RESEARCH ARTICLE

Biodegradation of high-molecular-weight polycyclic aromatic hydrocarbons by a wood-degrading consortium at low temperatures

Raquel Simarro[1], Natalia González[1], Luis Fernando Bautista[2] & Maria Carmen Molina[1]

[1]Department of Biology and Geology, ESCET, Universidad Rey Juan Carlos, Madrid, Spain; and [2]Department of Chemical and Environmental Technology, ESCET, Universidad Rey Juan Carlos, Madrid, Spain

Correspondence: Natalia González, Department of Biology and Geology, ESCET, Universidad Rey Juan Carlos, E-28933 Móstoles, Madrid, Spain. Tel.: +34 914888501; fax: +34 916647490; e-mail: natalia.gonzalez@urjc.es

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Abstract

This study evaluates the ability of two bacterial consortia (C2PL05 and BOS08), extracted from very different environments, to degrade low- (naphthalene, phenanthrene, anthracene) and high- (pyrene, perylene) molecular-weight polycyclic aromatic hydrocarbons (PAHs) at high (15–25 °C) and low (5–15 °C) temperature ranges. C2PL05 was isolated from a soil in an area chronically and heavily contaminated with petroleum hydrocarbons and BOS08 from decomposing wood in an unpolluted forest, free of PAHs. Bacterial consortia were described by cultivable and noncultivable techniques (denaturing gradient gel electrophoresis). Fungal DNA was not observed within the wood-decomposing consortium and fungal activity was therefore negligible during most of the PAH degradation process. PAH-degrading bacterial populations, measured by most probable number enumeration, increased during the exponential phase. Toxicity estimated by the Microtox method was reduced to low levels and final PAH depletion, determined by HPLC, confirmed the high degree (54% and 99%, respectively) of low- and high-molecular-weight PAH degradation capacity of the two consortia. PAH-degrading capacity was also confirmed at low temperatures, and especially by consortium BOS08 not previously exposed to those toxic compounds, where strains of *Acinetobacter* sp., *Pseudomonas* sp., *Ralstonia* sp. and *Microbacterium* sp. were identified.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) constitute a diverse class of organic compounds formed by two or more aromatic rings in several structural configurations, and they have carcinogenic, mutagenic and toxic properties. Therefore, environmental contamination by PAHs is of current concern, and it has been shown that bioremediation is the most efficient means by which to restore original ecosystem conditions (Haritash & Kaushik, 2009). However, high-molecular-weight PAHs (HMW-PAHs), such as pyrene, benzo[*a*]pyrene and benzo[*b*]fluoranthene, are generally recalcitrant and resistant to microbial attack due to their low solubility and bioavailability. Therefore, these compounds are highly persistent in the environment and are bioaccumulated in organisms (Lafortune *et al.*, 2009). The biodegradation of PAHs with fewer than three aromatic rings has been the subject of many reviews (e.g. Sutherland *et al.*, 1995). However, there is currently a lack of knowledge regarding the biodegradation of HMW-PAHs (Kanaly & Harayama, 2000, 2010).

Microbial communities play an important role in the biological removal of pollutants from soils (MacNaughton *et al.*, 1999). Therefore, changes in environmental conditions may alter species diversity of the soil microbiota and their metabolic rates (Margesin & Schinner, 2001). In areas chronically polluted by PAHs there are abundant bacteria able to degrade these toxic contaminants by using them as their sole carbon and energy sources (Taketani *et al.*, 2010). Recent studies (Tian *et al.*, 2008; Surridge *et al.*, 2009; Couling *et al.*, 2010) report the potential ability to degrade PAHs by microorganisms that have apparently not been previously exposed to such toxic compounds. This is extensively

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known for lignin-degrading white-rot fungi that produce a set of extracellular enzymes, such as oxidases and peroxidases (Wong, 2009), with low substrate specificity that expand their oxidative action beyond lignin, being capable of degrading other complex phenolic compounds and PAHs (i.e. Canet *et al.*, 2001). Although less extensively than in fungi, the PAH degradation capacity of bacteria has also been reported in this type of environment for genera such as *Pseudomonas* (Zimmermann, 1990; McMahon *et al.*, 2007), *Actinobacteria*, *Bacillus* and *Ralstonia*. However, according to Couling *et al.* (2010), the widespread capacity of microbial communities to degrade PAHs even from unpolluted soils can be explained by the fact that PAHs are ubiquitously distributed by natural processes throughout the environment at low concentrations, sufficient for bacteria to develop degrading capacity.

Regardless of these issues, there are some abiotic factors, such as temperature, that greatly influence biodegradation processes. It has been shown (Mohn & Stewart, 2000) that, although biodegradation of PAHs is more efficient in the range 20–30 °C, it can continue even in colder (< 5 °C) environments (Eriksson *et al.*, 2001). At low temperature, diffusion rates and solubility, and thus bioavailability, of PAHs decrease (Haritash & Kaushik, 2009). Simultaneously, microbial metabolism slows down, increasing the lag period (Atlas & Bartha, 1972; Eriksson *et al.*, 2001). However, according to the hypothesis that degrading microorganisms are present in most ecosystems, there exist bacteria adapted to low temperatures (Yakimov *et al.*, 2003; Brakstad & Bonaunet, 2006) that can express degrading capacity. The study of biodegradation at low temperatures is important because anthropogenic discharges have sometimes occurred and may continue to occur in cold environments such as Alaska (Bence *et al.*, 1996).

