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ORIGINAL PAPER

Biodegradation of phenanthrene and analysis of degrading cultures in the presence of a model organo-mineral matrix and of a simulated NAPL phase

Lucia Cavalca · Maria A. Rao · Silvana Bernasconi · Milena Colombo · Vincenza Andreoni · Liliana Gianfreda

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Abstract Two mixed bacterial cultures ($C_{B\text{-}BT}$ and $C_{I\text{-}AT}$) degraded phenanthrene when it was: (i) in the presence of either hexadecane as a non aqueous phase liquid or a montmorillonite–Al(OH)x-humic acid complex as a model organo-mineral matrix; (ii) sorbed to the complex, either alone or in the presence of hexadecane. The cultures had different kinetic behaviours towards phenanthrene with or without hexadecane. The degradation of Phe alone as well as that of Phe in hexadecane ended in 8 and 15 days with $C_{B\text{-}BT}$ and $C_{I\text{-}AT}$ cultures, respectively. Hexadecane increased Phe bioavailability for $C_{I\text{-}AT}$ bacteria which degraded Phe according to first-order kinetics. The same effect was observed for $C_{B\text{-}BT}$ bacteria, but with an initial 2 days lag phase and in accordance with zero-order kinetics. The presence of hexadecane did not affect the degradation of phenanthrene sorbed and aged on the complex by $C_{I\text{-}AT}$ culture. This capability was exhibited also after experimental aging of 30 days. The dynamics of the bacterial community composition was investigated through PCR-DGGE (denaturing gradient gel electrophoresis) of 16S rRNA gene fragments. Individual bands changed their intensity during the incubation time, implying that particular microbe's relative abundance changed according to the culture conditions. Isolation of phenanthrene and/or hexadecane degraders was in accord with cultivation-independent data. Growth-dependent changes in the cell surface hydrophobicity of the two cultures and of the isolates suggested that modulation of cell surface hydrophobicity probably played an important role for an efficient phenanthrene assimilation/uptake.

Keywords Biodegradation · Phenanthrene · Organo-mineral matrix · NAPL

Abbreviations

DGGE	Denaturing gradient gel electrophoresis
k	Degradation rate or degradation rate constant
MAH	Montmorillonite–Al(OH) x-humic acid complex
M1, M2, M3	Microcosms
NAPL	Non aqueous phase liquid

L. Cavalca · M. Colombo · V. Andreoni (✉)
Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche, Università degli Studi di Milano, Via Celoria 2, 20133 Milan, Italy
e-mail: vincenza.andreoni@unimi.it

M. A. Rao · L. Gianfreda
Dipartimento di Scienze del Suolo, della Pianta e dell'Ambiente, Università di Napoli Federico II, 80055 Portici, Napoli, Italy

S. Bernasconi
Dipartimento di Chimica Organica ed Industriale, Università degli Studi di Milano, 20133 Milan, Italy

PAH	Polycyclic aromatic hydrocarbon
Phe	Phenanthrene
Phe-MAH	Phenanthrene sorbed to Montmorillonite–Al(OH) x-humic acid complex
Phe-NAPL	Phenanthrene dissolved in hexadecane
Phe-NAPL-MAH	Phenanthrene sorbed to montmorillonite–Al(OH) x-humic acid complex in the presence of hexadecane

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are widespread environmental contaminants with severe, harmful effects on the environmental and human health. Consequently, their removal from the environment is presently a priority.

PAH biodegradation by microorganisms is a well known phenomenon (Cerniglia 1993; Harayama 1997). In soil, PAHs may, however, accumulate because of their low availability to microbial degradation. Besides the intrinsic low biodegradability of their molecular structures, the low availability of these compounds to microorganisms is also the result of their binding to organic (humic substances) and inorganic (clays, (hydr) oxides) soil fractions (Ruggiero et al. 2002). Soil-PAH contact time (i.e. aging), a naturally occurring process, increases the interactions between PAHs and soil matrix, and strongly decreases the amount of bioavailable pollutants.

Contradictory results have been often observed on the availability of organic molecules to the bacterial cells, depending on the extent and the intensity of their binding with the organic or inorganic soil particles. For instance, Theng et al. (2001) demonstrated that Phe was not completely degraded when intercalated into alkylammonium-montmorillonite clay. By contrast, Ortega-Calvo and Saiz-Jimenez (1998) found that a higher Phe mineralization occurred in the presence of humic fractions or clays. To our knowledge, less evidence is available on the bioavailability of Phe when sorbed to organo-mineral matrices that in soils are expected to be more common than isolated humic or clay components (Violante et al. 1999).

The PAH bioavailability is further complicated when PAHs interact with non-aqueous phase liquids (NAPL) and soil colloids, thus resulting less or completely unavailable for microorganisms. Microorganisms adopt several strategies to release the xenobiotic at a faster rate: release of extracellular enzymes, regulation of uptake systems, production of biosurfactants, depletion of the xenobiotic concentration in the surrounding volume, change of cell surface hydrophobicity (Baveye and Bladon 1999; Beal and Betts 2000; Bouchez-Naitali et al. 1999; Pirog et al. 2004). Therefore, both the slow mass transfer to the degrading microorganisms and the lack of biochemical or genetic factors may reduce bioavailability of pollutants.

Significant interest in the potential of bioremediation to restore PAH-polluted environments has promoted studies concerning the bioavailability of these compounds to different microbial organisms under different experimental conditions (Guerin and Boyd 1992; Alexander 2000; Amellal et al. 2001). The dynamics of microbial community composition in response to different environmental conditions can be monitored through molecular microbial ecology approaches such as PCR-DGGE (denaturing gradient gel electrophoresis) of 16S rRNA gene fragments. Although it is uncertain whether the bands represent the most abundant, the most extractable or the most active species (Müller et al. 2002), the relative prominence of different microbial species (phylotypes) traced by the PCR-DGGE patterns can be a useful tool to understand how microbial communities shape in response to the environmental heterogeneity of pollutant bioavailability (Fromin et al. 2002).

