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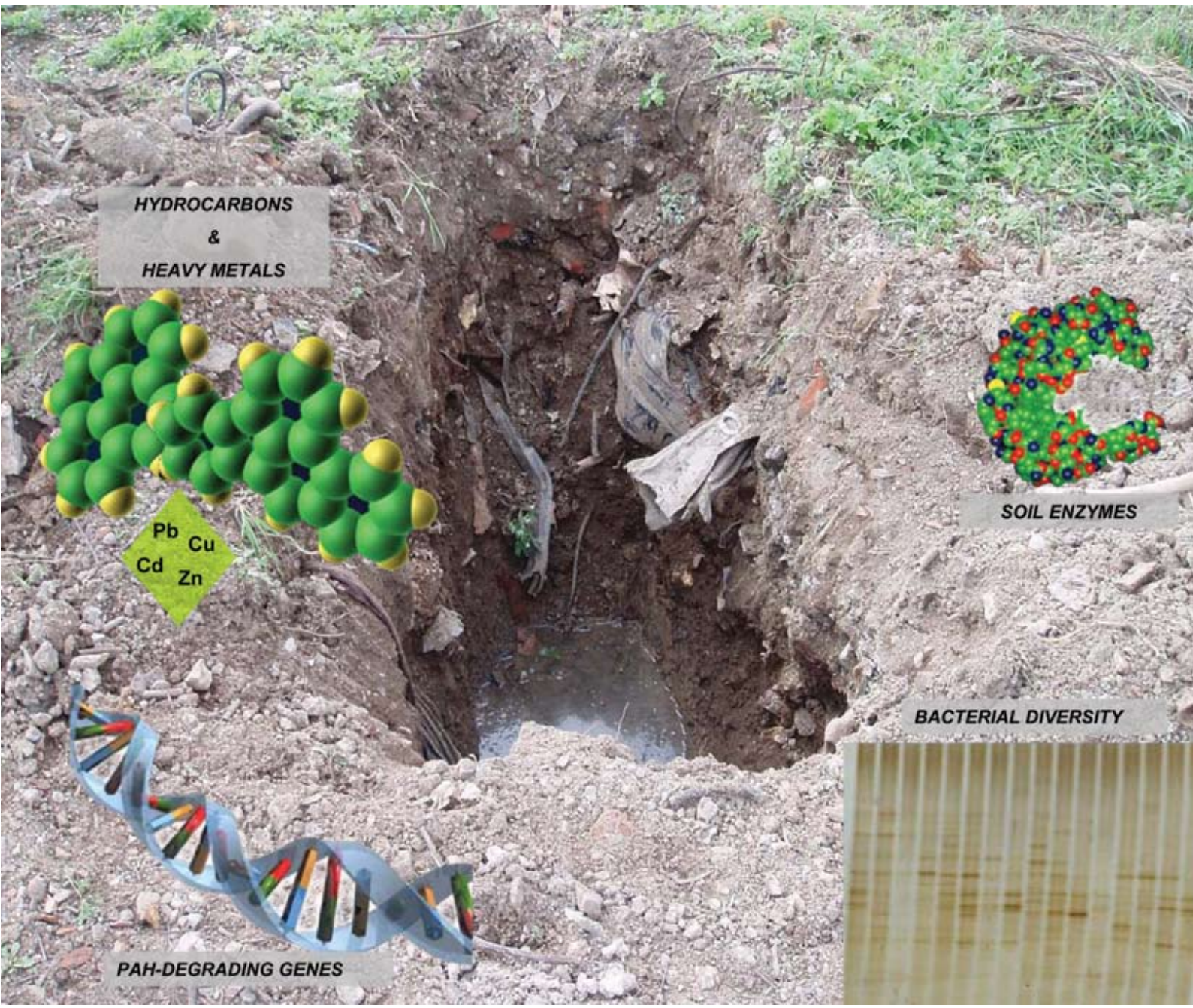

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Metabolic and bacterial diversity in soils historically contaminated by heavy metals and hydrocarbons

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The aim of this study was to characterize soils contaminated by different levels of heavy metals and hydrocarbons (Madonna Dell'Acqua, Pisa, Italy). The soils were chemically and biochemically analysed by measuring the standard chemical properties and some enzyme activities related to microbial activity (dehydrogenase activity) and the soil carbon cycle (total and extracellular β-glucosidase activities). The metabolic capacities of soil microorganisms to degrade hydrocarbons through catechol 2,3-dioxygenase were also described. The microbial diversity of contaminated and uncontaminated soils was estimated by denaturing gradient gel electrophoresis (DGGE) of amplified 16S rDNA sequences. The PCR/single-strand conformation polymorphism (PCR/SSCP) method was used to estimate the genetic diversity of PAH-degrading genes in both contaminated and uncontaminated soils. A greater bacterial diversity and lower catechol 2,3-dioxygenase activity was detected in unpolluted soils. The complexity of the microbial community (Shannon and Simpson indices) as well as the dehydrogenase soil activity negatively correlated with contamination levels. The greatest PAH-degrading gene diversity and the most intense catechol 2,3-dioxygenase activity were found in the soils with the highest levels of hydrocarbons. Heavy metals and hydrocarbon pollution has caused a genetic and metabolic alteration in microbial communities, corresponding to a reduction in microbial activity. A multi-technique approach combining traditional biochemical methods with molecular-based techniques, along with some methodological improvements, may represent an important tool to expand our knowledge of the role of microbial diversity in contaminated soil.

Introduction

Pollution by heavy metals and hydrocarbons has attracted much attention in recent decades. Besides their natural occurrence, heavy metals may enter ecosystems due to human causes, such as mining, smelting, sewage sludge disposal, application of pesticides and inorganic fertilizers, and atmospheric emissions.[1] Polycyclic aromatic hydrocarbons (PAHs) are a ubiquitous group of hazardous organic pollutants which exhibit strong carcinogenic and toxic properties. The main anthropogenic sources include industrial processing power and heat generation in waste incineration and traffic emissions.[2] PAHs can enter the soil *via* atmospheric deposits, and it is estimated that more than 90% of the total PAH load is found in surface soils.[3] Metals are strongly associated with PAHs, and soils contaminated by PAHs also often contain large amounts of heavy metals.[4,5] Both pollutants in the soil influence the microorganisms through changes in enzyme activities.[6] As microorganisms are in close contact with the soil environment, they are regarded as the best indicators of soil pollution.