Our main goal here was to study the effect of low temperatures on HMW-PAH degradation rate by considering two different consortia isolated from two different environments: one from decaying wood in an unpolluted forest (consortium BOS08) and the other from a polluted soil exposed to hydrocarbons (C2PL05). We also describe the microbial dynamics through the biodegradation process as a function of temperature and inoculum sources used.

Materials and methods

Chemicals and media

Naphthalene, phenanthrene, anthracene, pyrene and perylene (all > 99% purity), purchased from Sigma-Aldrich and Fluka, were prepared in a stock solution in n-hexane (Fluka) to obtain a final concentration of 0.02 g L^{-1} for naphthalene, phenanthrene and anthracene, 0.01 g L^{-1} for pyrene and 0.005 g L^{-1} for perylene. Tween-80, purchased from Sigma-Aldrich, was added according to a previously described method (Bautista *et al.*, 2009). Composition of optimized Bushnell Haas Broth medium (BHB) (Simarro *et al.*, 2010) was (g L^{-1}): 0.2 $MgSO_4{\cdot}7H_2O$, 0.02 $CaCl_2{\cdot}2H_2O$, 0.088 KH_2PO_4, 0.088 K_2HPO_4, 2.09 aNO_3 and 0.015 $Fe_2(SO_4)_3$.

Physicochemical characterization of soils and isolation of bacterial consortia

Consortium C2PL05 was isolated from a permanently polluted soil from a petroleum refinery (Ciudad Real, Spain) with a range of environmental temperatures from 10 °C in winter to 25 °C in summer. Consortium BOS08 was extracted from dead wood in a pristine Atlantic forest in Fragas do Eume Natural Park, Galicia, Spain (43°41.75′N, 8°06.83′W), in which *Quercus robur* and *Corylus avellana* were the dominant flora species and temperatures ranged from 10 °C in winter to 18 °C in summer. To obtain the microbial consortia, sieved soil and the wood sample were suspended in phosphate-buffered saline (PBS; 1 : 10) and stirred overnight at 25 °C. Then, 1.5 mL of each culture was inoculated in 50 mL BHB at pH 7.0 with Tween-80 1% (v/v) as surfactant and naphthalene, phenanthrene, anthracene, pyrene and perylene (each at 500 mg L^{-1}) as carbon sources. Each culture was incubated in an orbital shaker at 150 r.p.m. and 25 °C in the dark until the exponential phase was completed (~5 days); cell density was monitored by absorbance at 600 nm in a spectrophotometer (Spectronic Genesys; Thermo Fisher Scientific).

To confirm that consortium BOS08 had not been previously exposed to PAHs, original forest soil samples were extracted successively with acetone, n-hexane and water according to Jørgensen *et al.* (2005). Quantification and identification of PAHs was performed by GC-MS analysis of the extract using a Varian CP3800 gas chromatograph equipped with a Varian VF-1 ms capillary column (15 m length, 0.25 mm i.d., 0.25 μm film thickness) and coupled to a Varian 1200 L quadrupole mass-spectrometer detector. Helium was used as carrier gas and the following temperature programme was used: 40 °C for 3 min, increased to 350 °C at a rate of 5 °C min^{-1}. In addition, total petroleum hydrocarbons (TPHs) within the range C10–C40 were extracted with acetone, n-heptane and water as described by Jørgensen *et al.* (2005). The analysis of TPH was performed using GC-flame ionization detection (GC-FID; Varian CP3800 gas chromatograph) and fitted with a Zebron ZB-1XT capillary column (10 m length, 0.53 mm i.d., 0.15 μm film thickness; Phenomenex).

Helium was again used as carrier gas and the following temperature programme was used: an increase from 35 to 400 °C at a rate of 10 °C min^{-1} and 400 °C for 10 min.

Experimental design and treatment conditions

Fifteen microcosms (triplicates for five different incubation times) were performed with consortium C2PL05 at a high temperature range (H): 16 h in the light at 25 °C, followed by 8 h in the dark at 15 °C. Another 15 microcosms using consortium C2PL05 were incubated at low temperature range (L): 16 h in the light at 15 °C, followed by 8 h in the dark at 5 °C. The same experiments were performed with consortium BOS08. Sieved (< 2 mm) river sand was used as substrate to form the microcosms. Prior to use, it was burned at 550 °C in a furnace to remove organic matter and microorganisms. Water holding capacity (WHC) of the river sand was measured following the method described by Wilke (2005). Microcosms consisted of aluminium foil trays incubated in suitable chambers equipped with control systems for temperature, light/darkness cycle and humidity (60%). Each microcosm contained 90 g of sterilized sand, 18 mL BHB (60% of WHC) with Tween-80 1% (v/v), 2 mL PAH stock solution in n-hexane (final amount of each PAH per tray was 20 mg naphthalene, 20 mg phenanthrene, 20 mg anthracene, 10 mg pyrene and 5 mg perylene) and 3.5 mL of bacterial consortium (0.088 AU = 2.75×10^4 cells mL^{-1} for C2PL05 and 0.051 AU = 2.86×10^4 cells mL^{-1} for BOS08).