In the present study we monitored phenanthrene biodegradation by two mixed cultures, named $C_{B\text{-}BT}$ and $C_{I\text{-}AT}$ according to Andreoni et al. (2004) in model systems simulating different Phe bioavailability conditions. Namely: (i) Phe in the presence of a simulated NAPL phase (hexadecane), (ii) Phe sorbed to a model organo-mineral matrix alone or in the presence of

hexadecane after an experimental aging of 30 days in darkness. Finally, a possible physiological response of Phe degrading cultures to the low bioavailability of Phe was studied by measuring cell hydrophobicity as indicator of potential cell affinity for hydrophobic substrates.

Materials and methods

Experimental design

Duplicate microcosms were set up: Phe dissolved in hexadecane, used as a simulated NAPL phase (microcosm M1, Phe-NAPL), Phe sorbed to MAH and experimentally aged for 30 days either as sole Phe (microcosm M2, aged Phe-MAH, Fig. 1) or in the presence of hexadecane (microcosm M3, aged Phe-NAPL-MAH, Fig. 1). Microcosms (500 ml bottle), supplemented with the appropriate Phe source (see below) and with 45 ml M9 mineral medium (Kunz and Chapman 1981), were inoculated with 10% (v/v) of $C_{B\text{-}BT}$ and $C_{I\text{-}AT}$ cultures. Microcosms M1 and M2 were inoculated with cultures grown in M9 with Phe; microcosm M3 with cultures grown in M9 with Phe and hexadecane. Original enrichment cultures were previously selected after eight transplants on Phe from PAH-polluted river sediment ($C_{B\text{-}BT}$) and from an Italian agricultural soil ($C_{I\text{-}AT}$) (Andreoni et al. 2004). In microcosm M1 the Phe source consisted of 0.2 ml of a sterile solution of Phe in hexadecane (50 mg ml^{-1}, i.e. 10 mg of Phe in a mass ratio 1:15, w/w). Bottles of microcosm M2, containing 10 mg of Phe and 250 mg of MAH (40 mg Phe g^{-1} MAH) in M9 medium, and bottles of microcosm M3, containing 10 mg of Phe dissolved in hexadecane (600 mg g^{-1} MAH) and 250 mg of MAH, were prepared and aged in the dark for 30 days under sterile conditions. The MAH was sterilized by 2 h exposure to UV light under a laminar flow hood. At increasing incubation times, the sterility of MAH was monitored by determination of viable bacterial cells by the spread-plate method. After aging, the bottles were processed according to the scheme reported in Fig. 1. The chemical distribution of Phe and hexadecane (total content, free and MAH-sorbed fractions) in uninoculated microcosms was determined by centrifuging the mixtures at 9,000 rpm for 20 min at 5°C to separate the supernatants from the MAH-sorbed fractions and washing it three times with 45 ml sterile H_2O. Supernatants, MAH-sorbed fractions, washing waters and residues adhered to the bottle inner surfaces were extracted and then gas-chromatographically analysed as described below. Microcosms M2 and M3 containing the MAH-sorbed fractions were freshly added with M9 (45 ml), inoculated with $C_{B\text{-}BT}$ (M2) and $C_{I\text{-}AT}$ (M2 and M3) cultures and incubated at 30°C in darkness with agitation on an orbital shaker at

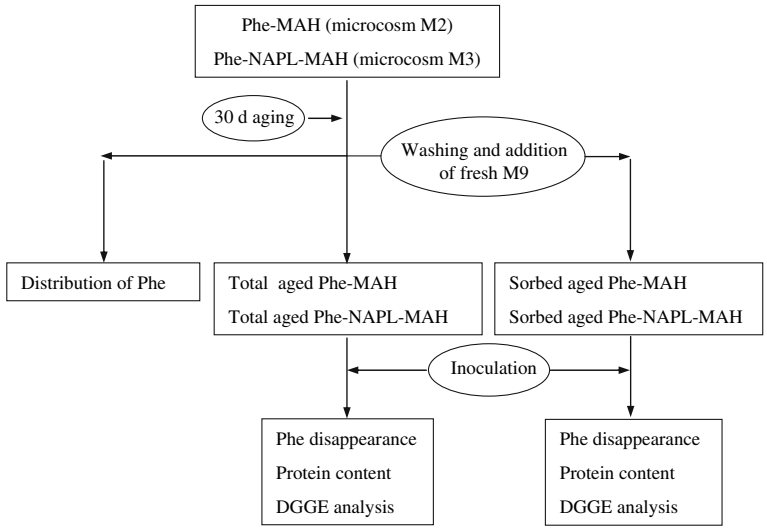

Fig. 1 Flow chart of the experimental design of aging procedure for microcosms M2 (Phe-MAH) and M3 (Phe-NAPL-MAH)

150 rpm. Non inoculated controls were included to determine the abiotic loss of Phe and/or hexadecane. Inoculated controls were constituted by bottles containing separately: 10 mg Phe dispensed as acetone solution, 150 mg hexadecane, 250 mg MAH with and without 10 mg Phe. In microcosms M2, in inoculated and non inoculated controls acetone was allowed to evaporate before adding M9 medium. All the bottles were Teflon-stoppered. If otherwise stated, at different incubation times two series of duplicate sacrificial samples of microcosms and controls were processed to quantify Phe and/or hexadecane, and to determine the protein content (Bradford 1976) and to perform DGGE analyses.

Phe disappearance was estimated by a time course analysis of degradation data according to zero- or first-order kinetics described by the equations $X_t = X_0 - kt$ and $X_t = X_0\ e^{-kt}$, respectively, where X_t is the amount of Phe in mg, t is time and k indicates the degradation rate (mg d^{-1}) or the degradation rate constant (d^{-1}) in the two equations, respectively.

Extraction and gas-chromatographic analyses of phenanthrene and hexadecane

Culture broths were extracted three times with petroleum ether (3 × 50 ml). The organic layers were collected, dried with Na_2SO_4, filtered and the solvent was removed under reduced pressure.