Few studies have considered the combined effect of heavy metals and PAHs on the structure of bacterial communities and enzyme activities in soil. Malizewska-Kordybach and Smreczak recently reported that the contamination of soils by PAHs (flourene, anthracene, pyrene, and chrysene) and heavy metals (Cd, Zn, and Pb) had an adverse effect on soil microorganism activity.[7] Gogolev and Wilke also demonstrated through the use of agar-plate experiments that fluoranthene might increase the toxicity of Zn, Cd and Cu for soil bacteria.[8] However, the effect of heavy metals and PAHs on soil microorganisms largely depends on their interaction with soil matrices.[9]

Despite the well-known bias of cultivation-based techniques, different cultivation methods have been used to determine whether native bacteria are capable of degrading organic contaminants. Molecular techniques using universal 16S rDNA gene primers allow researchers to examine microbial communities without changing cultivation techniques. Denaturing gradient gel electrophoresis profiling-sequence analysis of PCR-amplified 16S soil rDNA fragments (PCR-DGGE) and PCR/single-strand conformation polymorphism (PCR-SSCP) have been particularly useful for studying bacterial diversity and detecting genes involved in the degradation of xenobiotic compounds.[10,11] The presence of PAH-degrading genes could enable us to estimate the intrinsic potential of the soil across microorganisms to degrade PAHs. The aerobic deterioration of polyaromatic compounds depends on the presence of a multicomponent enzyme system, the initial PAH dioxygenase which catalyses the hydroxylation of substrates to the corresponding

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cis-dihydrodiol. Extradiol breaking and subsequent deterioration through the phased removal of aromatic rings completes the upper pathway, leading finally to the production of catechol, one of the key components of PAH deterioration. *Meta*-cleavage of catechol catalysed by catechol 2,3-dioxygenase (EC 1.13.11.2) appears to be the most common pathway in the subsequent phases of PAH deterioration (lower pathway).[12]

The study of soil enzyme activities enables us to monitor the decontamination process.[13] The study of catechol 2,3-dioxygenase activity in PAH-contaminated soils, which has not so far been analysed, could therefore provide accurate information on the capacity of the soil to degrade these organic components and on potential soil recovery.

The present study was conducted to determine a possible relationship between bacterial-community structure, PAH-degrading gene diversity and the degree of pollution by hydrocarbons and heavy metals. In addition, this study describes a method capable of measuring the metabolic capacities of soil microorganisms for PAHs degrading soil through catechol 2,3-dioxygenase (EC 1.13.11.2) activity.

Experimental

Soils

The site studied was located in the Madonna dell'Acqua province of Pisa (Tuscany, Italy) and covered an area of about 5000 m^2. The site has been important for over 20 years due to vehicle emissions and the presence of waste and iron material landfill sites. These activities have led to heterogeneous soil pollution caused by heavy metals and hydrocarbons, showing a spatial variability in the quality and quantity of pollutants. Nine samples, which included an off-site controlled experiment, were randomly taken from across the site from the 0–20 cm soil horizon, with triplicate samples taken from each sampling site (over a 0.5 m^2 area). Samples were sealed in plastic bags, and then transported on ice to the laboratory, where they were kept at a temperature of 4 °C until analysed. The relevant chemical characteristics of the soils (sandy-loam texture) are given in Tables 1 and 2.

Total hydrocarbons analysis

The total hydrocarbons were determined by the 1664 gravimetric method[14,15] using n-pentane instead of n-hexane, as modified by Ceccanti and collegues.[16]

The soil samples (1 g) were air-dried and mixed with Na_2SO_4 to remove residual water. Total hydrocarbons were extracted three times with 5 ml of pentane in an ultrasound bath for 15 min. Total hydrocarbon content is estimated by weighing the dry residue after solvent evaporation under nitrogen flow.

Chemical parameters

Electrical conductivity (EC) and pH were measured in a 1/10 (w/v) aqueous solution. Total carbon and nitrogen were determined by dry combustion with an RC-412 multiphase carbon and an FP-528 protein/nitrogen determinator respectively (LECO Corporation).

Table 1 Chemical characteristics of soil samples. Means values and ± standard deviation

Soil	pH	EC/dS m^{-1}	F^-/mg kg^{-1}	Cl^-/mg kg^{-1}	NO_3^-/mg kg^{-1}	PO_4^{3-}/mg kg^{-1}	SO_4^{2-}/mg kg^{-1}	N (%)	TOC (%)	TEC/mg kg^{-1}	WSC/mg kg^{-1}
S1	8.56	80	5.96 ± 0.12	40.00 ± 0.58	25.62 ± 1.00	[a]Nd	20.72	0.25	4.25 ± 0.09	4985.00	570.64
S2	8.85	120	5.28 ± 0.08	44.33 ± 2.03	23.22 ± 0.93	[a]Nd	22.54	0.17	1.87 ± 0.02	1442.33	402.62
S3	8.24	110	7.93 ± 0.11	77.33 ± 2.73	13.01 ± 0.37	8.16 ± 0.30	24.63	0.23	2.51 ± 0.08	3195.67	591.64
S4	8.98	110	5.94 ± 0.16	35.00 ± 1.15	20.23 ± 0.75	4.02 ± 0.14	10.46	0.16	2.63 ± 0.08	1638.00	3531.92
S5	8.85	150	9.09 ± 0.18	57.33 ± 1.45	4.87 ± 0.07	[a]Nd	34.94	0.02	1.78 ± 0.03	1316.00	329.12
S6	8.74	110	8.16 ± 0.10	48.67 ± 1.86	15.48 ± 0.45	[a]Nd	37.34	0.15	2.22 ± 0.06	1505.33	119.10
S7	8.56	100	6.47 ± 0.20	34.33 ± 1.33	20.15 ± 0.77	[a]Nd	39.46	0.13	2.04 ± 0.08	1306.00	297.61
S8	8.75	150	6.10 ± 0.17	43.67 ± 0.88	8.43 ± 0.25	[a]Nd	22.69	0.13	2.39 ± 0.09	1549.00	308.11
C	8.88	50	6.19 ± 0.18	48.67 ± 0.88	1.67 ± 0.08	[a]Nd	18.37	0.02	1.43 ± 0.04	402.67	591.64

[a] Nd: not detected; TOC: total organic carbon; TEC: total extractable carbon; WSC: water soluble carbon.