Bacterial growth, MPN and toxicity assays

Bacterial density during the PAH degrading process was monitored at 0, 11, 33, 66, 101 and 137 days. One gram of wet soil was suspended in 10 mL PBS and stirred overnight in an orbital shaker. Two aliquots of supernatant (1 mL) were used to measure cell density by absorbance at 600 nm in a spectrophotometer (Spectronic Genesys; Thermo Electron Corp.). One of the aliquots was used as blank and was centrifuged (200 ***g***) for 5 min before measurement. From the absorbance data, the intrinsic growth rate in the exponential phase was calculated according to:

$$\Delta A_i = \left(\frac{\ln(A_i) - \ln(A_{i-1})}{t_i - t_{i-1}}\right)$$

where A is the absorbance at 600 nm, t is the time elapsed in hours and the subscript i corresponds to each sample or sampling time. Increments were normalized by absorbance measurements taken at the initial time (day 0) to correct the difference of the initial densities.

Heterotrophic and PAH-degrading microbial populations from the consortia were estimated by a miniaturized most probable number (MPN) technique in 96-well microtitre plates with eight replicate wells per dilution (Wrenn & Venosa, 1996). The total heterotrophic microbial population was estimated in 180 μL Luria–Bertani medium with glucose (15 g L^{-1}) and 20 μL of the microbial consortium. The PAH-degrading population in the inoculum was estimated in 180 μL BHB medium containing Tween-80 (1%, v/v), 10 μL of PAH stock mix solution as the only carbon source (n-hexane was allowed to evaporate prior to inoculation) and 20 μL of the microbial consortium in each well. To extract bacteria, 1 g of the microcosm substrate was suspended in PBS (1 : 10, w/v) and shaken overnight in an orbital shaker.

Acute toxicity to bacteria during PAH degradation was also monitored through screening analysis of the samples following the Microtox method, which studies the inhibition of the luminiscence of the bacterium *Vibrio fischeri* (former *Photobacterium phosphoreum*) following the protocol suggested by Microbics Corporation (1992). The lyophilized bioluminescent bacteria were hydrated with NaCl to a final concentration of 2%, generating light that could be measured with a luminometer. The toxicity decreased proportionately to the percentage of the light inhibition of *V. fischeri* after 15 min of incubation at 15 °C.

Monitoring of PAH biodegradation

All five PAHs studied were extracted from microcosms at the initial and end time points of the experiment. Thus, 10 g of soil from each replicate was dried overnight at room temperature and PAHs were extracted with 100 mL dichloromethane over a period of 3 h in a Soxhlet device. The solvent was removed in a rotary evaporator and the residue was dissolved in 1 mL dichloromethane. Remaining PAHs were analysed by GC-FID in a Varian CP3800 gas chromatograph using helium as carrier gas and a Varian VF-5 ms capillary column (30 m length, 0.25 mm i.d., 0.25 μm film thickness). The temperature programme was as follows: 80 °C for 2 min, increasing to 300 °C at a rate of 10 °C min^{-1} and 300 °C for 15 min. Naphthalene, phenanthrene, anthracene, pyrene and perylene concentrations were calculated from their corresponding standard curves.

Control experiments were carried out in a previous work (Simarro *et al.*, 2010) without addition of microbial inoculum, and the content of naphthalene, phenanthrene and anthracene was monitored. Results determined that only naphthalene showed some degree of abiotic depletion, yielding an apparent first-order abiotic constant rate of $2.7 \times 10^{-3} \pm 7 \times 10^{-5}$ h^{-1}. This value allowed for the calculation of the biotic biodegradation rate of naphthalene.

DNA extraction from cultured bacteria and phylogenetic analysis for characterization of the PAH-degrading consortium

Cultivable microorganisms were identified in each microcosm at 0, 33 and 101 days of the biodegradation process after suspending 1.5 g of soil in PBS (1 : 10). The suspension was incubated overnight in an orbital shaker at 150 r.p.m., maintaining the same temperature and light conditions as during the incubation process. To obtain a minimum of 10 PAH-degrading colonies, 100 μL of the supernatant was placed on Petri plates with BHB medium and purified agar and sprayed with a stock mix solution of naphthalene, phenanthrene, anthracene, pyrene and perylene (final concentration 500 mg L^{-1}) as carbon source and incubated at the same temperature conditions.

Total DNA of the PAH-degrading isolated cultures (DIC) was extracted using the UltraClean Microbial DNA Isolation Kit (MoBio Laboratories). DNA extracted was loaded on to agarose gel (1%) to estimate the amount of DNA extracted by comparing the intensity of the DNA band with a molecular weight marker of known concentration.

