation kinetics, the production of CO_2 in microcosms with phenanthrene increased linearly during the first 30 days (Fig. 2B), non exhibiting differences between uninoculated microcosms and those inoculated with strain AP1 and reaching maximum values of approximately 18% of mineralization for this PAH (once the intrinsic

Table 1 GC retention times and electron impact mass spectral properties of the oxidation products detected in soil acidic extracts from the microcosms inoculated with *Mycobacterium* sp. AP1

Product	R_t	Abundance* %	Ion fragments *m/z*	Identification
I	17.7	2.4	270(15), 255(34), 223(10), 197(18), 181(100), 167(6)	ND
II	18.3	20.1	270(2), 239(3), 211(100), 196(19), 180(14), 168(7), 152(15), 139(10), 126(2), 76(18), 59(9)	Diphenic acid[a]
III	22.6	73.3	322(3), 291(2), 263(1), 231(4), 202(5), 178(100), 113(9), 101(13), 85(21)	ND

*Relative abundance in the GC chromatograms of the acidic extracts from the microcosms at the end of incubation (60d)

[a] Identified by comparison with analytical data for authentic standard

respiration is subtracted). Thereafter the increase in CO_2 production is similar to that observed for the intrinsic respiration (microcosms without phenanthrene).

No metabolites were detected in non-inoculated microcosms, which is consistent with the similar percentages obtained for phenanthrene degradation and mineralization. In inoculated microcosms, two acidic metabolites were detected (Table 1): metabolite I (2.4% in GC at 60 days), which remains to be identified, and metabolite II (20%) which has been identified as diphenic acid. A third compound (73%) was tentatively identified as a Diels-Alder adduct and could be the result of spontaneous reactions of possible phenanthrene oxidation products. The accumulation of all those products increased linearly throughout the incubation time (data not shown), diphenic acid reaching maximum values of 1.2 mg. Figure 3 shows the evolution, in equivalents of phenanthrene, of the amount of diphenic acid accumulated in relation to that of phenanthrene loss and CO_2 production. Equivalents in moles of phenanthrene were calculated as the moles of initial phenantrene that had been converted to the found moles of diphenic acid and CO_2, considering that to produce one molecule of diphenic acid one molecule of phenantrene needs to be converted, and that the mineralization of a whole molecule of phenantrene would produce 14 molecules of CO_2. According with these data, at the end of incubation approximately 10% of the depleted phenanthrene (48 μmol) was recovered as diphenic acid (4.6 μmol) while 50% (24 μmol) had been mineralized. Considering the relative abundances observed in GC for compounds I and III, it can be concluded that the detected oxidation products, which are also major products accumulated in AP1 liquid cultures growing on phenanthrene (non-published results), would account for an important part of the difference between the degradation and the mineralization percentages in the inoculated microcosms. Work is in progress for a definitive identification of compounds I and III.

That in non-inoculated microcosms all the depleted phenanthrene seems to be mineralized would indicate that the degradation of phenanthrene does not result in an increase in the biomass of the microbial soil population. This is consistent with the hypothesis of Johnsen et al. (2005), in stating that, PAH-degrading populations in soil are probably mostly not growing, but they are in a pseudo-stationary phase where transient growth only replaces decaying cells. On the other hand, the inoculation of a specific degradative and active growing population, such as strain AP1, in a medium with a considerable amount of bioavailable phenanthrene may result in a fast up-take of the substrate in the early phases of incubation, this resulting in a relative high production, and subsequent diffusion outside the cells, of the less readily metabolizable intermediates or dead end products. In fact, strain AP1 does not utilize diphenic acid in liquid cultures, which may be due to the lack of enzymes for its further degradation or to a lack of transport mechanisms associated to the up-take of this compound. The partially oxidated products that diffuse to the soil may be then susceptible of degradation by other populations present as has been demonstrated in the laboratory (Casellas et al. 1998). This effect is not appreciable in the conditions of the present study, since once the degradation of phenanthrene has stopped (30 days) diphenic acid continues to accumulate and there is not a substantial reduction in the rest of oxidated compounds. Besides the risk derived of their potential toxicity, which in the case of diphenic acid has been seldom studied, it has been described that the accumulation of oxidated products may inhibit the biodegradation of the parent PAH (Casellas et al. 1998).

Conclusions

This work is a contribution to the general knowledge of the microbial processes involved in the biodegradation of PAHs in soils. The designed system and analytical protocol proved to be useful for both, the basic study of the processes that determine the environmental fate of PAHs in polluted soils, and the tractability studies to evaluate the implementation of strategies to enhance the performance of indigenous or inoculated bacteria into the environment controlling all critical factors to establish a successful bioremediation procedure of a contaminated environment.

The identification and accumulation kinetics of diphenic acid confirms the relevance of the study of microbial processes involved in PAH degradation in soils to perform a complete risk assessment of

bioremediation processes. This is the first study on the degradation and mineralization of a given PAH in soil static systems that reports the formation and persistence of bacterial metabolites.

Further studies using the developed system will be performed to study the fate of other PAHs in soils. The identification of the metabolites formed, including those for which authentic standards are not commercially available, will be facilitated by the wide analytical database for the identification of PAH metabolites available in our laboratory.

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