Table 2 Total hydrocarbons (TPH) and metals content (mg kg $^{-1}$) of soil samples. Means values and ± standard deviation

Soil	TPH	Cu	Pb	Ni	Zn	Cd	Cr
S1	2.07 ± 0.13	412 ± 16.9	2361 ± 122	126 ± 4.15	530 ± 20.8	12.7 ± 0.18	154 ± 5.2
S2	4.84 ± 0.38	269 ± 10.4	1022 ± 38.4	120 ± 2.63	460 ± 23.9	8.8 ± 0.10	186 ± 7.2
S3	2.48 ± 0.11	112 ± 5.1	770 ± 9.5	95.2 ± 2.07	120 ± 3.26	26.3 ± 1.03	125 ± 5.3
S4	1.63 ± 0.08	144 ± 5.7	445 ± 1.0	100 ± 1.57	270 ± 12.5	8.9 ± 0.19	94.3 ± 1.8
S5	0.51 ± 0.04	112 ± 2.2	238 ± 5.8	103 ± 2.31	120 ± 5.14	3.1 ± 0.01	112 ± 4.9
S6	1.59 ± 0.40	236 ± 11.4	1134 ± 30.7	110 ± 2.78	230 ± 7.59	4.1 ± 0.03	75.4 ± 1.5
S7	1.27 ± 0.28	360 ± 14.1	1620 ± 81.4	125 ± 4.78	720 ± 16.6	6.9 ± 0.40	193 ± 3.4
S8	1.19 ± 0.07	409 ± 16.6	26500 ± 904	104 ± 4.11	280 ± 9.28	5.2 ± 0.16	279 ± 11.2
C	0.84 ± 0.13	236 ± 9.9	80 ± 0.6	59.5 ± 0.88	50.1 ± 1.98	4.3 ± 0.13	27.2 ± 0.8

Soluble organic carbon (WSC) and pyrophosphate-extractable carbon (PEC) were determined by dichromate oxidation.[17] Inorganic anions were measured on soil-water extract by ionic chromatography using a DIONEX chromatograph. Heavy metals were determined by atomic absorption spectroscopy after microwave acid digestion extraction.[18]

Enzyme activities

Dehydrogenase and β-glucosidase activity was determined using 1 g of soil, following ref. 19 modified by ref. 20 and ref. 21, respectively.

For the analysis of extracellular β-glucosidase activities, total pyrophosphate-extractable carbon (PEC) was extracted with $Na_2P_4O_7$ (0.1 M, pH 7.1) in a 1 : 10 solid–liquid ratio by mechanical shaking at 37 °C for 24 h and then centrifuged at 8000 *g* and filtered through a 0.22 μm Millipore membrane. The extract was dialysed against distilled water and then concentrated 10 times at 35 °C using a Savant Speed Vac concentrator. (GMI, Inc., Minnesota, USA). The β-glucosidase activity was determined using 0.5 ml of dialysed concentrated pyrophosphate extract.[21]

The C contents of PEC were determined by acid digestion with 1 N potassium dichromate and sulfuric acid cc. at 160 °C for 30 min. A spectrophotometric method was used to quantify the Cr^{3+} produced by the reduction of Cr^{6+} ($\lambda = 590$ nm).[22]

For the determination of the catechol 2,3 dioxygenase (C23DO) activity, the following procedure was followed.[23] Five grams of soil were mixed with 25 ml of 0.1 M phosphate buffer (pH 7), and the solution was shaken at 28 °C for 72 h in the dark. Then, 5 ml of the extracts were incubated in 100 ml of minimum mineral medium (MMM) for growth and enzyme-induction purposes. Catechol was then added to MMM to reach a final concentration of 0.27 g l^{-1}, whereupon the cultures were shaken and then incubated at 28 °C for 7 days in the dark. Thereafter, cell-extracts were centrifuged at 8000 *g* for 15 min at 4 °C. Cell pellets were washed three times in solution saline 0.85% NaCl and centrifuged at 8000 *g* for 15 min at 4 °C. The pellets were resuspended in a lysozyme buffer (100 mM EDTA, 50 mM NaCl pH 6.9 and 1 mg ml^{-1} lysozyme; Sigma), and then incubated at 30 °C for 90 min to disrupt the cells. Cell debris was removed by centrifugation (12000 *g*, 25 min) to achieve a clarified extract. To determine the C23DO activity, samples (300 μl) were added to the reaction mixture containing 1 ml of 0.3 mM catechol (final volume: 5 ml) and then incubated at 37 °C for 20 h. The 2,3-dioxygenase was assayed in a 0.1 M phosphate buffer (pH = 7.0) and the formation of 2- hydroxymuconic semialdehyde ($\lambda = 375$ nm; $\varepsilon_m = 33400$ M^{-1} cm^{-1}) monitored. Assays without soil and without catechol were carried out simultaneously as controls. Results were expressed as μmol product g^{-1} h^{-1}.

Soil-DNA extraction

Total DNA was extracted from subsamples of 250 mg of soil by the bead-beating method according to manufacturer's instructions for the MoBio UltraClean Soil DNA Isolation kit (MoBio Laboratories Inc., Solana Beach, CA, USA) with a few modifications, including the repetition of the second step (Inhibitor Removal Solution) to remove trace concentrations of PCR inhibitors. The DNA samples were checked for concentration and quality using the NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

PCR-DGGE analysis

PCR was performed with 16S rDNA universal bacterial denaturing gradient gel electrophoresis (DGGE) primers (synthesized by TIB®MOLBIOL, Berlin, Germany) (primer 1, primer 2 and primer 3) throughout to amplify the V3 hypervariable region of 16S rDNA genes. The nucleotide sequences of the primers are as follows: primer 1 (P1 5′-CCT ACG GGA GGC AGC AG- 3′) primer 2 (P2 5′-ATT ACC GCG GCT GT GG- 3′) and primer 3 (P3 5′- CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG - 3′). Primer 3 (P3) contains the same sequence as primer 1 (P1) but has at its 5′ end an additional 40-nucleotide GC-rich sequence (GC clamp).[24] Two successive amplifications were carried out with the following modifications: a hot start of 5 min at 94 °C; 19 cycles consisting of 94 °C for 15 s, 65–55 °C for 15 s, decreasing the temperature by 0.5 °C each cycle (touchdown), and 72 °C for 30 s; 14 cycles consisting of 94 °C for 15 s, 55 °C for 15 s, and 72 °C for 30 s; and a final step of 10 min at 72 °C. The total reaction mixture of the first PCR consisted of 25 μl with the following ingredients: 1 μl volume (approx. 10 ng) of extracted DNA, 1 μM primer P1, 1 μM primer P2, 10 μl Eppendorf® Master Mix (2.5X) and sterile Milli-Q water to a final volume. The second amplification was performed by using 1 μl of the products of the first reaction as template. In this, primers P2 and P3 were used under the same conditions described above. PCR products were analysed by electrophoresis in 2% agarose gels stained with ethidium bromide.