Amplification of the 16S rRNA coding region of the DNA was performed using primers 16S F27 and 16S R1488 (Lane, 1991). The method used was: denaturing for 9 min at 94 °C, 30 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1.5 min, followed by 10 min of final primer extension at 72 °C. PCR chemicals were applied at the concentration indicated by the ExTaq HS DNA polymerase protocol to a DNA volume of 2 μL. The DNA concentration used by the PCR analyses was around 0.1–0.4 μg and it was quantified by loading the PCR product into an agarose gel and comparing the intensity of the PCR product with a molecular weight marker of known concentration. Sequences were edited and assembled using CHROMASPRO software version 1.42 (Technelysium Pty Ltd) to check for reading errors and, when possible, to resolve ambiguities. BLASTn analyses (Zheng Zhang *et al.*, 2000; available at http://ncbi.nlm.nih.gov/BLAST/) were used to find nearly identical sequences for the 16S rRNA gene sequences determined. Sequences were aligned using the Q-INS-i algorithm (Katoh & Toh, 2008a) of the multiple sequence alignment software MAFFT version 6.611 (Katoh & Toh, 2008b), aligning sequences in a single step.

All sequences identified (using cultivation and noncultivation techniques) and more similar sequences downloaded from GenBank were used to construct the phylogenetic tree. Due to the lack of quality control on taxa names given to sequences deposited in GenBank, type strains of identified species were used as quality control of the sequence. Sequence divergence was computed in terms of the number of nucleotide differences per site between sequences according to the Jukes & Cantor (1969) algorithm. The distance matrix for all pairwise sequence combinations was analysed with the neighbour-joining (NJ) method of phylogenetic tree construction with 1000 bootstrap replicates using PAUP 4.0B10 (Swofford, 2003). In addition, maximum parsimony (MP) was also analysed (Molina *et al.*, 2009). Sequences of *Aquifex piruphilus* and *Hydrogenobacter hydrogenophylus* were used as the outgroup.

Denaturing gradient gel electrophoresis of microbial consortia during PAH degrading process

A nonculture-dependent molecular technique, denaturing gradient gel electrophoresis (DGGE), was performed to study the effect of temperature on the community structure of both microbial consortia during the PAH degradation process by comparing the treatment at day 0, 33 and 101 with the initial composition of the consortia. Total DNA was extracted from 0.25 g of the samples using a Microbial Power DNA isolation kit (MoBio Laboratories) and amplified using the primers 16S 338F-GC and 16S 518R according to the ExTaq HS DNA polymerase protocol (Laboratorios Conda). The PCR conditions were as follows: 9 min of initial denaturing at 95 °C; 30 cycles at 94 °C for 1 min and 55 °C for 1 min (annealing), and 72 °C for 1.5 min (polymerization temperature); followed by 10 min of final primer extension. The PCR product (5–20 μg g^{-1} of soil) was loaded onto a 10% (w/v) polyacrylamide gel with a denaturing gradient from 35% to 65% denaturant. Gels were stained with SYBR-Gold and viewed under UV light; predominant bands in the DGGE gel were excised. The inability to reamplify bands may be due to exposure to UV light and subsequent degradation of DNA. DNA from the bands was cloned in the pGEM-T Easy Vector (Promega), giving 20 clones per band, to identify the possible different ribotypes. Bands from different treatments migrating the same distance were cloned to test that they correspond to the same sequence.

Uncultured bacteria (DUB) were edited and assembled as described above and included in the matrix for constructing the phylogenetic tree. Images of DGGE were digitalized and processed using the UN-Scan-It gel analysis software version 6.0 (Silk Scientific).

To identify the presence of fungi in consortium BOS08 during the process, total DNA was extracted from the samples using a Microbial Power DNA isolation kit (MoBio Laboratories) and amplified with the primer set 18S ITS1F and ITS4 according to the Qiagen Multiplex PCR kit protocol. The PCR conditions were as follows: 5 min of initial denaturing at 95 °C; 30 cycles at 95 °C for 1 min and

55 °C for 0.5 min (annealing), and 72 °C for 1.5 min (polymerization temperature); followed by 10 min of final primer extension. DNA of *Clitocybe metachroa* was extracted using a DNeasy Plant Mini Kit (Qiagen) from the mushroom, for use as a PCR positive control. PCR products were visualized under UV light, on an agarose gel (1%) using SYBR-Gold as intercalating agent.

Statistical analysis

To evaluate the effects of inoculum source (consortium from contaminated soil vs. consortium from wood) and temperature on the final percentage of PAH depletion and on the intrinsic growth rate (μ), a bifactorial analysis of variance (ANOVA) was used. Variances were checked for homogeneity by a Cochran's test. A Student–Newman–Keuls (SNK) test was used to discriminate among different treatments after a significant F-test, representing this difference by letters in the graphs. Data were considered significant at $P < 0.05$. All tests were performed using the software STATISTICA 6.0 for Windows. Differences in microbial assemblages were graphically evaluated for each factor combination (time, consortium and temperature) with non-metric multidimensional scaling (MDS) using PRIMER software (Clarke, 1993). The SIMPER procedure (Clarke, 1993) was used to identify the percentage contribution (%) of each band in DGGE to the similarity in microbial assemblages at each treatment. Ordinations were made on the basis of the Bray–Curtis dissimilarity matrix. Based on Viejo (2009), bands were considered 'highly influential' if they contributed to the first 60% of the cumulative percentage of average dissimilarity/similarity between/within combinations of factors.