For the Phe analysis, the organic residue was solved in 2 ml of ethyl acetate and 4 ml of a solution of dodecanol in ethyl acetate (5 mg ml^{-1}) were added as internal standard for gas chromatographic analyses. For the hexadecane analysis, the organic residue was solved in 20 ml of ethyl acetate. Two ml of this solution were added to 4 ml of a solution of dodecanol in ethyl acetate (5 mg ml^{-1}) and used as internal standard for gas chromatographic analyses. This analytical method detected 0.1 mg ml^{-1} of Phe.

Gas-chromatographic analyses were carried out using a DANI 1000 Gas-chromatograph equipped with a FID detector (hydrogen 0.9 bar, air 1.0 bar and nitrogen 1.0 bar) and a fused silica capillary column WCOT-CP-SIL 8 CB Chrompack (25 m × 0.32 mm ID), carrier helium (0.8 bar), and injection temp. 300°C, detection 300°C, initial oven temp. 140°C (3 min), temperature increase 10°C min^{-1}, final isotherm 250°C, and injection volume 2 µl. The dodecanol Rt was 6.3 min, the hexadecane Rt was 7.9 min and the Phe Rt was 10.6 min. Detector signal output was monitored by computer and all chromatograms and data were generated and processed by Dani Data Station version 1.7 software.

Chemicals

Reagent grade Phe (>96% purity) and HPLC-grade solvents were purchased from Sigma Aldrich (Germany). All other chemicals, reagent grade, were supplied by Analar, BDH Ltd, (Germany), unless otherwise stated.

A synthetic Montmorillonite–Al(OH)x-Humic acid complex (MAH) was obtained as described by Violante et al. (1999). The complex had a C.E.C. of 36 cmol(+) kg^{-1}, a specific surface of 167 $m^2\ g^{-1}$ and 0.6% of organic carbon.

Molecular methods

At each incubation time, DNA was extracted from replicate sacrificial samples of $C_{I\text{-}AT}$ and $C_{B\text{-}BT}$ cultures by G-NOME kit (Bio-101, UK) according to the manufacturer's instructions. Cell suspension solution (1.85 ml) was added to the pellet (50 mg of fresh weight) obtained from a freshly grown liquid culture and 50 µl of RNase were mixed to the sample. Then the sample was incubated at 55°C for 2 h in the presence of 100 µl cell lysis solution and 25 µl of proteinase mix. Samples were precipitated in the presence of 8 ml 100% ethanol and the DNA pellet was re-suspended in 200 µl TE (10 mM Tris–HCl pH 8.0 and 1 mM Na_2EDTA pH 8.0). DNA from isolates was obtained by proteinase K digestion of cells as follows: cell suspensions (100 µl OD_{600} = 2.0) were centrifuged at 13,000 × g for 7 min and suspended in 100 µl of sterile MilliQ water, 100 µl of 10 mM Tris–HCl buffer pH 8.0 and 13 µl of Proteinase K (1 mg ml^{-1}). The mix was incubated for 2 h at 55°C then boiled for 10 min and centrifuged at 13,000 × g for 5 min. The DNA-containing supernatant was withdrawn and put in sterile microtubes.

The complete 16S rRNA gene was amplified from mixed culture and isolated strain total DNA (20 ng) by using eubacterial universal primers P27f and P1495r referred to *Escherichia coli* nucleotide sequence of 16S rDNA gene according to Weisburg et al. (1991). Amplification of hyper variable region V3 of the 16S rRNA gene was performed using a nested PCR approach by using 100-folds diluted PCR products of 16S rDNA as template for the amplification reaction by V3 forward/V3 reverse primer pair (Muyzer et al. 1993). PCR mixtures contained in a final volume of 50 µl: 10 ng of 16S rDNA, 1U of *Taq* polymerase, 0.5 µM of each primer (Invitrogen, UK), 0.2 µM of deoxynucleoside triphosphate (dNTPs), and 1 × PCR buffer. *Taq* polymerase, dNTPs, and PCR buffer were purchased from Fermentas (Ontario, Canada). The samples were firstly denatured for 3 min at 94°C, and then subjected to 19 cycles consisting of 1 min 20 s at 94°C, 1 min 30 s at 65°C and 1 min 10 s at 72°C; the annealing temperature was decreased by 1°C every second cycle until a touchdown at 55°C, at which temperature 7 additional cycles were carried out. The last step included an extension of 5 min at 72°C. PCR was performed on T-Gradient Biometra apparatus (Germany). The PCR products were checked on 2% agarose gel.

For DGGE analysis V3 amplicons were run on polyacrylamide gel (8%) with a denaturing gradient of 40–80% (where 100% denaturant gels contain 7 M urea and 40% formamide). DGGE run was performed on samples of 10 µl-volume at a constant voltage of 70 V for 16 h in 1 × TAE (4.84 g l^{-1} of Tris base, 1.14 ml l^{-1} of glacial acetic acid, 2 ml l^{-1} of 0.5 M EDTA solution pH 8.0) running buffer at 60°C in the DCODE Universal Mutation Detection System (Biorad, USA). After electrophoresis, the gels were stained with 0.5 mg l^{-1} ethidium bromide solution and photographed under UV light and analysed with digital GelDoc System (Biorad). As control, the V3 region of the 16S rDNA fragments of single isolates were run on DGGE gels, and they were checked to co migrate with prominent bands present in the two cultures.

Strains were identified on the basis of sequence analysis of the complete 16S rRNA gene by using eubacterial universal primers P27f and P1495r. Dominant bands within the enriched mixed culture profiles from each microcosm conditions were excised by a sterile scalpel from DGGE gels under UV light and suspended in 200 µl deionised water. A 10 µl volume of the DNA solution was then used as a template for V3 amplification by V3 forward/V3 reverse primer pair without GC clamp. PCR products were directly sequenced by using the *Taq* DyeDeoxy terminator cycle-sequencing procedure according to the ABI Prism protocol (Applied Biosystems, USA). The forward and the reverse sample were run on a 310A sequence analyser (Applied Biosystems, USA). A similarity analysis of the sequences was obtained by using the Advanced Blast Search program (GenBank, NCBI) to determine their phylogenetic type.