DGGE analyses were conducted using 20 μl of this latter PCR product loaded into a 40 to 65% urea–formamide–polyacrylamide gel. An INGENYphorU System (Ingeny

International BV, The Netherlands) was run at 75 V for 17 h at 58 °C to separate the fragments. Gels were silver stained with the Bio-Rad Silver Stain according to the standard DNA-staining protocol and photographed under UV light ($\lambda = 254$ nm) using an UVItec Gel Documentation system (UVitec Limited, Cambridge, UK).

Isolation, PCR amplification and sequencing of DNA fragment from DGGE

DNA fragments from DGGE were isolated as previously described.[25] PCR was performed with 1–2 μl of this solution (5– 10 ng for reaction) and primers P2 and P3 using the same method as above.

PCR fragments were purified by MBL-Agarosa Quickclean (Dominion-MBL SL, Spain). The cloning experiments were carried out following the instructions of the manufacturer of the pMBL-T-vector (Dominion-MBL SL, Spain). Clone cells with recombinant plasmid DNAs were selected on Luria-Bertani plates,[26] and then subjected to PCR with primers M13f and M13r.[27] A touchdown approach modified from that of Watanabe and colleagues[28] was employed, consisting of 94 °C for 3 min, 94 °C for 30 s, 54 °C for 30 s, 72 °C for 1 min 30 s, cycled 30 times for 30 s at 94 °C, and a final extension for 7 min at 72 °C. PCR reaction was performed in 25 μl volume containing 0.4 μM for each primer, 0.2 mM deoxynucleoside triphosphates, 2.5 μl of 10× PCR buffer, 2 mM $MgCl_2$, 10 ng of genomic DNA, and 0.025U BIOTAQ DNA polymerase (Bioline, Luckenwalde, Germany).

PCR products were purified by the MBL-PCR QuickClean kit (Dominion-MBL SL, Spain) and direct sequencing using an automatic DNA sequencer (ABI-PRISM 310; Applied Biosystems, Inc.).

Sequences recovered from excised bands were analysed for chimeric character by using the Ribosomal Database Project II (RDP II) Chimera Check Program (http://rdp.cme.msu.edu/seqmatch/). Sequences that appeared chimeric were excluded from further analysis. The names and accession numbers of organisms that most closely matched each of the clones in the 16SrRNA gene sequence, as well as their tentative phylogenetic placement, are given in Table 3.

PCR-SSCP analysis diversity of the PAH degrading genes

For analysing the PAH-degradation potential of bacterial populations from PAH-contaminated soils, the soil DNA was amplified using the primers P1.1f and P2.2r and the PCR conditions previously described.[29]

The PCR products were purified by 1.5% (w/v) agarose-gel electrophoresis. The targeted bands were excised and extracted using an MBL-Agarosa Quickclean (Dominion-MBL SL, Spain) and eluted with 20 μl 1X TE (10 mM Tris-HCl at pH 8,1 mM EDTA).

The cloning experiments were carried out following the same procedure as described above for DGGE. Clones of appropriate sizes (*ca.* 293 bp) were selected by electrophoretic analysis and the corresponding DNA subjected to nested PCR, using primers P1.1f and P2.2r.[29] The PCR products were then purified using the MBL-PCR QuickClean kit (Dominion-MBL SL, Spain), and 4 μl of the purified PCR product were mixed with 6 μl denaturing loading buffer (95% formamide, 10 mM NaOH, 0.25% bromophenol blue, 0.25% xylene cyanol), denatured at 95 °C for 2 min and immediately chilled on wet ice for 5 min.[25] SSCP screening of the PCR products was then conducted at a constant power of 2 W for 12 h at 22 °C in an INGENYphorU System (Ingeny International BV, The Netherlands). Four microlitres of the denatured samples were loaded into the slots of the gel composed of 0.7x MDE gel (Cambrex Bio Science Rockland, Inc. Rockland, ME USA) and 0.6x TBE buffer (89 mM Tris, 89 mM boric acid and 2.0 mM EDTA, pH 8.0).[26] The gel was silver stained and thereafter dried directly on filter paper at room temperature. Clones with different banding profiles were analysed.

Data analyses

All results are the means of three replicates. The data was subjected to variance analysis, and Duncan's Multiple Range Test as a post hoc test was used to separate the means with an overall significance level of 0.05. Correlations were calculated by Spearman's correlation coefficient (SPSS for Windows, version 15.0).

The DDGE band patterns were compared in different lanes using UVImap Analysis software (UVitec Limited, Cambridge, UK). The lanes were normalized to contain the same amount of total signal after background subtraction, and the gel images were straightened and aligned to give a densitometric curve. Band positions were converted to Rf values between 0 and 1, and profile similarity was calculated by determining Nei and Li's similarity coefficients[30] for the total number of lane patterns from the DGGE gel. The similarity coefficients calculated were then used to construct a dendrogram using the unweighted pair-group method with arithmetical averages (UPGMA).

The species richness on DGGE gels (R) was calculated as the mean number of bands presents.[31] Index of dominance S and Shannon index H' were used to evaluate the biodiversity and predominance of microbial groups in the soils.[32,33]

Results and discussion

Chemical properties

Many contaminated sites contain a variety of toxicants. Risk assessment and the development of soil-quality criteria therefore require information on the interaction between toxicants. Interactions with heavy metals are relatively well studied, but little is known about interactions between heavy metals and polycyclic aromatic hydrocarbons.

Tables 1 and 2 summarize the chemical properties of the contaminated soils and the quantities of organic and inorganic pollutants.

