Biodegradation (2007) 18:607–616
DOI 10.1007/s10532-006-9093-3

ORIGINAL PAPER

Degradation of polycyclic aromatic hydrocarbons in soil by a two-step sequential treatment

Leticia Pizzul · Åsa Sjögren · María del Pilar Castillo · John Stenström

Received: 30 June 2006 / Accepted: 8 November 2006 / Published online: 11 January 2007

Abstract The objectives of this work were to isolate the microorganisms responsible for a previously observed degradation of polycyclic aromatic hydrocarbons (PAH) in soil and to test a method for cleaning a PAH-contaminated soil. An efficient PAH degrader was isolated from an agricultural soil and designated as *Mycobacterium* LP1. In liquid culture, it degraded phenanthrene (58%), pyrene (24%), anthracene (21%) and benzo(*a*)pyrene (10%) present in mixture (initial concentration 50 μg ml^{-1} each) and phenanthrene (92%) and pyrene (94%) as sole carbon sources after 14 days of incubation at 30°C. In soil, *Mycobacterium* LP1 mineralised ^{14}C-phenanthrene (45%) and ^{14}C-pyrene (65%) after 10 days. The good ability of this *Mycobacterium* was combined with the benzo(*a*)pyrene oxidation effect obtained by 1% w/w rapeseed oil in a sequential treatment of a PAH-spiked soil (total PAH concentration 200 mg kg^{-1}). The first step was incubation with the bacterium for 12 days and the second step was the addition of the rapeseed oil after this time and a further incubation of 22 days. Phenanthrene (99%), pyrene (95%) and anthracene (99%) were mainly degraded in the first 12 days and a total of 85% of benzo(*a*)pyrene was transformed during the whole process. The feasibility of the method is discussed.

Keywords *Mycobacterium* · Polycyclic aromatic hydrocarbons · Rapeseed oil · Soil bioremediation

L. Pizzul (✉) · Åsa Sjögren · M. P. Castillo · J. Stenström
Department of Microbiology, Swedish University of Agricultural Sciences, Box 7025, SE-750 07 Uppsala, Sweden
e-mail: Leticia.Pizzul@mikrob.slu.se

Introduction

Polycyclic aromatic hydrocarbons (PAH) are widely distributed environmental pollutants from natural and anthropogenic sources that include e.g. combustion processes and production and use of creosote and coal-tar (Howsan and Jones 1998). PAH are degraded by a wide range of microorganisms (Cerniglia 1992; Mueller et al. 1996), among which several actinomycetes are represented. The ability of members of this group to degrade PAH could be explained in part by their capacity to transform natural complex organic substances, such as lignin-related compounds (Ball et al. 1989), to produce biosurfactants or emulsifiers (Bicca et al. 1999; Kim et al. 2000) and by the hydrophobicity of the cell surfaces, which allows the close contact with or adhesion to low water soluble or lipophilic molecules and which favours the uptake process (Bastiaens et al. 2000). Some rhodococci, for

instance, degrade naphthalene, phenanthrene and anthracene, in many cases accompanied by the production of biosurfactants (Allen et al. 1997; Dean-Ross et al. 2001; Andreoni et al. 2004) and several mycobacteria are able to mineralise anthracene and pyrene, a process that is facilitated by the presence of mycolic acids in their cell wall, which allows better attachment to the hydrophobic compounds (Heitkamp et al. 1988; Dean-Ross and Cerniglia 1996; Wick et al. 2002).

Low molecular weight PAH (2–3 aromatic rings) are relatively easily metabolised but those with high molecular weight (more than 3 rings) are slowly transformed due to the increase in their hydrophobicity (Kanaly and Harayama 2000) and the decrease in bioavailability due to their absorption into the organic matter, strong binding to soil particles and sequestration inside micropores (Hatzinger and Alexander 1995). Bioremediation techniques often include the addition of supplements to enhance bioavailability and biodegradation of the PAH by indigenous or inoculated microorganisms. Carmichael and Pfaender (1997) divide the supplements into three general categories according to their impact on the microbial processes: surfactants that enhance the availability of the pollutants (Carriere and Mesania 1995), specific carbon sources or inducers that promote the growth of a particular group of degraders (Wong et al. 2002; Lee et al. 2003), and organic and inorganic nutrients that increase the size and activity of the soil microbial community. Some PAH are degraded only in the presence of an additional co-substrate, sometimes another PAH, by cometabolism or by stimulation of biodegradation (Bouchez et al. 1999; Kanaly et al. 2000).

Vegetable oils may play an important role in PAH degradation. The presence of peanut oil has been shown to enhance degradation of anthracene in liquid medium and soil slurry (Pannu et al. 2003), while rapeseed and babassu oil stimulate the production of biosurfactants (Sim et al. 1997; Vance-Harrop et al. 2003), which in turn can enhance the solubilisation of PAH (Page et al. 1999). Peanut, sunflower and rapeseed oils are also efficient in solubilising and extracting PAH from contaminated soils (Berg Schuur and Mattiasson 2003; Pannu et al. 2004; Gong et al. 2005).