Growth characteristics of isolates and determination of cell surface hydrophobicity

Different colonies were isolated, on the basis of the colony morphology, from the two cultures plated onto ten-fold diluted Tryptic Soy agar after 8 days of incubation. The isolates were cultured in sterile vials containing M9 mineral medium supplemented separately with Phe and hexadecane at 200 mg l^{-1}. At the occurrence, 1 g l^{-1} glucose was added to the medium.

Cell surface hydrophobicity of both $C_{I\text{-}AT}$ and $C_{B\text{-}BT}$ cells and of some isolates was measured by their affinity towards hexadecane in three independent experiments (Rosenberg et al. 1980). Cells were grown on glucose or Phe, harvested, washed twice with phosphate buffer (10 mM, pH 7.0), and suspended again in the same buffer to obtain cell suspensions with a final $OD_{400\ nm}$ of 1.0. The 5 ml assay mixture contained 4 ml of cell suspension and 1 ml of hexadecane. After 5 min of pre-incubation, the mixture was vortexed for 60 s and incubated for an additional 30 min at room temperature. The OD of the aqueous phase was measured at 400 nm. The cell surface hydrophobicity was expressed as the percentage of cells transferred to the hexadecane phase by measuring the OD of the aqueous phase before and after mixing and was calculated according to: 100 × (1 – OD of the aqueous phase/OD of the cell suspension).

Results

Phenanthrene biodegradation in different microcosms

Phe-NAPL (Microcosm M1)

The degradation of Phe in hexadecane as well as that of Phe alone ended in 8 and 15 days for $C_{B\text{-}BT}$ and $C_{I\text{-}AT}$ cultures, respectively (Fig. 2). The two cultures showed, however, a different response to the presence of the NAPL phase and a different kinetic behaviour. Both the curves for $C_{I\text{-}AT}$ were well fitted by first-order kinetics according to the equations: [Phe] = 9.28 $e^{-0.12t}$, R^2 = 0.902); [Phe – NAPL] = 10.8 $e^{-0.21t}$, R^2 = 0.960 (Fig. 2B), thus suggesting that hexadecane increased the Phe bioavailability for the $C_{I\text{-}AT}$ bacteria (k = 0.21 d^{-1} against 0.12 d^{-1}). For $C_{B\text{-}BT}$ a linear degradation of only Phe took place which ended at day 8, thus indicating a zero-order kinetics ([Phe] = 9.59–1.21 t, R^2 = 0.970), whereas a sort of a 2 day lag phase was observed in the presence of hexadecane. Afterwards, a linear degradation of Phe by $C_{B\text{-}BT}$ occurred, still ending at day 8, with a behaviour describable by the expression: [Phe – NAPL] = 13.3–1.66 t, R^2 = 0.997 (Fig. 2A). In the same 2 day time span, $C_{I\text{-}AT}$ degraded 6% of hexadecane, whereas $C_{B\text{-}BT}$ did not degraded it.

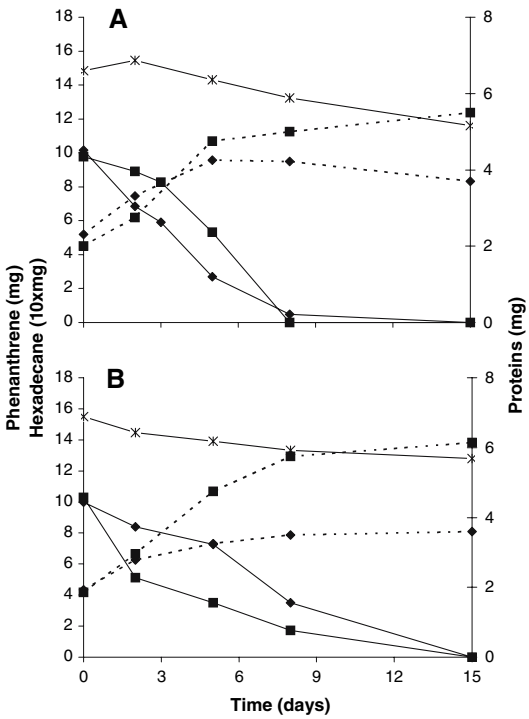

Fig. 2 Phenanthrene degradation in microcosm M1 (Phe-NAPL) by $C_{B\text{-}BT}$ (**A**) and $C_{I\text{-}AT}$ (**B**) cultures. Disappearance of: Phe (–♦–), Phe dissolved in hexadecane (NAPL phase) (–■–), hexadecane (–✻–); corresponding cell growth on: Phe (- -♦- -), Phe dissolved in hexadecane (- -■- -). Each value is the mean of two independent determinations which varied in the range of 2-10%

In non inoculated controls negligible decreases (<0.5%) of Phe and hexadecane was measured indicating the absence of not-controlled handling losses from the systems. The higher protein contents of the two cultures growing on Phe-NAPL were due to the concomitant utilization of the two carbon sources.

Aged Phe-MAH (Microcosm M2) and aged Phe-NAPL-MAH (Microcosm M3)

In preliminary experiments the effect of freshly added MAH on Phe biodegradation by the bacterial cultures was evaluated and the results indicated that MAH did not affect substantially the shape of Phe degradation curve. At 21 day incubation, no more Phe was measured in the samples containing or not fresh MAH. The amount of extractable Phe at zero incubation time was 10% lower than that added, thus indicating a fairly small initial adsorption of Phe on MAH surfaces (data not shown).

The chemical distribution of Phe and hexadecane in uninoculated M2 and M3 microcosms is summarized in Table 1. Data indicated that no abiotic loss of Phe occurred during aging. In a relatively short aging time (30 days), approximately 50% of the initial Phe was sorbed to MAH. The remaining amount was distributed among the different free Phe forms. The presence of a NAPL phase shifted the distribution of Phe between sorbed Phe-MAH and unsorbed amounts and 2.57 mg of the initially added Phe was sorbed to the solid matrix.