At the measured pH range soil microbial growth and its activity are usually favoured. Soil pH can provide valuable information on the presence and toxicity of several elements, including Fe, Al, Mn, Cu, Cd and others, to plants and microorganisms.[34,35] In this study, the pH values measured the alkaline nature of the soil. The electrical conductivity (EC) ranged from 80 to 150 μS cm^{-1}. The high EC found in soil sample S5 could be due to the high concentration of fluoride,

Table 3 Nearest match identification of bacterial 16S rDNA gene sequences isolated from soil samples

	Closer relative by homology with BLAST				
Query	Organism	Accession number	Identity (%)	Phylogenetic class	Phylogenetic taxa
B2	Unidentified *Eubacterium*	AJ232846	89	Firmicutes	Clostridia; Clostridiales; Eubacteriaceae
B3	Uncultured *Eubacterium*	AF495406	89	Firmicutes	Clostridia; Clostridiales; Eubacteriaceae
B4	Uncultured *Bacterium* clonesJC2701R_55	AY349521	93	Bacteria	Environmental sample
B5	Proteobacterium UMB 38	DQ147584	100	Proteobacteria	Unclassified
B6	Uncultured soil *Bacterium* clonesM25_Pitesti	DQ378245	99	Bacteria	Environmental sample
B7	*Thermoactinomyces sacchari*	AJ251779	95	Firmicutes	Bacilli; Bacillales; Thermoactinomycetaceae; Laceyella
B8	*Cinetobacter.calcoaceticus*	X81668	99	Proteobacteria	Gammaproteobacteria; Pseudomonadales; Moraxellaceae; Acinetobacter
B9	Uncultured soil bacterium clone C129s	AF507687	98	Bacteria	Environmental samples
B10	Uncultured Firmicutes bacterium clone GASP-WC2W3_D03	EF075358	98	Firmicutes	Environmental samples
B11	Monochloroacetic-acid-degrading bacterium ′Band C′	AF532191	96	Bacteria	Unclassified bacteria
B12	Cellvibrio sp. R-20952	AJ786793	98	Proteobacteria	Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae
B13	Uncultured actinobacterium clone LW7s	DQ973232	100	Actinobacteria	Environmental samples
B15	*Roseomonas* sp. 1 LOT M4	AY624051	100	Proteobacteria	Alphaproteobacteria; Rhizobiales; Methylobacteriaceae; Roseomonas
B18	Bacterium Ellin 5273	AY234624	96	Actinobacteria	Acidimicrobidae; unclassified Acidimicrobidae
B21	Bacteroidetes bacterium MH2-1	EF187349	97	Bacteroidetes/Chlorobi group	Bacteroidetes; unclassified
B22	Bacterium TSA-5.4	AF240150	96	Bacteria	Unclassified bacteria
B23	Uncultured earthworm casting bacteriums clone c251	AY154605	96	Bacteria	Unclassified bacteria
B25	Uncultured Firmicutes bacteriums clone GASP-WB2S1_H07	EF073821	99	Firmicutes	Environmental samples
B26	Caulobacter subvibrioides (ATCC 15264)	X94470	96	Proteobacteria	Alphaproteobacteria; Sphingomonadales
B28	Sphingobacteriaceae str. Ellin 160	AF409002	90	Bacteroidetes/Chlorobi group	Bacteroidetes; Sphingobacteria
B29	*Spirosoma* sp. RODSPM9	EF451725	92	Bacteroidetes/Chlorobi group	Bacteroidetes; Sphingobacteria; Sphingobacteriales; Flexibacteraceae; Spirosoma
B31	*Mycobacterium doricum* strain DSM	AF547917	98	Actinobacteria	Actinobacteridae; Actinomycetales; Corynebacterineae; Mycobacteriaceae; Mycobacterium

chloride and sulfates. Only phosphates were detected in soil samples S3 and S4 (Table 1).

Nevertheless, the size of the sampled area (about 5000m^2), the percentage of organic carbon ranging from 4.25 to 1.43% (Table 1), and a consequent overvaluation due to organic pollutants should be taken into account.[16,35] Also, total extractable and water-soluble carbon varied greatly between samples, with soil sample control (C) and soil sample S6 showing the lowest values.

Total hydrocarbon content (TPH) in the soil samples are shown in Table 2. S2 recorded the highest content (4.84 mg kg^{-1}), while S5 and control soil C had the lowest values (0.51 mg kg^{-1} and 0.84 mg kg^{-1}, respectively).

In this study, all soils showed heavy metal levels above permissible limits for lead, cadmium and zinc (Table 2). The highest values for lead content were detected in the samples S1, S2, S6, S7 and S8. Also, samples S1 and S3 showed very high values for cadmium, while S7 recorded the highest zinc content. It is possible that heavy metal pollution is strongly associated with PAHs, and soils contaminated by PAHs also often contain large amounts of heavy metals.[4,5]

Enzyme activities

Enzyme activities have often been regarded as indicators of microbial activity and are also useful for determining the intensity of microbial metabolism in soil. Nannipieri and colleagues showed that the measurement of the activity of several enzymes in soil may be a good method for estimating the overall microbial activity and its response to widespread pollution.[13] Enzymes, in fact, are the catalysts of important metabolic functions, including the decomposition and detoxification of contaminants.[16,36] Dehydrogenase activity, which indicates the biological oxidation processes in soils and other systems, has been used as an indicator of overall microbial activity.[20] Measurement of dehydrogenase activity is therefore usually related to the presence of viable microorganisms.

As it was previously found in heavy metal and hydrocarbon-contaminated soils,[7,8] in our study dehydrogenase activity in soil may be more affected by high levels of heavy metals than by TPH levels. This activity in the soil samples ranged from 3 to 5 μg INTF g^{-1} h^{-1} (Fig. 1a), while in the control soil (C), intense enzyme activity was found 9.308 μg INTF g^{-1} h^{-1}. Gogolev and Wilke showed that the high concentrations of heavy metals on cell surfaces probably suppress interactions of fluoranthene (0.2 mg l^{-1}) with lipophilic compounds of bacterial membranes.[8] It can be concluded from our agar-plate experiments that fluoranthene may enhance the toxicity of heavy metals to soil bacteria. Given the low solubility of most PAHs and their high adsorption rates for organic material, it is extremely unlikely that adverse effects of heavy metals will be enhanced by the presence of PAHs in contaminated soils.