In a previous work we studied the degradation of a mixture of PAH by *Rhodococcus wratislaviensis* in soils amended with rapeseed oil (Pizzul et al. 2006b). We found that two distinct processes, one biological and one abiotic, resulted in degradation of PAH. Biological processes degraded phenanthrene and pyrene to a large extent, either by *R. wratislaviensis* or by the indigenous microflora. The abiotic process, which occurred in the presence of 1% (w/w) rapeseed oil, mainly transformed the benzo(*a*)pyrene but the biological process also contributed to a small extent. Anthracene was degraded by the biological activity or oxidised by the abiotic process. The total degradation obtained probably was not optimal when the two processes occurred simultaneously, since the microorganisms were negatively affected by the high concentration of rapeseed oil needed for the abiotic process. Therefore, a two-step biological-abiotic process was proposed as a remediation technique in PAH-contaminated soils. In the same experiment, high mineralisation and degradation levels for some of the PAH were obtained in the control soil, indicating the presence of potential indigenous PAH-degrading microorganisms.

In the first part of the present work we describe the isolation, identification and characterisation of a PAH-degrading microorganism from an agricultural soil. This microorganism was used in the second part of the study, where we report the results of the application of the proposed sequential biological-abiotic treatment to a PAH-spiked soil and discuss the feasibility of the method.

Materials and methods

Chemicals

Anthracene (>96%) and phenanthrene (>97%) were purchased from Merck (Hohenbrunn, Germany), pyrene (98%) was purchased from Aldrich, anthraquinone (>99%) was supplied by Fluka (Steinheim, Germany) and benzo(*a*)pyrene (97%) was supplied by Sigma-Aldrich (Steinheim, Germany). The labelled compounds [9-^{14}C] phenanthrene (specific activity 55.7 mCi mmol^{-1}; radiochemical purity 98.9%) and [4, 5, 9, 10-^{14}C]

pyrene (specific activity 55 mCi $mmol^{-1}$; radiochemical purity 97.8%) were supplied by Sigma (USA) and [7,10-^{14}C] benzo(*a*)pyrene (specific activity 61.0 mCi $mmol^{-1}$; radiochemical purity 98.9%) was supplied by Amersham Biosciences (UK). Toluene (HPLC grade) was provided by Fisher Scientific (UK). Rapeseed oil was a gift from JTI-Swedish Institute of Agricultural and Environmental Engineering (Uppsala, Sweden).

Soil

The soil used in the experiments was an agricultural topsoil containing 1% organic carbon and 0.11% nitrogen and with a pH_{water} of 6.6. It was collected in Uppsala, Sweden, sieved (<2 mm) and stored at 4°C until used.

Isolation of pyrene-degrading bacteria from soil

The soil was spiked with phenanthrene, anthracene, pyrene and benzo(*a*)pyrene, incubated for 49 days at 30°C and then stored at –20°C until used. A sample (1 g) of thawed soil was added to a 100 ml Erlenmeyer flask containing 25 ml of minimal salt medium (modified from Mandelbaum et al. 1993) with pyrene as the only carbon source (80 μg ml^{-1}) and Delvocid® (Gist-Brocades, The Netherlands) (1 g l^{-1}) to reduce fungal growth, and incubated at 30°C and 150 rpm. The culture was maintained by consecutive transfers into fresh medium at 8-day intervals. After the third transfer, dilutions of the enrichment culture were spread on Tryptic Soy Broth (3 g l^{-1}) agar plates and incubated at 30°C. Single isolates were selected based on colony morphology and tested for degradation of pyrene. Each isolate was grown in GYM *Streptomyces* medium (4 glucose l^{-1}, 4 g yeast extract l^{-1} and 10 g malt extract l^{-1}; pH 7.2) for 4 days on a shaker (150 rpm) at 30°C. The culture was centrifuged (10 min, 970 × *g*), the supernatant discarded and the pellet washed twice with sterile tap water. The culture was then diluted to an OD_{600} of 0.500 and 1 ml was added to tubes containing pyrene (25 μg ml^{-1}) as the sole carbon source in 10 ml of minimal salt medium (modified from Mandelbaum et al. 1993) and glass beads, as described in Pizzul et al. (2006a). The microorganisms were incubated at 30°C for 10 days and the concentration of the remaining pyrene was measured at the end of the incubation time. A sterile control was included. The experiment was conducted in duplicate.

Identification of Mycobacterium LP1

Cells were incubated in GYM medium at 30°C for 4 days. The FastDNA® Spin Kit for soil (Qbiogene, Carlbac, CA, USA) was used to extract DNA with the following modifications. A 1.5 ml cell suspension was transferred to an 1.5 ml Eppendorf tube and the cells were centrifuged for 1 min at 16,000 × *g* using a Heraeus® BIOFUGE pico (DJB Labcare, Newport Pagnell, Buckinghamshire, UK). The supernatant was removed and 975 μl phosphate buffer and 125 μl MT-buffer from the kit were added. Bead beating was performed in a FastPrep® instrument for 60 s at speed 5.0. All following centrifugation steps were performed at a speed at 16,000 × g. The DNA was eluted in 50 μl DES (DNA Elution Solution-Ultra Pure Water, supplied with the kit) and its concentration determined using a NanoDrop® ND-1000 Spectrophotometer (Baylor College of Medicine, Huston, Texas, USA). The PCR was performed using a 'touchdown' protocol developed to amplify the 16S ribosomal rDNA from actinobacteria (Stach et al. 2003). Ten to 100 ng of DNA and 10 pmol of the primers S-C-Act-235-a-S-20 and S-C-Act-878-a-A-19, synthesised by Invitrogen™ (Invitrogen Ltd, Paisley, UK), were used for PCR amplifications. The FailSafe™ PCR System with PreMix B (Epicentre, Madison, Wisconsin, USA) was used according to the instructions, except that the final reaction volume was 50 μl. All PCR reactions were conducted in a GeneAmp® PCR System 9700 (PE Applied Biosystems, Norwalk, CT, USA). The PCR products were purified using MiniElute PCR Purification Kit (Quiagene, Wesr Sussex, UK), and sequenced in both directions with the primers used in the PCR reactions using BigDye™ Terminator chemistry (Applied Biosystems, Foster City, CA, USA). All (two forward and two reverse) sequence chromatograms were assembled with the Staden Package (University of Cambridge, UK). The consensus sequences