Results

Hydrocarbons in soils

Figure 1 shows the results of GC-MS analysis of the hydrocarbons extracted from samples where the two consortia were isolated. Soil samples where the C2PL05 consortium was isolated contained 6.4 wt% (on a dry weight basis) TPHs (< C40). However, no PAHs or any other petroleum hydrocarbons were detected within samples where the BOS08 consortium was obtained.

Cell growth, intrinsic growth, MPN and toxicity assays

Figure 2a and b shows the growth of the two microbial consortia during the PAH biodegradation process. Lag phases were absent and long exponential phases (until approximately day 66) were observed in all treatments

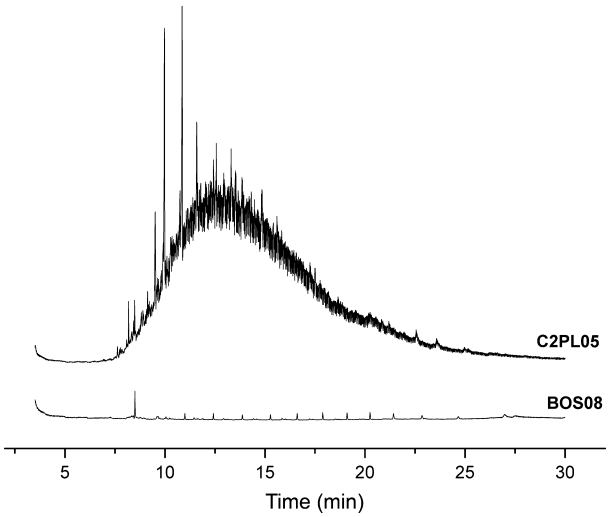

Fig. 1. Chromatogram showing TPH extracts from soils where consortia C2PL05 and BOS08 were isolated. The analysis was performed using a GC-flame ionization detector fitted with a Zebron ZB-1XT capillary column. Helium was used as carrier gas.

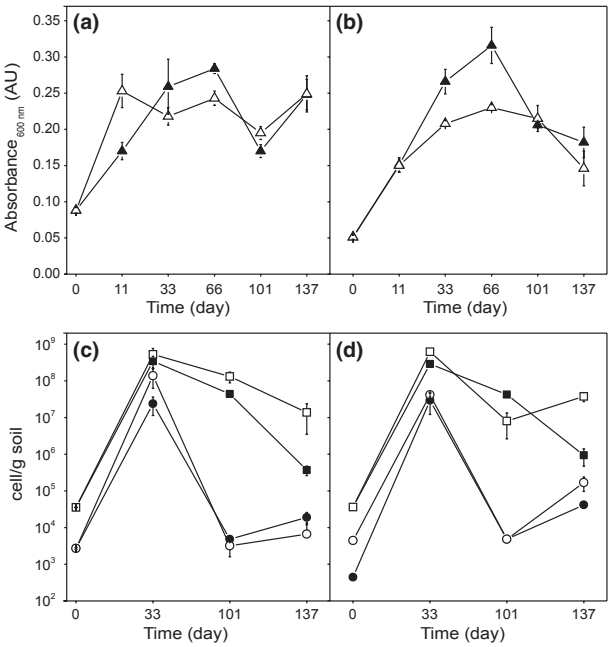

Fig. 2. Cell growth of consortia C2PL05 (a) and BOS08 (b) at high (25–15 °C; filled triangles) and low (15–5 °C; empty triangles) temperature range during PAH biodegradation; and MPN [cells (g soil)$^{-1}$, fresh weight] for consortia C2PL05 (c) and BOS08 (d) of heterotrophic (squares) and PAH-degrading (circles) bacteria cultivated at high (filled symbols) and low (empty symbols) temperature range.

except for the C2PL05 consortium at low temperature (completed at day 11). In general, higher cell densities were achieved in those microcosms incubated at the higher temperature range. Despite the similar cell densities reached by both consortia and at both temperature

Table 1. Intrinsic growth rate (μ) and biodegradation percentage of phenanthrene (Phe), anthracene (Ant), pyrene (Pyr), perylene (Per) and total PAH (Tot) at the final treatment point for consortia C2PL05 and BOS08 at high (H) and low (L) temperature range

	μ		PAH biodegradation (%)									
Treatment	d^{-1} ($\times 10^{-3}$)	±SD ($\times 10^{-3}$)	Phe	±SD	Ant	±SD	Pyr	±SD	Per	±SD	Total	±SD
C2PL05 H	15.8^{b}	0.9	95.4	±0.4	99.3	±0.1	27.0	±6.2	98.6	±0.1	98.9^{c}	±0.4
C2PL05 L	10.5^{a}	1.7	80.1	±6.1	45.9	±15.8	47.0	±11.8	53.8	±19.0	54.3^{a}	±10.9
BOS08 H	24.1^{c}	1.7	93.8	±2.7	99.3	±0.4	47.2	±13.0	86.4	±6.1	$86.6^{b,c}$	±6.0
BOS08 L	18.9^{b}	1.2	94.0	±3.1	57.9	±3.1	54.2	±10.2	69.1	±13.7	$67.7^{a,b}$	±7.7

Superscript letters (a–c) show differences between groups ($P < 0.05$, SNK).