Total β-glucosidase activity was greater in soil samples S1, S3, and S4 and lower in S2, S5, S7, and control soil C (Fig. 1b). The β-glucosidase activity measured in the extractable carbon fraction was significantly higher in sample S1 but was not detected in control soil (C) and soil sample S8 (Fig. 1c). Specific β-glucosidase activity per unit of extractable carbon was greater in soil samples S1 and S7 due to the C-cycle of humic carbon in these soils (Fig. 1d). Spearman's correlation coefficient between TPH and both β-glucosidase activities showed a strong positive correlation (0.759 and 0.731; $p < 0.01$). This could be interpreted as reflecting the transitory state of intense microbiological and biochemical activity which may be due to hydrocarbon pollutants constituting a degradable substrate capable of stimulating the proliferation of some of the soil's microflora.[37,38] In addition, PAHs may stimulate β-glucosidase activity and partially inhibit the leucine-aminopeptidase activity.[39] We think that the TPH mixture could actually have modified the microbial metabolism. Moreover, changes in microbial diversity, especially in relation to the development and adaptation of hydrocarbon degraders, might partially explain the modification of activity levels.

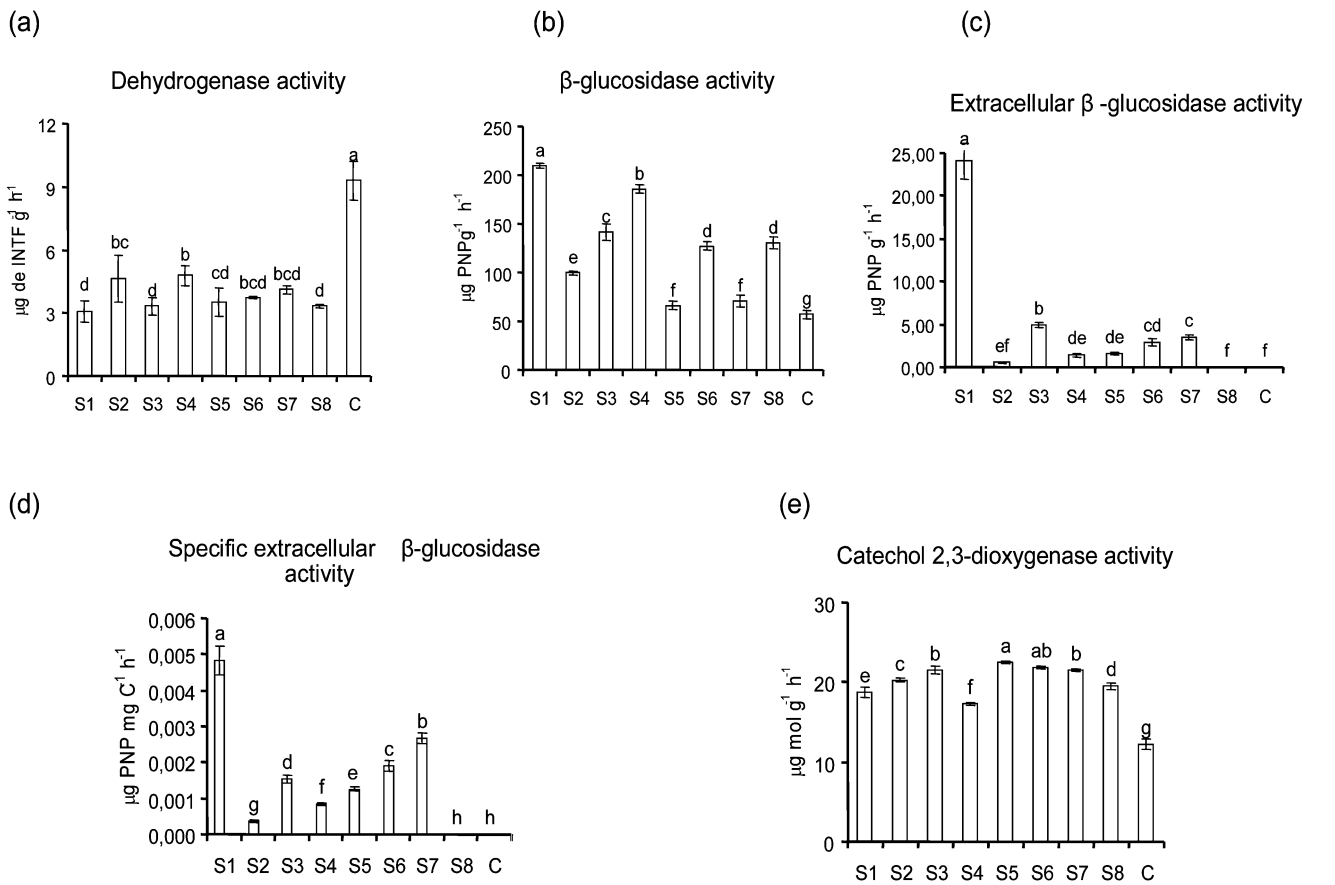

Fig. 1 Soil enzyme activities. (a) Dehydrogenase activity, (b) β-glucosidase activity, (c) extracellular β-glucosidase activities, (d) specific β-glucosidase activity, (e) catechol 2,3-dioxygenase activity. Vertical bars represent standard errors. Mean values not sharing a letter differ significantly according to Duncan's multiple range test ($p < 0.05$).

The soil activity indicated an active microbial degradation of the hydrocarbons present in the soils. The control (C) soil presented the lowest catechol 2,3-dioxygenase activity (12.20 μmol g^{-1} h^{-1}), whereas other soil samples with medium and higher hydrocarbon totals on the surface showed higher levels of this activity (Fig. 1e). The C23DO activity in soil positively correlated with the level of TPH detected in these soils (0.858; $p < 0.01$). This could indicate a possible degradation in the TPH present in the soils. The C23DO is a key enzyme in PAH degradation, as catechol is a central intermediary in the PAH-degradation pathway. Thus, it appears that the detection and screening of this enzyme activity could be a useful tool for monitoring the degradation of PAHs in soils.

Therefore, a significant effect of TPH in soil was to decrease the overall microbial activity and to increase the PAH-degrading activity. As expected, a negative correlation (−0.337; $p < 0.05$) between dehydrogenase and C23DO activity was detected.

Soil bacterial diversity

The intrinsic capability of contaminated soils to degrade PAHs is related to the presence of a possibly heterogeneous but metabolically active microbial community.[40]

Bacterial-community profiles were made on the basis of amplified soil DNA (Fig. 2a, b), and two main clusters in a UPGMA dendrogram analysis were formed (Fig. 2b). The first cluster was made up of more polluted soils. After analysis, this cluster was subdivided into three groups. Soil samples S1 and S3, with medium levels of contamination, formed the first group. The second group included soil samples S4, S5 and S6 with low levels of contamination and the third group was formed only by soil sample S2, which showed the highest level of contamination by TPH. The second cluster was made up of two groups. The first contained soils S7 and S8, while the second group contained only the control soil, which was very different from the other soils. These three soils showed the lowest values of TPH.