were compared with the 16S rDNA sequences from GenBank at the National Centre for Biotechnology Information (NCBI—http://www.ncbi.nih.gov/) using the basic local alignment search tool (BLAST) (Altschul et al. 1997).

Characterisation of Mycobacterium LP1

The ability of *Mycobacterium* LP1 (10^5 cfu ml^{-1}) to degrade phenanthrene, anthracene, pyrene and benzo(*a*)pyrene, alone or in a mixture, was tested in minimal salt medium (modified from Mandelbaum et al. 1993) containing 50 μg ml^{-1} of each of the PAH, as previously described (Pizzul et al. 2006a). The concentrations of the PAH were determined after 14 days and the values compared to sterile controls. The experiment was conducted in duplicate.

The ability of *Mycobacterium* LP1 to mineralise ^{14}C-phenanthrene, ^{14}C-pyrene and ^{14}C-benzo(*a*)pyrene in soil spiked with a mixture of phenanthrene, anthracene, pyrene and benzo(*a*)pyrene (50 mg kg^{-1} each) was tested as previously described (Pizzul et al. 2006b). The soil was autoclaved twice (20 min, 121°C) before the addition of the PAH and the inoculum (10^5 cfu g^{-1}). In brief, mineralisation was determined by collecting $^{14}CO_2$ from the degradation of the PAH in a NaOH solution and measuring the radioactivity in a liquid scintillation counter (Beckman LS 600 series, USA), after mixing with 4 ml of Insta-Gel Plus (Perkin–Elmer, USA). Mineralisation was expressed as $^{14}CO_2$ produced as a percentage of the initial radioactivity. The experiment was run in triplicate.

Sequential treatment of PAH-contaminated soil

A soil sample (200 g) was spiked with a mixture of the following PAH: anthracene, phenanthrene, pyrene and benzo(*a*)pyrene (50 mg kg^{-1} of each) in an acetone solution (Brinch et al. 2002). After evaporation of the acetone, the soil was inoculated with *Mycobacterium* LP1 to a final concentration of 10^6 cfu g^{-1}. Soil without addition of microorganisms was used as the control. After 12 days of incubation, rapeseed oil (1% w/w) was added to half the samples. The soil was incubated at 30°C and the water content was kept at 60% of the water-holding capacity. Samples were taken at the beginning of the experiment, 5 and 10 days after the inoculation and 5, 10 and 22 days after the addition of rapeseed oil, and the PAH concentrations determined. The experiment was conducted in triplicate.

Analytical methods

PAH in liquid media were extracted by adding 10 ml of toluene to the culture tubes and shaking vigorously for 1 h on a shaking table (adapted from Karstensen 1997). PAH in soil were extracted by adding 10 ml of toluene and 10 ml of a 0.05 M $Na_4P_2O_7$ solution to tubes containing 5 g of soil and shaking vigorously for 16 h. In both cases, the extracts were centrifuged for 10 min at $358 \times g$ and a portion of the toluene layer was centrifuged again at $5411 \times g$ and analysed directly by GC-MS. The recovery was higher than 95% for all the compounds. The GC-MS analysis was performed as previously described (Pizzul et al. 2006a).

Results

Isolation of pyrene degraders

In a previous work we found that the autochthonous microflora in an agricultural soil was able to degrade phenanthrene, anthracene and pyrene to a large extent (Pizzul et al. 2006b). In order to identify the PAH-degraders, a sample of the control soil from that experiment was used as inoculum for an enrichment culture using pyrene as the sole carbon source. Seven distinct isolates (A, B, C, D, E, F or G) based on different colony morphology were obtained. Three of the strains degraded pyrene to different extents. Isolates F and G degraded 6 and 23% respectively, while the highest degradation was obtained with isolate D (65%). Isolates A, B, C and E did not degrade pyrene. From these results it was concluded that isolate D could be one of the main microorganisms responsible for the degradation of pyrene in the soil and this strain was further identified and characterised.

Identification of Mycobacterium LP1

A 594 base-pair sequence was obtained from the 16S ribosomal RNA gene using the primers S-C-Act-235-a-S-20 and S-C-Act-878-a-A-19. Sequence similarity searches were performed against publicly available ribosomal RNA sequences. Our sequence was found to be identical to ten sequences in the database (*Mycobacterium gilvum*; AF544637, *M. gilvum*; AF544636, *M. gilvum*; AF544635, *M. gilvum*; AF544634, *M. gilvum*; AF544633, *Mycobacterium* sp.; AY694989, *Mycobacterium* sp.; AJ245704, *Mycobacterium* sp.; AJ245703, *Mycobacterium* sp.; X81891, *M. gilvum*; ATCC 43909). Accordingly, our isolate belongs to the genus *Mycobacterium*, and probably represents a *M. gilvum* strain, although the resolution of our sequence analysis is insufficient to differentiate on the level of strains. The metabolic traits of our isolate indeed agree with previous studies of *M. gilvum* (Habe et al. 2004; Mutnuri et al. 2005). *Mycobacterium* spp. are well known PAH-degraders that commonly are isolated from PAH-contaminated soil (Cheung and Kinkle 2001).