Table 2. Analysis of variance (ANOVA) of the effects of temperature and type of consortium on (a) intrinsic growth rate (μ), (b) total PAH biodegradation and (c) biodegradation of pyrene and perylene

Factor	d.f.	Sum of squares	*F*	*P*
(a) μ				
Temperature[†]	1	0.0036	593.1	**
Consortium[‡]	1	8.3×10^{-5}	13.6	***
Temperature × consortium	1	2.0×10^{-4}	34.3	n.s.
Error	8	4.9×10^{-5}	0.001	
(b) Total PAH biodegradation (%)				
Treatment[§]	3	3526	7.3	*
Error	8	1281		
(c) Biodegradation of pyrene and perylene (%)				
Treatment[§]	3	1124.9	1.1	n.s.
PAHs[¶]	1	8509.8	25.1	***
Treatment × PAH	3	3194.9	3.1	n.s.
Error	16	5422.5		

Asterisks indicate significant differences at: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

[†]High (15–25 °C) or low (5–15 °C) temperature range.

[‡]Consortium C2PL05 or BOS08.

[§]C2PL05 at high and low temperature range or BOS08 at high and low temperature range.

[¶]Naphthalene, phenanthrene, anthracene, pyrene and perylene.

levels, intrinsic growth rate (μ) during the exponential phase (Table 1) showed significant differences between consortia and incubation temperatures but not in the interaction between the two (Table 2a). Differences between treatments showed that the highest μ was obtained at high temperatures (25–15 °C) and with the BOS08 consortium.

Figure 2c and d shows that the initial number of PAH-degrading bacteria was at least one order of magnitude lower than heterotrophic bacteria in both consortia. The highest heterotrophic bacterial concentration was achieved after 33 days of incubation, reaching a final value of approximately 10^8–10^9 cells g^{-1} soil (fresh weight), four orders of magnitude above the initial values. The highest contribution of PAH-degrading bacteria to total heterotrophic bacteria was observed at 33 days of incubation. No differences were observed between temperature ranges. From 33 days, both types of populations started to decrease but PAH-degrading bacteria in the consortia increased again at 101 days, reaching values by the end of the process that were similar to initial values.

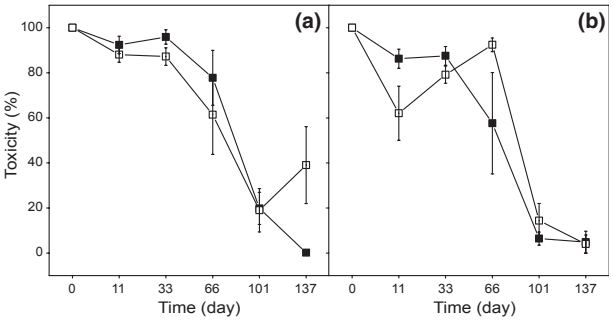

Fig. 3. Toxicity of microcosms with consortium C2PL05 (a) and BOS08 (b) incubated at high (25–15 °C; filled squares) and low (15–5 °C; empty squares) temperature range during PAH biodegradation process. Toxicity was measured using the Microtox assay, which measures the luminescence inhibition of *Vibrio fischeri*.

With regard to toxicity values (Fig. 3), complete detoxification was achieved at the end of each treatment, except for consortium C2PL05 (percentage of toxicity, 40%) incubated at low temperature (Fig. 3a). When consortium BOS08 was incubated at low temperature, toxicity increased over a time period between 11 and 66 days (Fig. 3b).

Biodegradation of PAH

PAH biodegradation results are shown in Table 1. PAH depletion showed significant differences (Table 2b) within the C2PL05 consortium, with highest values at high temperature and the lowest at low temperature (Table 1). There was no effect of temperature on PAH depletion within the BOS08 consortium, and PAH depletion showed average values within C2PL05. Regarding each individual PAH, naphthalene was completely degraded by the end of treatment, 80% of phenanthrene was depleted in all treatments, and anthracene and perylene were further reduced at high (> 85%) rather than low (> 50%) temperature. However, pyrene was consumed at a significantly lower level by the consortia than by perylene (Tables 1 and 2c).

Phylogenetic analyses

Phylogenetic relationships among the degrading isolated cultured and noncultured bacteria are shown in Fig. 4. The aligned matrix contains 1349 unambiguous nucleotide position characters, 505 of which are parsimony-informative and 173 characters are excluded. Parsimony analysis of the data matrix yielded 87 parsimonious trees with CI = 0.756, RI = 0.945 and a length of 1096. Figure 4 also shows the topology of the NJ tree. No inconsistencies were found when analysing NJ and MP boostrap values.