It has to be noted that in several cases, bands that migrate to the same position represent the same or very closely related species, even belonged to the same genus.[41–44] However, some strains may generate more than one band in DGGE.[45] Due this limitation, care must be exercised when estimating species numbers and diversity from the DGGE community fingerprints, but this technique can give us a semiquantitative measure of bacterial diversity of soils and could be used to analyse bacterial natural abundance, diversity and dynamics.[42,46] Table 4 summarizes the richness index (R), the Shannon index of general diversity (H') and Simpson index of dominance (S). Richness varied from relatively low values for soil S2, with R averaging 19, to significantly higher values for control soil where R was 41. According to our results, R, H' and D varied significantly with the degree of TPH and heavy metals. Soil contamination affected genetic diversity, with soil S2 showing the lowest diversity ($R = 19$; $H' = 1.74$), and control soil recording the highest one ($R = 41$; $H' = 2.78$). In this study, biodiversity was directly related to dehydrogenase activity, indicating a metabolically and functionally active bacterial community. Nevertheless, the Simpson index was lowest in the control soil (0.019), indicating an absence of dominant

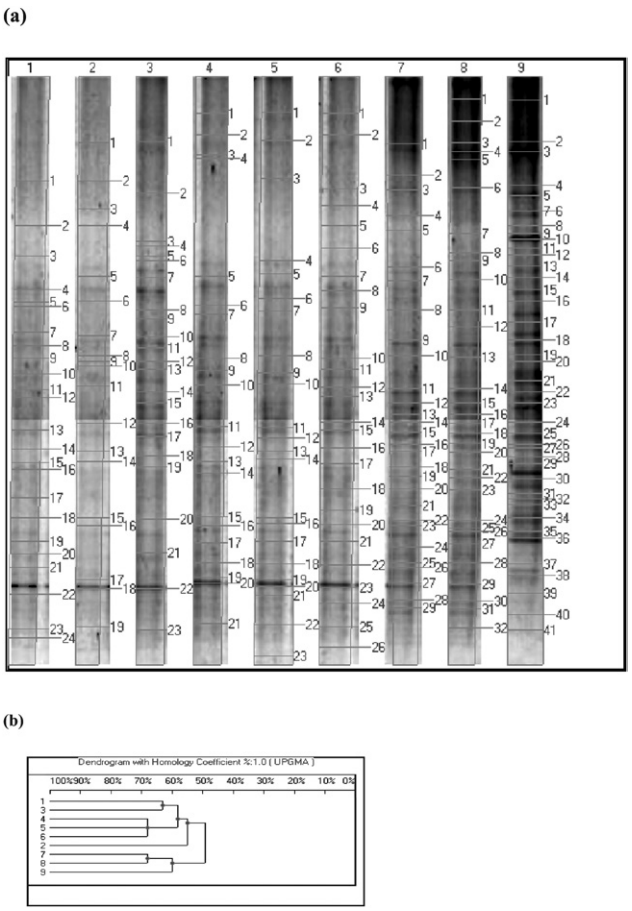

Fig. 2 (a) DGGE analysis of V3 fragments obtained from the soil samples: S1 (lane 1), S2 (lane 2), S3 (lane 3), S4 (lane 4), S5 (lane 5), S6 (lane 6), S7 (lane 7), S8 (lane 8) and control soil (lane 9); (b) cluster analysis of bacterial DGGE profiles.

populations. High dominance indices were found in the other more polluted soils. These indicate the supremacy of populations which may be metabolically more active due to the presence of pollutants.

Sequence analysis of DGGE bands

The partial 16S rRNA sequences of a total of 32 DNA bands were successfully determined but only 21 sequences revealed an over 89% match in terms of homology, showing the degree of sequence similarity to other known organisms (Tables 3 and 4). Phylogenetic classes (*Proteobacteria*, *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, and *Chlorobi*) were found in these soils. These sequences correspond to PAH-degrading bacteria already described by other authors.[47,48]

Band 8, which presented 99% homology with respect to *Cinetobacter calcoaceticus*, was detected in all soils. Band 2 (89% *Firmicutes* class), Band 9 (98% for environmental bacteria samples isolated from oil-contaminated soil), Band 18 (96% for *Actinobacteria*), and Band 25 (99% for *Firmicutes*) were also found in all soils except in the control soil.

Band 28 (90% *Sphingobacteria*) was exclusively found in soil S5. *Roseomonas* sp. 1 LOT M4 (100% homology with Band 15)

Table 4 Species richness (R), Shannon (H') and Simpson (D) diversity indexes values and bands retrieved from the DGGE profiles. For R, H' and D indexes, mean values not sharing a letter differ significantly according to Duncan's multiple range test ($P < 0.05$)

Soil	*R*	*H′*	*S*	Bands retrieved from the DGGE profiles
S1	24 bc	−2.03b	0.030 b	B2,B4,B8,B9,B12,B15,B18,B25,B26
S2	19 c	−1.74 c	0.026 bc	B2,B4,B8,B9,B12,B15,B18,B25,B26
S3	23 bc	−1.93 bc	0.023 c	B2,B4,B8,B9,B12,B15,B18
S4	21 bc	−1.91 bc	0.024 bc	B2,B4,B5,B8,B9, B15,B18,B25,B29
S5	23 bc	−2.71 ab	0.024 bc	B2,B4,B5,B8,B9,B13,B15,B18,B22,B22,B25,B26,B28,B29,B31
S6	26 bc	−1.96 bc	0.037 a	B2,B4,B8,B9,B12,B15,B18,B23,B26
S7	29 bc	−2.19 ab	0.039 a	B2,B4,B8,B9,B12,B15,B18,B23,B26
S8	32 b	−2.09 ab	0.026 bc	B2,B4,B8,B9,B12,B15,B18
C	41 a	−2.78 a	0.019 d	B3,B4,B5,B6,B7,B9,B10,B11,B12,B13,B21,B22,B22,B26

was not present in either soil S5 or control soil C. These soils were the only ones where Band 13, which showed 100% homology with *Actinobacteria*, was found.