The strain was designated *Mycobacterium* LP1.

Characterisation of Mycobacterium LP1

Mycobacterium LP1 was tested for its ability to degrade PAH in liquid cultures. When the compounds were added individually as the sole carbon source, *Mycobacterium* LP1 degraded 92% of phenanthrene and 94% of pyrene, whereas no degradation of anthracene or benzo(*a*)pyrene was observed (Table 1). When the PAH were present in a mixture, the degradation of phenanthrene and pyrene was 58% and 24%, respectively. Moreover, anthracene (21%) and some benzo(*a*)pyrene (10%) were degraded after 14 days in the presence of the other PAH.

The ability of *Mycobacterium* LP1 to mineralise PAH was tested in soil spiked with the four PAH and with ^{14}C-phenanthrene, ^{14}C-pyrene or ^{14}C-benzo(*a*)pyrene. The soil was sterilised before treatment to avoid overestimations, since the soil used as inoculum for the isolation of *Mycobacterium* LP1 and the soil used in the mineralisation study were collected from the same site. *Mycobacterium* LP1 mineralised 45% of the ^{14}C-phenanthrene and 65% of ^{14}C-pyrene after 39 days, most of the mineralisation occurring in the first 10 days (Fig. 1). No mineralisation of ^{14}C-benzo(*a*)pyrene was observed. However, as the concentration of the remaining PAH was not determined at the end of the trial, we do not know the final levels of degradation of each of the PAH and how anthracene and benzo(*a*)pyrene were affected by the presence of the other PAH in the soil. Furthermore, because the PAH were added in a mixture, we do not know whether the mineralisation of the PAH could have been higher if they were added singly, as was observed for the degradation in the liquid cultures.

Sequential treatment of PAH-contaminated soil

Degradation of PAH after inoculation (the 'biological' step)

The first step in the sequential treatment was the inoculation of the soil with *Mycobacterium* LP1. The degradation values for the different PAH after the first 10 days of incubation are shown in Table 2. Higher degradation of phenanthrene, pyrene and anthracene was obtained in the inoculated soils than in the control soils. The degradation of benzo(*a*)pyrene was similar in both soils.

Degradation of PAH after addition of rapeseed oil (the 'abiotic' step)

The second step of the treatment started with the addition of rapeseed oil (1% w/w) at day 12. The final degradation values obtained at the end of the incubation time are summarised in Table 3.

The degradation values for phenanthrene and pyrene in the inoculated soils at day 34 (Table 3) did not differ greatly from those at day 10 (Table 2) since these compounds had been almost completely degraded during the first step. However, there was an effect of the rapeseed oil on the degradation of these two compounds by the autochthonous microflora in the control soil (Table 3). The inhibitory effect of the rapeseed oil was reflected in the lower degradation values for phenanthrene (85%) and especially pyrene (33%) (Table 3) compared to the control soil without rapeseed oil, where the indigenous

Table 1 Degradation of phenanthrene, anthracene, pyrene and benzo(*a*)pyrene, alone or in a mixture, by *Mycobacterium* LP1 in liquid culture after 14 days of incubation at 30°C

	Degradation (%)	
	Alone	Mixture
Phenanthrene	91.8*	58.1 ± 6.2
Anthracene	4.7 ± 7.6	20.8 ± 3.12
Pyrene	93.7 ± 0.04	23.8 ± 5.3
Benzo(*a*)pyrene	–	10.3 ± 4.7

Values are means ± SD (n = 2)

* n = 1

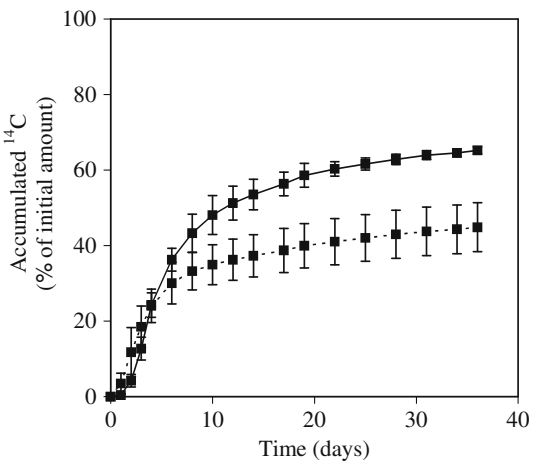

Fig. 1 Phenanthrene (dashed line) and pyrene (solid line) mineralisation (as % of added ^{14}C) by *Mycobacterium* LP1, in sterile soil incubated at 30°C. Values are means ± SD (n = 3)

microorganisms degraded 99% of phenanthrene and 92% of pyrene.