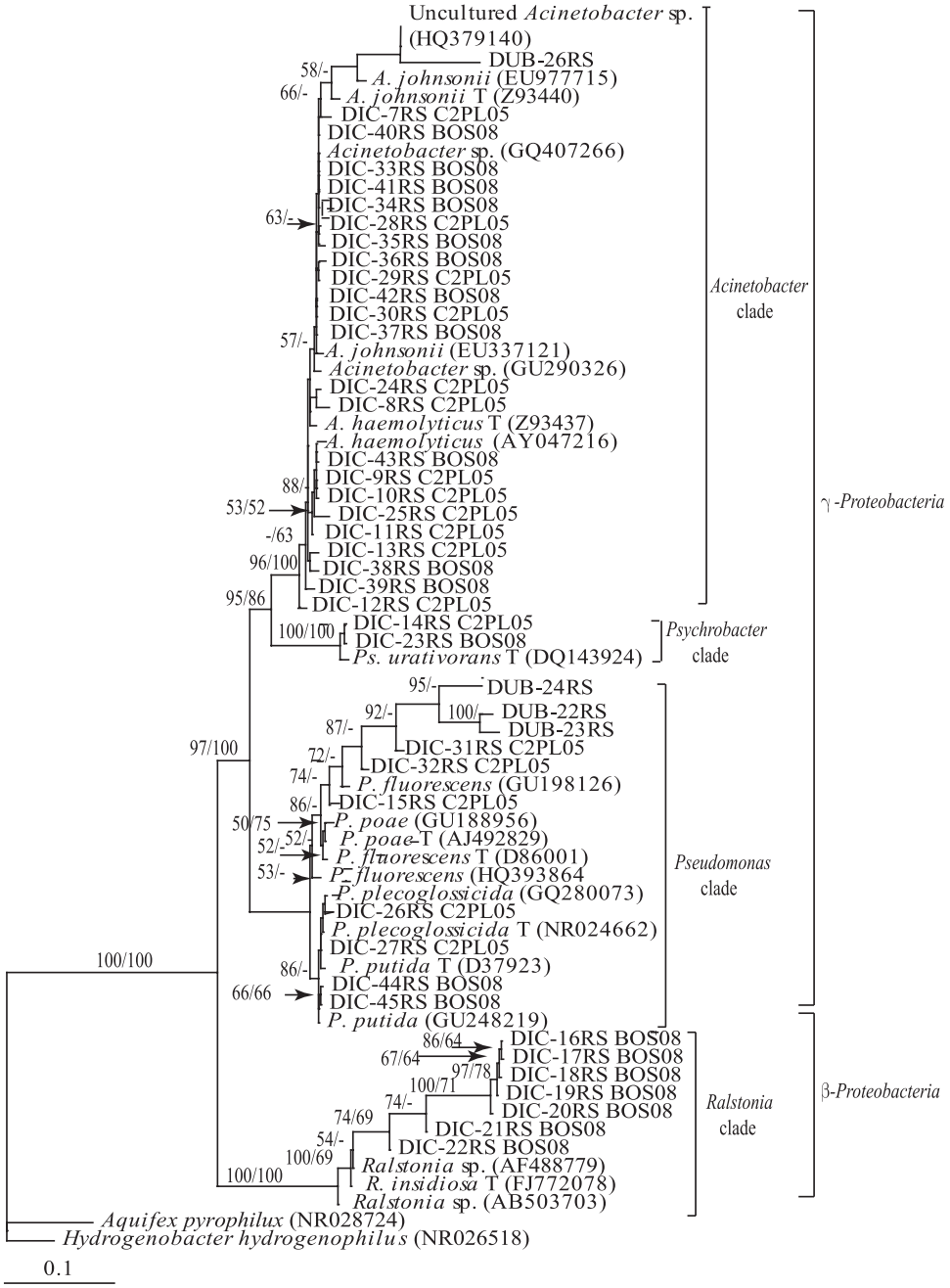

Fig. 4. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationship of the PAH-DIC and DUB, obtained from DGGE of the consortia and cultivable identification process at day 0, 33 and 101. Bootstrap values based on neighbour-joining (NJ) and maximum-parsimony (MP) analyses are shown at branch points (NJ/MP). No incongruence between the MP and NJ topology was detected. *P.*, *Pseudomonas*; *Ps.*, *Psychrobacter*; *A.*, *Acinetobacter*; *R.*, *Ralstonia*.