Band 5 (100% *Proteobacteria*) was present in S4, S5, and C, probably due to the closeness of the soil plots. However, Band 29 (92% homology with *Spirosomona* sp.) appeared only in S4 and S5.

The main matches described above were also identified for control soil C. This soil also displayed the following bands: Band 3 (89% homology with *Firmicutes* class), Band 7 (95% homology with *Termoactinomyces saccari*), Band 10 (98% homology with a non-cultivable *Firmicutes* bacterium) and Band 11 (96% homology with monochloroacetic-acid-degrading bacterium isolated from environmental samples). Band 31 was exclusively found in the control soil and matched with the *Mycobacterium doricum* DSM strain (98% homology).

Several studies have shown that contamination by PAH induces changes in the taxonomic and physiological diversity of the native microbial communities of contaminated ecosystems.[49–51] In our study, the percentage of *Proteobacteria* was higher in soils where there was greater contamination by hydrocarbons. *Firmicutes* and *Actinobacteria* were also found in these soils.

When contamination by hydrocarbons was reduced, the *Proteobacteria* tended to decrease, going down to 39% of the total. Moreover, in contaminated soils, there was a tendency for new taxonomic groups to appear, as in the case of *Bacteroidetes* (*Chlorobi* group) in soils S5 and C which had lower hydrocarbon contamination levels. The opposite tendency occurred with *Actinobacteria*, which tended to disappear as pollutants decreased.

The low percentage of *Proteobacteria* and *Actinobacteria* could explain the narrow diversity and/or absence of PAH-degrading genes in soils S5 and C. A possible relationship exists between the polluting-agent concentration, the number and percentage of selective taxonomic groups, and presence of PAH-degrading genes. This same reduction in *Proteobacteria* and *Actinobacteria* could similarly explain the low catechol 2,3-dioxygenase activity detected in control soil C. The reduction in these two groups could reflect the decrease in catechol 2,3-dioxygenase activity. Selectivity among hydrocarbon-resisting bacterial communities, where some populations appeared to degrade hydrocarbons according to the results presented, might be assumed.

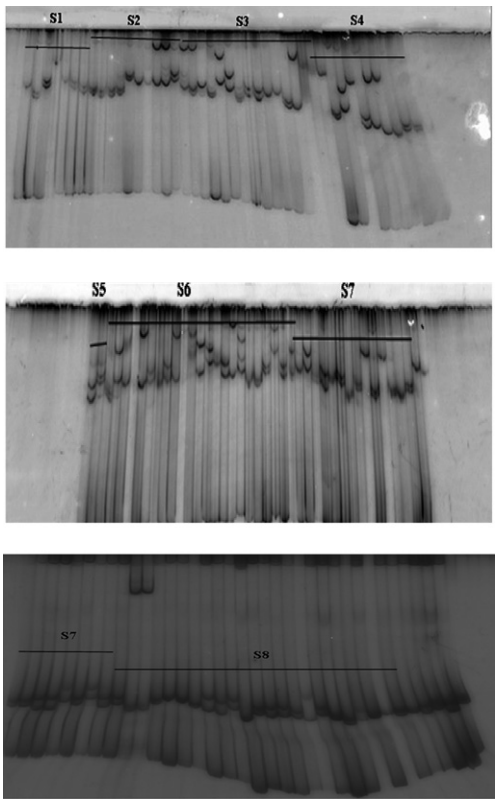

Fig. 3 SSCP profiles PAH-degrading genes from the soil samples.

PAH-degrading genes

Fig. 3 shows the SSCP soil profiles. DNA fragments were amplified in all soils, although the low intensity of the band detected in control soil C was inadequate for later obtaining clones for SSCP analysis.

In the soils with lower pollutant levels, a smaller number of clones were found for this fragment, only two were detected in soil 5 (S5) (0.51 ± 0.04mg TPH kg^{-1}). The highest number of PAH-degrading genes were found in soils S6, S7 and S8 (23, 24 and 23 respectively) as shown in Fig. 3, corresponding to soils showing medium-level contamination by TPH (1.1–1.5 mg kg^{-1}). In soils with TPH levels (S1, S2, S3 and S4) between 1.6 and 4.0 mg TPH kg^{-1}; 7, 10, 15 and 10 clones, respectively, were detected.

The genetic potential for PAH degradation in the environment has been limited by the narrow range of PCR primers used to screen for PAH dioxygenases which detect only a fraction of the PAH degraders in the environment. It is important to note that the degenerated primers were developed from *Pseudomonas* sp. and most of the identified species do not belong to this group. Thus, the limited amplification of bands in some soils may be due to primer non-specificity, although the degenerated primers are based on highly conserved amino-acid sequences; these primers frequently detect only dioxygenases from *Pseudomonas* and fail to detect dioxygenases from other genera known to degrade PAHs. The development of new pools of primers for the screening of enzymatic activities should enable us to describe the degrading potential of soil more accurately.

Conclusions

This study of heavy metals and the effect of hydrocarbons on biological soil activity aimed to analyse the impact of both polluting agents on the diversity of the bacterial community structure and its functionality by measuring the presence of PAH-degrading genes and the activities of soil enzymes.

Genetic fingerprinting using DGGE analysis of PCR amplified 16S rDNA provided a deeper insight into the effects of pollution on soil with respect to the composition of microbial communities. The sequences found in these soils corresponded to sequences of PAH-degrading bacteria (Phyla: *Proteobacteria*, *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, *Chlorobi*) already described by other authors.

The control soil showed non-dominance of species. Bands of PAH-degrading genes and a low C23DO activity were not detected in this soil, indicating a relationship between enzymes that attack the initial compounds and the degradation of the intermediary metabolites formed. The soil most contaminated with TPH (S2) showed the lowest diversity of microorganisms, high numbers of PAH degrading clones and intense catechol 2,3-dioxygenase activity. This indicates that the parameters of biodiversity, PAH-degrading genes, and catechol 2,3-dioxygenase activity analysed in this study may be appropriate tools for monitoring contamination and bioremediation in soils.

Thus, a multi-technique approach that combines traditional biochemical methods with molecular-based techniques, along with some methodological improvements, may represent an important tool to broaden our knowledge of the role of microbial diversity in soil ecosystems. This approach shows that heavy metals and hydrocarbon pollution has caused a genetic and metabolic alteration in microbial communities, corresponding to a reduction in microbial activity.

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