The degradation of anthracene was similar for all the treatments (Table 3). Anthraquinone was formed as a product of the oxidation of anthracene in the presence of rapeseed oil, as previously reported (Pizzul et al. 2006b). The concentration of this metabolite rapidly increased after the addition of oil to 14.2 mg kg^{-1} in the inoculated soil and to 43.5 mg kg^{-1} in the control soil (data not shown). The transformation of anthracene into anthraquinone was almost stoichiometric, and therefore the smaller production of anthraquinone in the presence of *Mycobacterium* LP1 was due to the lower concentration of anthracene present in the soil at the time of addition of the oil (Table 2).

The addition of rapeseed oil favoured the transformation of benzo(*a*)pyrene. After 34 days of incubation, 85% of the compound was transformed in the treatments with the oil, compared to 48% in treatments without oil (Table 3).

Discussion

Characterisation of Mycobacterium LP1

Mycobacterium LP1 is able to use phenanthrene and pyrene as sole carbon sources. However, the presence of other PAH is required for anthracene and benzo(*a*)pyrene degradation. Substrate interactions for PAH mixtures (e.g. competitive inhibition and cometabolism) have often been observed (Walter et al. 1991; Bouchez et al. 1999; Dean-Ross et al. 2002) and they are difficult to predict (Guha et al. 1999). These interactions may play an important role in the degradation of PAH in contaminated soils, where the compounds are usually present in mixtures. However, as the aim of our work was to obtain a general overview of the ability of *Mycobacterium* LP1 to degrade different PAH, these complex interactions were not investigated.

In 1988, Heitkamp and co-workers reported for the first time the isolation of a bacterium able to extensively mineralise pyrene, which was identified as belonging to the genus *Mycobacterium*. Since then, mineralisation of pyrene and other PAH by different mycobacteria has been reported by several authors (e.g. Boldrin et al. 1993; Dean-Ross and Cerniglia 1996). As in the case of *Mycobacterium* LP1, all the isolated PAH-degrading mycobacteria belong to the 'fast-growing mycobacteria', i.e. they form colonies from dilute inocula within 7 days under optimal conditions (Hartmans and De Bont 1991) and are distinct from the slow-growers, which include the pathogenic species (Leys et al. 2005). *Mycobacterium* LP1 was found to be closely related to *M. flavescens* (reclassified as *M. gilvum* by Kim et al. 2005), which was isolated from a polluted sediment and was able to mineralise pyrene (39%) and phenanthrene (18%) in liquid culture but did not utilise anthracene or benzo(*a*)pyrene (Dean-Ross and Cerniglia 1996). Even though the results

Table 2 Degradation of phenanthrene, anthracene, pyrene and benzo(*a*)pyrene in soils inoculated with *Mycobacterium* LP1 or without inoculum (control soil) after 10 days of incubation at 30°C, before the addition of rapeseed oil

Treatment	Degradation (%)			
	Phenanthrene	Anthracene	Pyrene	Benzo(*a*)pyrene
Inoculated soil	96.3 ± 0.1	78.4 ± 1.1	92.2 ± 1.4	22.7 ± 4.9
Control soil	72.2 ± 3.7	34.2 ± 3.4	17.6 ± 1.6	23.0 ± 4.3

The values are means ± SD (n = 6)

are not completely comparable because they were obtained under different growth conditions, both strains showed the same tendency for a higher mineralisation of pyrene compared to phenanthrene and no mineralisation of benzo(*a*)pyrene. However, the mineralisation levels observed for *Mycobacterium* LP1 were double those reported for *M. flavescens*.

Some mycobacteria are able to degrade PAH with more than four aromatic rings. For instance, *Mycobacterium* sp. strain RJGII-135, isolated from a former coal gasification site, degraded benzo(*a*)anthracene and benzo(*a*)pyrene (Schneider et al. 1996) and *Mycobacterium* PYR-1 - degraded benzo(*a*)pyrene (Heitkamp and Cerniglia 1988), in both cases cometabolically in the presence of yeast extract, peptone and starch. *Mycobacterium* LP1 did not degrade benzo(*a*)pyrene as sole carbon and energy source but a small (10 %) degradation was observed in the presence of the other PAH. Additional studies using different co-substrates are needed to determine whether the cometabolic processes reported for other mycobacteria are also performed by Mycobacterium LP1.

Interestingly, *Mycobacterium* LP1, which was as efficient as the pyrene-degrading strains reported in the literature, was isolated from a non-polluted soil. In a study of the PAH degradation capacity of soil microbial communities, Johnsen and Karlson (2005) showed that ^{14}C-pyrene was mineralised in all analysed samples from Danish soils, regardless of whether they were from contaminated sites or not. Those authors suggested that the background level of pyrene in soils in industrialised areas was enough to maintain the degradation traits in the gene pool of soil microorganisms. They also compared the PAH degradation potential of the Danish soils according to the time needed to achieve mineralisation of 10% and 50% of the added ^{14}C label, and reported values of 36 and 54 days respectively for the agricultural soil with the shortest lag phases, and 3 and 44 days for an industrial soil. When expressing our results in the same way, we obtained values of 15 and 23 days, closer to those in a highly contaminated soil than in a diffusely contaminated one (Pizzul et al. 2006b).