Figure 3 shows the time courses of total and sorbed Phe degradation by $C_{I\text{-}AT}$ culture in M2 and M3 microcosms. All the degradation data were analysed by zero-order kinetics, according

Table 1 Distribution of Phe (A) and Phe dissolved in hexadecane (B) in aged Phe-MAH system

Fraction		Phe (mg)	Hexadecane (mg)
A	Total Phe	9.81	
	Phe sorbed to the washed MAH	5.58	
	Free Phe in supernatant	0.78	
	Free Phe adhered to bottle surfaces	2.30	
	Free Phe in water washings	0.74	
	Total recovery	9.39	
B	Total Phe and hexadecane	10.30	126.0
	Phe and hexadecane sorbed to the washed MAH	2.57	6.5
	Phe and hexadecane in supernatant	3.83	85.6
	Phe and hexadecane adhered to bottle surfaces	1.03	14.1
	Phe and hexadecane in water washings	1.21	15.3
	Total recovery	8.64	121.5

Each value is the mean of two independent determinations which varied in the range of 2-10%

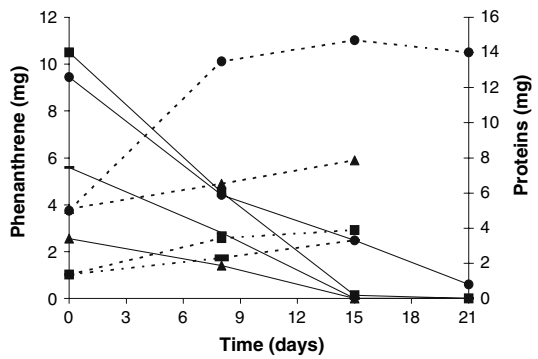

Fig. 3 Sorbed phenanthrene degradation (Phe-MAH) by $C_{I\text{-}AT}$ in microcosm M2 (aged Phe-MAH) and M3 (aged Phe-NAPL-MAH). Disappearance of: total aged Phe-MAH (–■–), sorbed aged Phe-MAH (–■–), total aged Phe-NAPL-MAH (–•–), sorbed aged Phe-NAPL-MAH (–▲–); corresponding cell growth on total aged Phe-MAH (- -■- -), sorbed aged Phe-MAH (- -■- -), total aged Phe-NAPL-MAH (- -•- -), sorbed aged Phe-NAPL-MAH (- -▲- -). Each value is the mean of two independent determinations which varied in the range of 2-10%

to the equations here reported: [Aged Phe-MAH] = 10.35 – 0.7 t, $R^2 = 0.970$; [Aged Phe-NAPL-MAH] = 9.1 – 0.48 t, $R^2 = 0.98$; [Aged sorbed Phe-MAH] = 5.61 – 0.38 t, $R^2 = 0.99$; [Aged sorbed Phe-NAPL-MAH] = 2.62 – 0.17 t, $R^2 = 0.98$. After 8 day incubation, the sorbed aged Phe-NAPL-MAH decreased from 2.56 mg to 1.4 mg, with a degradation rate of 0.17 mg d^{-1} lower than that calculated for sorbed aged Phe-MAH (0.38 mg d^{-1}). In the same time span, a higher decay (0.7 mg d^{-1}) of total aged Phe-MAH was measured. While the degradation of Phe sorbed to MAH completed within 15 days either in the absence (M2) or in the presence (M3) of the NAPL, detectable levels of Phe were still measured in the total Phe-NAPL-MAH system. The utilization of Phe by cells was supported by the corresponding increase of proteins that paralleled the Phe utilization patterns measured (Fig. 3). A similar behaviour was observed with $C_{B\text{-}BT}$ culture (data not shown). This culture grew and degraded sorbed aged Phe-MAH, although a slower decay of Phe bound to MAH than Phe occurred.

Monitoring of Phe degrading cultures under different bioavailability conditions

Analysis of prominent DGGE bands amongst cultures growing on Phe, Phe-NAPL, NAPL, and both total and sorbed aged Phe-MAH and Phe-NAPL-MAH showed a similarity among the bacterial community structures of the treatments and only band intensity differed. Individual bands got brighter or fainter during the incubation time, implying that particular microbe's relative abundance changed according to the culture conditions.

During the time course of the experiments, prominent bands were: B2 in $C_{B\text{-}BT}$ (Fig. 4) and B3 in $C_{I\text{-}AT}$ (Fig. 5) belonging to *Sphingomonas* sp. (98% nucleotide sequence identity to Acc. no X87164 and 97% to Acc. no AJ438177) and B5 in $C_{B\text{-}BT}$ and B6 in $C_{I\text{-}AT}$ belonging to *Rhodococcus* sp. (100% nucleotide sequence identity to Acc. no

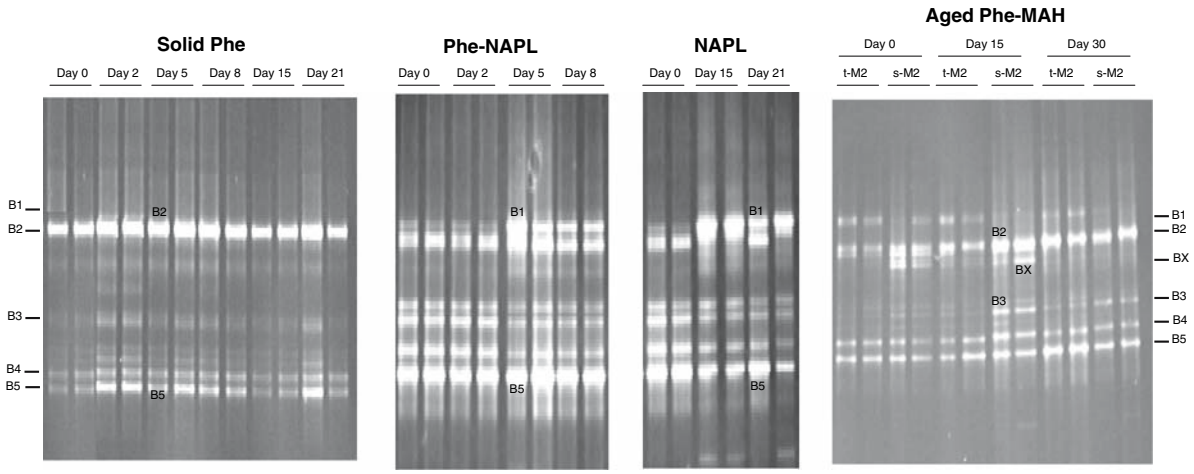

Fig. 4 Molecular profiles of $C_{B\text{-}BT}$ culture during degradation of: Phe, Phe dissolved in hexadecane (Phe-NAPL), hexadecane (NAPL), total aged Phe-MAH (t-M2) and sorbed aged Phe-MAH (s-M2). Bands: B1 = *Stenotrophomonas* sp., B2 = *Sphingomonas* sp., Bx = unidentified band, B3 = *Alcaligenes* sp., B4 = *Achromobacter* sp., B5 = *Rhodococcus* sp.. Replicates are shown for each incubation time