Sequential treatment

In a previous work we studied the effect of the addition of rapeseed oil on the degradation of PAH in soil by *Rhodococcus wratislaviensis* (Pizzul et al. 2006b). In the absence of rapeseed oil, phenanthrene, anthracene and pyrene were degraded by *R. wratislaviensis* and/or the indigenous microflora. The presence of 1%

Table 3 Degradation of phenanthrene (PHE), anthracene (ANT), pyrene (PYR) and benzo(a)pyrene (BaP) in soils with different treatments, after 34 days of incubation at 30°C

Treatment	Degradation (%)			
	PHE	ANT	PYR	BaP
Inoculated soil with rapeseed oil	98.9 ± 0.2	98.9 ± 0.4	94.5 ± 0.9	85.0 ± 3.9
Inoculated soil without rapeseed oil	99.0 ± 0.1	97.9 ± 0.9	96.6 ± 0.6	45.5 ± 4.3
Control soil with rapeseed oil	85.3 ± 1.3	97.8 ± 0.4	32.9 ± 1.6	85.5 ± 0.9
Control soil without rapeseed oil	98.8 ± 0.02	96.4 ± 0.5	91.8 ± 0.3	50.4 ± 2.9

The values are means ± SD (n = 3)

rapeseed oil led to a high transformation of anthracene and benzo(*a*)pyrene, mainly as a result of abiotic processes, since similar transformation levels of these compounds were obtained in sterile vs. inoculated liquid media (Pizzul et al. 2006a) and sterile vs. non sterile soil (unpublished data). However the high amount of oil restrained the biological processes and the degradation of phenanthrene and pyrene was inhibited. Therefore, the two-step sequential treatment was tested as an alternative that exploits both mechanisms.

The results obtained in the present work support the application of the sequential treatment to clean up a PAH-contaminated soil. In the first step, the inoculation with *Mycobacterium* LP1 promoted the biological transformation of phenanthrene, anthracene and pyrene. The PAH remaining in the soil after the biological degradation, especially benzo(*a*)pyrene, were transformed abiotically with the addition of rapeseed oil (1% w/w). This transformation may be due to a process of lipid peroxidation occurring in the presence of rapeseed oil (Pizzul et al. 2006b) but the mechanisms behind the oil effect have not yet been elucidated.

The biological treatment of PAH-contaminated soils is often limited by the low bioavailability of the pollutants. The combination of biodegradation processes and the use of environmentally friendly solvents to increase solubility has been proposed as a way to overcome this problem. The addition of paraffin oil to a slurry-type bioreactor doubled the mineralisation of pyrene by a *Mycobacterium* strain (Jimenez and Bartha 1996). Due to the hydrophobic surface of their cell walls, the bacteria adhered to the emulsified solvent droplets, which also contained the dissolved pyrene, and in this way the uptake was enhanced. High degradation rates of a mixture of PAH by *Mycobacterium* PYR-1 were also obtained in a two-phase partitioning bioreactor after the addition of a biodegradable solvent, bis (ethyl-hexyl) sebacate, which increased the solubilisation of the hydrophobic compounds (MacLeod and Daugulis 2003).

Vegetable oils have been proposed as natural, cost-effective and non-toxic solvents. Since high removal efficiency (>90%) can be obtained, remediation schemes have been developed based on the solubilisation and extraction of the PAH from the soils using, for example, peanut and sunflower oil (Pannu et al. 2004; Gong et al. 2005; Gong et al. 2006). However, these systems are rather complex since (a) special containers or columns are used for the extraction; (b) high volumes of oil are required; (c) the oil has to be recycled and a cleaning step, e.g. using activated carbon, has to be included for the system to be cost-effective; and (d) the activated carbon has to be regenerated by microbial degradation or conventional thermal treatment.

Berg Schuur and Mattiasson (2003) proposed a two-step remediation treatment of PAH-contaminated soil that included first biodegradation by PAH-degraders in a bioreactor, and then extraction of the remaining PAH with rapeseed oil in a slurry system, using 1.5 to 7.5 ml of rapeseed oil for 28 g soil (~5 to 26% v/w). The total concentration of PAH decreased from 2470 mg kg^{-1} to 1366 mg kg^{-1} (44%) after 30 days in the bioreactor and to 343 mg kg^{-1} (86%) after the oil extraction. Even though high degradation levels were obtained with this treatment, it involves the use of bioreactors, which has the disadvantage that the soil has to be removed and transported to special facilities that require relatively high capital investment and that have high operating costs (Mueller et al. 1996). In this case too, it is important to regenerate and re-use the oil in order to make this approach economically viable (Berg Schuur and Mattiasson 2003).

Pannu et al. (2003) reported the effects of small amounts of vegetable oil on the biodegradation of PAH. The presence of 0.2% (v/v) of peanut oil had a stimulatory effect on the degradation of PAH in a soil slurry system, where 96% of phenanthrene, 74% of anthracene, 65% of pyrene and 70% of benzo(*a*)pyrene were degraded after 24 days of incubation at 30°C. The beneficial effect was attributed to the solubilisation of the PAH in the oil and subsequent transfer to the bacterial cells associated with the oil drops and to the promotion of biosurfactant production. Rather high degradation values may be obtained with this system but, as described above, it has the same drawback of involving the use of bioreactors.

The sequential treatment described in the present study has the distinguishing characteristic

that it is directly applicable to the soil and there is no need for bioreactors or special equipment. Moreover, the pollutants remaining after the biological degradation are transformed chemically in the soil matrix after the oil addition in the second step. The method has potential for scaling up as it is simple, relatively fast and recycling of the oil is not required since the amounts applied are low. More studies are needed to better understand the processes behind the oil effect and to optimise the method.

References

Allen CCR, Boyd DR, Larkin MJ, Reid K, Sharma ND, Wilson K (1997) Metabolism of naphthalene, 1-naphtol, indene, and indole by *Rhodococcus* sp. strain NCIMB 12038. Appl Environ Microbiol 63:151–155

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

Andreoni V, Cavalca L, Rao MA, Nocerino G, Bernasconi S, Dell'Amico E, Colombo M, Gianfreda L (2004) Bacterial communities and enzyme activities of PAHs polluted soils. Chemosphere 57:401–412

Ball AS, Betts WB, McCarthy AJ (1989) Degradation of lignin-related compounds by actinomycetes. Appl Environ Microbiol 55:1642–1644

Bastiaens L, Springael D, Wattiau P, Harms H, deWachter R, Verachtert H, Diels L (2000) Isolation of adherent polycyclic aromatic hydrocarbon (PAH)-degrading bacteria using PAH-sorbing carriers. Appl Environ Microbiol 66:1834–1843

Berg Schuur JH, Mattiasson B (2003) Separation of coal-tar constituents from soil particles in a two-liquid-phase slurry system. Environ Technol 24:755–765

Bicca FC, Fleck LC, Ayub MAZ (1999) Production of biosurfactant by hydrocarbon degrading *Rhodococcus ruber* and *Rhodococcus erythropolis*. Rev Microbiol 30:231–236

Boldrin B, Tiehm A, Fritzsche C (1993) Degradation of phenanthrene, fluorene, fluoranthene and pyrene by a *Mycobacterium* sp. Appl Environ Microbiol 59:1927–1930

Bouchez M, Blanchet D, Bardin V, Haeseler F, Vandecasteele J-P (1999) Efficiency of defined strains and of soil consortia in the biodegradation of polycyclic aromatic hydrocarbon (PAH) mixtures. Biodegradation 10:429–435

Brinch UC, Ekelund F, Jacobsen CS (2002) Method for spiking soil samples with organic compounds. Appl Environ Microbiol 68:1808–1816

Carmichael LM, Pfaender FK (1997) The effect of inorganic and organic supplements on the microbial degradation of phenanthrene and pyrene in soils. Biodegradation 8:1–13

Carriere PPE, Mesania FA (1995) Enhanced biodegradation of creosote-contaminated soil. Waste Manage 15:579–583

Cerniglia CE (1992) Biodegradation of polycyclic aromatic hydrocarbons. Biodegradation 3:351–368

Cheung P-Y, Kinkle BK (2001) *Mycobacterium* diversity and pyrene mineralization in petroleum-contaminated soils. Appl Environ Microbiol 67:2222–2229

Dean-Ross D, Cerniglia CE (1996) Degradation of pyrene by *Mycobacterium flavescens*. Appl Microbiol Biot 46:307–312

Dean-Ross D, Moody J, Cerniglia CE (2002) Utilization of mixtures of polycyclic aromatic hydrocarbons by bacteria isolated from contaminated sediment. FEMS Microbiol Ecol 41:1–7

Dean-Ross D, Moody JD, Freeman JP, Doerge DR, Cerniglia CE (2001) Metabolism of anthracene by a *Rhodococcus* species. FEMS Microbiol Lett 204:205–211

Gong Z, Alef K, Wilke BM, Li P (2005) Dissolution and removal of PAHs from a contaminated soil using sunflower oil. Chemosphere 58:291–298

Gong Z, Wilke BM, Alef K, Li P, Zhou Q (2006) Removal of polycyclic aromatic hydrocarbons from manufactured gas plant-contaminated soils using sunflower oil: Laboratory column experiments. Chemosphere 62:780–787

Guha S, Peters CA, Jaffé PR (1999) Multisubstrate biodegradation kinetics of naphyhalene, phenanthrene, and pyrene mixtures. Biotechnol Bioeng 65:491–499

Habe H, Kanemitsu M, Nomura M, Takemura T, Iwata K, Nojiri H, Yamane H, Omori T (2004) Isolation and characterization of an alkaliphilic bacterium utilizing pyrene as a carbon source. J Biosci Bioeng 98:306–308

Hartmans S, De Bont JA (1991) The genus *Mycobacterium*-Nonmedical. In: Ballows A, Trüper H, Dworkin M, Harder W, Schleifer KH (eds) The Prokaryotes, vol 2. Springer-Verlag, New York, pp 1214–1237

Hatzinger PB, Alexander M (1995) Effect of aging of chemicals in soil on their biodegradability and extractability. Environ Sci Technol 29:537–545

Heitkamp MA, Cerniglia CE (1988) Mineralization of polycyclic aromatic hydrocarbons by a bacterium isolated from sediment below an oil-field. Appl Environ Microbiol 54:1612–1614

Heitkamp MA, Franklin W, Cerniglia CE (1988) Microbial metabolism of polycyclic aromatic hydrocarbons: isolation and characterization of a pyrene-degrading bacterium. Appl Environ Microbiol 54:2549–2555

Howsan M, Jones KJ (1998) Sources of PAHs in the environment. In: Hutzinger O (ed) The Handbook of Environmental Chemistry, vol 3. Springer-Verlag, Berlin, pp 137–174