AF447392 and to AF447391); band B4, affiliated to *Achromobacter* sp. (98% nucleotide sequence identity to Acc. no AF411021), was visible in both cultures. Moreover, bands B1 related to *Stenotrophomonas* sp. (100% nucleotide sequence identity to Acc. no AF273079) and B3 to *Alcaligenes* sp. (98% nucleotide sequence identity to Acc. no AF508102) were detected in $C_{B\text{-}BT}$ and bands B1 – B2 related to *Pseudomonas* sp. (99% nucleotide sequence identity to Acc. no AB007999) were in $C_{I\text{-}AT}$. The communities were stable during the degradation of Phe alone and the appearance of new bands was not observed.

In the presence of Phe dissolved in hexadecane (Phe-NAPL) the bands which were present on Phe persisted and other got brighter, particularly those affiliated to *Stenotrophomonas* sp. (B1) and *Rhodococcus* sp. (B5) in $C_{B\text{-}BT}$ (Fig. 4) and to *Pseudomonas* sp. (B1-B2) in $C_{I\text{-}AT}$ (Fig. 5). The observation that these populations were stimulated during the growth on the sole hexadecane (NAPL) could indicate a hexadecane degrading capacity of strains affiliated to these species.

In the presence of total aged Phe-MAH the composition of the two cultures did not vary when compared with that grown on Phe, probably due the presence of free forms of Phe (Table 1). On the contrary, in the presence of sorbed aged Phe-MAH bands B2 in $C_{B\text{-}BT}$ (s-M2 Fig. 4) and B3 in $C_{I\text{-}AT}$ (s-M2 Fig. 5) corresponding to *Sphingomonas* sp. became more prominent. Moreover, in the first culture two bands, one related to *Alcaligenes* sp. (B3) and one unidentified (BX), were detected with stronger intensity than in presence of total aged Phe-MAH at day 15. Finally a band related to *Pseudomonas* sp. (B1) got brighter in $C_{I\text{-}AT}$ in the presence of sorbed aged Phe-NAPL-MAH (s-M3 Fig. 5), suggesting a probable involvement of this population in the degradation of sorbed aged hexadecane.

Characterization of isolates from $C_{I\text{-}AT}$ and $C_{B\text{-}BT}$ cultures

The bacteria isolated from the two cultures were analyzed for their capability to grow separately on Phe and hexadecane as only C source (Table 2). Among the isolates from the $C_{B\text{-}BT}$ culture, *Rhodococcus aetherovorans* B22, *Alcaligenes* sp. B14 and *Methylobacterium* sp. B12 grew on Phe but only the first utilized hexadecane. On the contrary, *Stenotrophomonas acidaminiphila* B8 grew only on hexadecane. In the $C_{I\text{-}AT}$, *R. aetherovorans* I3, *Sphingomonas flava* I3R and I51 and *Achromobacter xylosoxidans* I6 grew on Phe, *Pseudomonas* sp. I3H on hexadecane, and *St. acidaminiphila* IE3 and *R. aetherovorans* I19 grew on both Phe and hexadecane.