Jimenez IY, Bartha R (1996) Solvent-augmented mineralization of pyrene by a *Mycobacterium* sp. Appl Environ Microbiol 62:2311–2316

Johnsen A, Karlson U (2005) PAH degradation capacity of soil microbial communities-Does it depend on PAH exposure? Microbial Ecol 50:488–495

Kanaly RA, Bartha R, Watanabe K, Harayama S (2000) Rapid mineralization of benzo[*a*]pyrene by a microbial consortium growing on diesel fuel. Appl Environ Microbiol 66:4205–4211

Kanaly RA, Harayama S (2000) Biodegradation of high-molecular-weight polycyclic aromatic hydrocarbons by bacteria. J Bacteriol 182:2059–2067

Karstensen KH (1997) Analysis of polyaromatic hydrocarbons Nordic Guidelines for Chemical Analysis of Contaminated Soil Samples. SINTEF, Espoo, Finland, pp 126–130

Kim SH, Lim EJ, Lee SO, Lee JD, Lee TH (2000) Purification and characterization of biosurfactants from *Nocardia* sp. L-417. Biotechnol Appl Biochem 31:249–253

Kim Y-H, Engesser K-H, Cerniglia C (2005) Numerical and genetic analysis of polycyclic aromatic hydrocarbon-degrading mycobacteria. Microbial Ecol 50:110–119

Lee PH, Doick KJ, Semple KT (2003) The development of phenanthrene catabolism in soil amended with transformer oil. FEMS Microbiol Lett 228:217–223

Leys NM, Ryngaert A, Bastiaens L, Wattiau P, Top EM, Verstraete W, Springael D (2005) Occurrence and community composition of fast-growing *Mycobacterium* in soils contaminated with polycyclic aromatic hydrocarbons. FEMS Microbiol Ecol 51:375–388

MacLeod CT, Daugulis AJ (2003) Biodegradation of polycyclic aromatic hydrocarbons in a two-phase partitioning bioreactor in the presence of a bioavailable solvent. Appl Microbiol Biotechnol 62:291–296

Mandelbaum R, Wacket L, Allan D (1993) Mineralization of the *s*-Triazine ring of atrazine by stable bacterial mixed cultures. Appl Environ Microbiol 59:1695–1701

Mueller JG, Cerniglia CE, Pritchard PH (1996) Bioremediation of environments contaminated by polycyclic aromatic hydrocarbons. In: Crawford RL, Crawford LD (eds) Bioremediation: principles and applications. Cambridge University Press, pp 125–194

Mutnuri S, Vasudevan N, Kaestner M (2005) Degradation of anthracene and pyrene supplied by microcrystals and non-aqueous-phase liquids. Appl Microbiol Biotechnol 67:569–576

Page CA, Bonner JS, Kanga SA, Mills MA, Autenrieth RL (1999) Biosurfactant solubilization of PAHs. Environ Eng Sci 16:465–474

Pannu J, Singh A, Ward O (2003) Influence of peanut oil on microbial degradation of polycyclic aromatic hydrocarbons. Can J Microbiol 49:508–513

Pannu J, Singh A, Ward O (2004) Vegetable oil as a contaminated soil remediation amendment: application of peanut oil for extraction of polycyclic aromatic hydrocarbons from soil. Process Biochem 39:1211–1216

Pizzul L, Castillo MdP, Stenström J (2006a) Characterization of selected actinomycetes degrading polyaromatic hydrocarbons in liquid culture and spiked soil. World J Microb Biot 22:745–752

Pizzul L, Castillo MdP, Stenström J (2006b) Effect of rapeseed oil on the degradation of PAH in soil by *Rhodococcus wratislaviensis*. Int. Biodeter. Biodegr. *In press* doi:10.1016/j.ibiod.2006.08.004

Schneider J, Grosser R, Jayasimhulu K, Xue W, Warshawsky D (1996) Degradation of pyrene, benz[a]anthracene, and benzo[a]pyrene by *Mycobacterium* sp. strain RJGII-135, isolated from a former coal gasification site. Appl Environ Microbiol 62:13–19

Sim L, Ward O, Li Z-Y (1997) Production and characterisation of a biosurfactant isolated from *Pseudomonas aeruginosa* UW-1. J Ind Microbiol Biotechnol 19:232–238

Stach JEM, Maldonado LA, Ward AC, Goodfellow M, Bull AT (2003) New primers for the class Actinobacteria: application to marine and terrestrial environments. Environ Microbiol 5:828–841

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

Vance-Harrop MH, de Gusmao NB, de Campos-Takaki GM (2003) New bioemulsifiers produced by *Candida lipolytica* using D-glucose and babassu oil as carbon sources. Braz J Microbiol 34:120–123

Wick LY, de Munain AR, Springael D, Harms H (2002) Responses of *Mycobacterium* sp. LB501T to the low bioavailability of solid anthracene. Appl Microbiol Biotechnol 58:378–385

Wilson SC, Jones KC (1993) Bioremediation of soil contaminated with polynuclear aromatic hydrocarbons (PAHs): a review. Environ Pollut 81:229–249

Wong JWC, Lai KM, Ma KK, Fang M (2002) Isolation and optimization of PAH-degradative bacteria from contaminated soil for PAHs bioremediation. Water Air Soil Poll 139:1–13