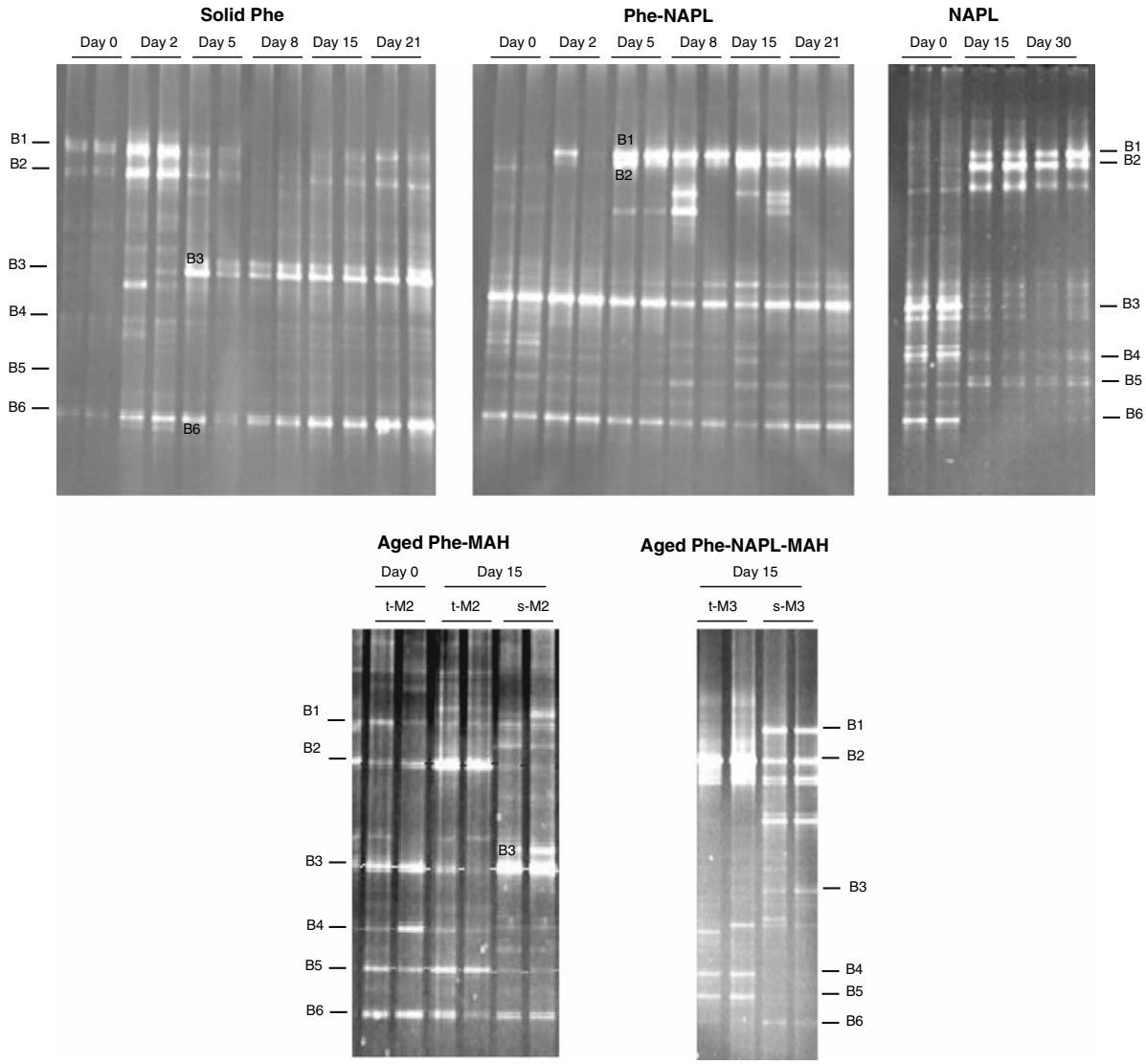

Fig. 5 Molecular profiles of $C_{I\text{-}AT}$ culture during degradation of: Phe, Phe dissolved in hexadecane (Phe-NAPL), hexadecane (NAPL), total aged Phe-MAH (t-M2) and sorbed aged Phe-MAH (s-M2), total aged Phe-NAPL-MAH (t-M3) and sorbed aged Phe-NAPL-MAH (s-M3). Bands: B1 and B2 = *Pseudomonas* sp., B3 = *Sphingomonas* sp., B4 = *Achromobacter* sp., B5 = *Pseudomonas putida*, B6 = *Rhodococcus* sp.. Replicates are shown for each incubation time

Comparison between isolate and DGGE band sequences revealed that the followings were overlapping: those belonging to Phe degrading *S. flava* and *R. aetherovorans* strains in both the cultures, *Pseudomonas* sp. and *A. xylosoxidans* strains in the $C_{I\text{-}AT}$ culture, *Stenotrophomonas* sp. and *Alcaligenes* sp. in the $C_{B\text{-}BT}$ culture. Although the bands indicate the presence of these genera in the enrichment cultures, they could belong to different strains or even to different species.

Changes in cell surface hydrophobicity of $C_{I\text{-}AT}$ and $C_{B\text{-}BT}$ cultures and of some isolates, with the exception of *R. aetherovorans* B22, were observed by analyzing cell affinity towards hexadecane (Table 2). The Phe-grown cells of the two cultures and of the isolates showed a higher affinity

Table 2 Growth characteristics and cell hydrophobicity of $C_{I\text{-}AT}$ and $C_{B\text{-}BT}$ cultures and of isolated strains

Culture	Strain	Identification[a] (GeneBank Acc. Num.)	Growth (OD_{600})		% adhered to hexadecane (mean ± SD) after growth on	
			Phe	Hexa	Glucose	Phe
$C_{I\text{-}AT}$			+	+	16 ± 4	31 ± 9
	I6	*A. xylosoxidans* (AF411021)	+	–	n.d.	n.d.
	I3H	*Pseudomonas* sp. (AB007999)	–	+	n.d.	n.d.
	I3	*R. aetherovorans* (AF447392)	+	–	n.d.	n.d.
	I5	*Kaistella koreensis* (AY299974)	–	–	n.d.	n.d.
	I19	*R. aetherovorans* (AF447391)	+	+	3 ± 2	73 ± 7
	I51	*S. flava* (X87164)	+	–	12 ± 2	28 ± 5
	I3R	*S. flava* (X87164)	+	–	10 ± 4	48 ± 8
	I3E	*St. acidaminiphila* (AF273080)	+	+	4 ± 3	21 ± 2
$C_{B\text{-}BT}$			+	+	17 ± 3	29 ± 2
	B14	*Alcaligenes* sp. (AF508102)	+	–	n.d.	n.d.
	B8	*St. acidaminiphila* (AF273079)	–	+	n.d.	n.d.
	B18	*S. flava* (AJ438177)	+	–	6 ± 2	21 ± 5
	B9	*Aquamicrobium defluvium* (Y15403)	–	n.d.	n.d.	n.d.
	B12	*Methylobacterium* sp. (AJ276806)	±	–	n.d.	n.d.
	B16	*A. xylosoxidans* (AF411020)	–	–	n.d.	n.d.
	B22	*R. aetherovorans* (AF447391)	±	+	40 ± 2	39 ± 3

[a] 16S rDNA sequence homology ≥98%; SD, standard deviation; Phe, phenanthrene; Hexa, hexadecane; + , $OD_{600} \geq 0.3$; –, $OD_{600} \leq 0.3$; ±, scarce growth; n.d., not determined. Each value is the mean of three independent determinations

towards hexadecane compared to glucose grown cells, suggesting that the carbon-dependent increase in cell surface hydrophobicity facilitated the direct contact between cells and Phe particles, and probably played an important role in Phe uptake/assimilation. When grown on Phe, *R. aetherovorans* I19 was found to be much more hydrophobic than the other tested isolates. A lower cell surface hydrophobicity, independent on growth substrates, was instead measured for *R. aetherovorans* B22. *Rhodococcus* sp. strains are recognized to show distinct levels of cell surface hydrophobicity depending on phase- and substrate-growth (Bredholt et al. 2002). Differences in cell wall properties found among *S. flava* strains suggest that *S. flava* I3R might contain additional functions for adhesion.

Discussion

The present work can address some of the questions still recently arisen, concerning the effects of NAPL phases on Phe biodegradation in complex systems simulating real conditions: presence of soil/soil simulating matrices and multiple microbial populations. The present results demonstrate that the two mixed bacterial cultures utilized Phe also when sorbed to a synthetic organo-mineral soil colloid, maintaining their kinetic features.

The capability of bacteria to attach to a NAPL phase cannot exclude that the Phe dissolved in hexadecane may be degraded directly from the organic phase (Efroymson and Alexander 1991). The initial slower biodegradation of Phe when dissolved in hexadecane by $C_{B\text{-}BT}$ (Fig. 2A) could be justified by an initial acclimation phase to hexadecane prior to Phe degradation rather than to be limited by the spontaneous Phe partitioning into water. The faster degradation of Phe dissolved in hexadecane by $C_{I\text{-}AT}$ culture might be due also to the bacterial capability to excrete products that either might increase the surface area of the NAPL droplets or decrease the interfacial tension by NAPL and water. At a similar Phe:hexadecane mass ratio, Sandrin et al. (2006) observed that hexadecane increased Phe mineralization rate by a *Burkholderia cepacia* isolate.

In agreement with what observed with whole soil (Nocerino 2003) and with a simulated organic soil colloid (Russo et al. 2005), the presence of an organo-mineral complex like MAH did not

restrict the bioavailability of Phe to microbial cells. Moreover, the solid matrix did not reduce the accessibility of the hydrocarbon to bacterial cells (Ortega-Calvo and Saiz-Jimenez 1998). After 30 day aging in the presence of MAH, 50% of the initial amount of Phe was bound to the matrix (Table 1). During aging phenomena in soil, a compound usually distributes itself between a fraction that can be rapidly desorbed, and another that is more slowly desorbed (Reid et al. 2000), being engaged within the micropores of soil colloids. Due to the sufficient open structure of MAH (Violante et al. 1999), sequestration sites for Phe may exist and might have facilitated the entrapment of Phe with the aging. Notwithstanding, Phe biodegradation occurred, probably due to the presence of microorganisms with capabilities to utilize differently available forms of Phe. As observed in studies with aged soils (Huesemann et al. 2004), the main constraint to degradation was the absence of catabolic features rather than the bioavailability of recalcitrant xenobiotics.

In the case of $C_{I\text{-}AT}$, although hydrophobic interactions could establish between hexadecane and humic acid on MAH surfaces, Phe bioavailability was not restricted. Possible cometabolic effects due to the utilization (though at a very less extent) of the humic acid contained in the MAH cannot be, however, ruled out. Although humic acid mainly interlayered into montmorillonite surfaces and thus it was protected against any possible release (Violante et al. 1999), very low amounts were, however, exposed onto the external surfaces, mainly at the edge of the montmorillonite structure, and as such available to microbial cells. Control tests performed by incubating MAH and $C_{B\text{-}BT}$ culture without any additional C source demonstrated a negligible growth of cells (data not shown).

In a system working with mixed community, it is difficult to directly correlate degradation kinetics with the state of Phe because of the population dynamics of the cultures. Prominent bands in DGGE profiles of the two Phe degrading cultures belonged to *Sphingomonas* sp. and *Rhodococcus* sp., recognised to be important biocatalysts for the remediation of PAH-polluted sites. The presence of Phe in different bioavailable forms determined differences in the relative abundance of different populations within the bacterial communities, indicating that particular bacteria were favoured by a particular form of Phe. This is of ecological significance in relation to the presence of different Phe-degrading bacteria, which can adapt to different micro-environmental niches (Friedrics et al. 2000). Band of *Sphingomonas* sp. got brighter in both cultures and that of *Alcaligenes* sp. in $C_{B\text{-}BT}$, when cultures grew on sorbed aged Phe-MAH, leading to hypothesize a role of these bacteria in the degradation of Phe under lower bioavailability conditions (Vacca et al. 2005). When Phe was dissolved in hexadecane, the composition of the Phe-degrading communities of the two cultures was not modified and the growth of hexadecane-degraders, such as *Pseudomonas* sp. strains in $C_{I\text{-}AT}$ culture and *Stenotrophomonas* sp. strains in $C_{B\text{-}BT}$, was stimulated. In particular, *Pseudomonas* sp. strains isolated from the $C_{I\text{-}AT}$ culture (i.e. strain I3H) were effective in the degradation of hexadecane, possibly helping in raising the degradation rate of Phe (Fig. 2B). The presence of *Sphingomonas* sp. and *Rhodococcus* sp. Phe degrading isolates in the two cultures is related to the composition of PAH degrading communities in hydrocarbon contaminated soils (Kastner et al. 1994; Bastiaens et al. 2000). Moreover ancillary species, incapable to degrade Phe but possibly participating in the degradation processes and/or in the utilization of derived metabolites, were members of the enrichment cultures.

The Phe degrading strains, as well as $C_{I\text{-}AT}$ and $C_{B\text{-}BT}$ cultures, showed an affinity for the organic phase, indicating a capability to develop hydrophobic surfaces, recognized to favour the adhesion to insoluble/solid compounds (van Oss 1994). For some strains a possible production of surfactants to enhance desorption of Phe from MAH cannot be excluded (Oberbremer et al. 1990). *Sphingomonas* spp. strains possess glycosphingolipids in their outer membrane resembling the structure of surfactants and they excrete the heteropolysaccharides ''sphingans'' which were found to increase the bioavailability of PAHs (Johnsen and Karlson 2004).

The results presented demonstrate that the two mixed bacterial cultures were able to utilize Phe when sorbed to a synthetic organo-mineral soil colloid and when dissolved in hexadecane as a simulated NAPL phase. The different phenanthrene bioavailability determined a modification in the relative frequency of some phylotypes in the two cultures. The answer to whether the isolated strains are actually able to degrade sorbed Phe awaits further research in order to consider them as ''true'' potential candidates for bioaugmentation of polluted soils with low PAH bioavailability. Investigations in soil with undisturbed structure to simulate the natural environment are in progress.

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