DIC-46-RS (*Rhodococcus* sp.), DIC-47RS (*Bacillus psychrodurans*) and DUB-25RS (*Microbacterium* sp.) were not included in the phylogenetic tree due to their high phylogenetic distance from most of the DIC and DUB. The phylogenetic tree comprised bacteria belonging to the *Gammaproteobacteria* and *Betaproteobacteria*. The group of *Gammaproteobacteria* consisted of the *Acinetobacter* clade, *Psychrobacter* clade and *Pseudomonas* clade, whereas the *Betaproteobacteria* group consisted solely of the *Ralstonia* clade. Within the *Acinetobacter* clade, although identity approximation (BLAST option, GenBank) revealed *Acinetobacter johnsonii* and *Acinetobacter haemolyticus* to be closest to some of the DIC and DUB, incorporation of the type strains of these species in the phylogenetic tree did not produce a clear monophyletic group. Thus, molecular identification of these strains (Fig. 5) was exclusively restricted to the genus level, i.e. *Actinobacter* sp. A similar criterion was taken for the *Pseudomonas* clade, where molecular identifications carried out through BLAST searches were not supported by the monophyletic hypothesis when type strains were included in the analysis. The *Psycrobacter* clade, a sister group of the *Acinetobacter* clade, is represented by the type strain of *Psychrobacter urativorans* (DQ143924) in which DIC-14RS and DIC-23RS are nested. The *Betaproteobacteria* consisted of just the *Ralstonia* clade, confirmed by the type strain of *R. insidiosa* (FJ772078), although DICs included in this clade were more closely related to the *Ralsonia* sp. strain (AF488779).

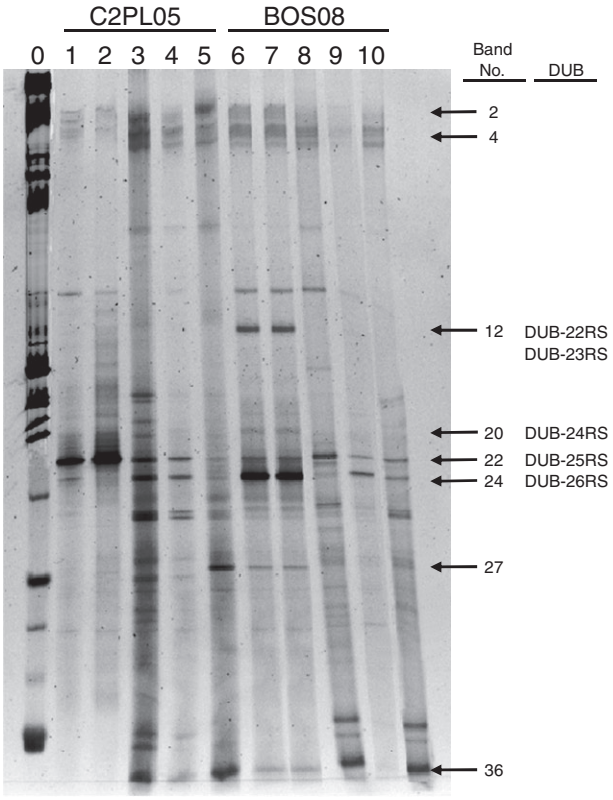

Fig. 5. DGGE (with a denaturing gradient from 35% to 65% denaturant) of PCR-amplified 16S rRNA gene fragments from consortium C2PL05 (lanes 1–5) and consortium BOS08 (lanes 6–10). Lane 0 contains DNA markers, lanes 1 and 6 correspond to the initial time, lanes 2 and 7 to the high-temperature range at day 33, lanes 3 and 8 to the high-temperature range at day 101, lanes 4 and 9 to the low-temperature range at day 33, and lanes 5 and 10 to the low-temperature range at day 101. Excised bands and clone ID (DUB) of successfully cloned bands are indicated.

Diversity and evolution of cultivated and uncultivated bacteria and dynamics during PAH biodegradation

PCR analysis to identify fungal DNA in BOS08 yielded negative results for the initial period of the biodegradation process at both temperature ranges. Fungal DNA was only positive at high temperatures and at the end of the biodegradation process (101 and 137 days).

A minimum of 10 colonies were isolated and identified by molecular techniques from the four treatments at days 33 and 101 by cultivation methods. The most influential DGGE bands, comprising 60% of the contribution based on similarity (Fig. 5) according to the results of PRIMER analysis, were cloned and identified with the exception of bands 2, 4, 27 and 36, which could not be cloned after several attempts, probably due to DNA degradation as a result of UV exposure. This effect was more notable in low-intensity bands with less DNA concentration. The results of the identification by cultivation- and noncultivation-based methods (Fig. 5) show that DUB-26RS, uncultured *Acinetobacter* sp., and DUB-25RS, uncultured *Microbaterium* sp., were always present in the two consortia (Fig. 5), both at high and at low temperatures. However, it should also be noted that *Rhodococcus* sp. strains were uniquely identified from the more significant bands of the C2PL05 consortium whereas *Ralstonia* sp. and *Bacillus* sp. were only found in the significant bands excised from consortium BOS08, comprising all of the above DIC strains (Fig. 5). In-depth analysis of the community of microorganisms, through DGGE fingerprinting and further identification of the bands, allowed us to establish those bands responsible for the similarities between treatments (Table 3) and the most influential factor. MDS (Fig. 6) showed that both time and temperature have important effects on C2PL05 microbial diversity, whereas only time had an effect on the BOS08 consortium. The highest average similarity (53.27%) was observed on day 101, with bands 36, 4 (unidentified) and 24 (DUB-26RS, *Acinetobacter* sp.) being responsible

Table 3. DGGE band contributing to approximately the first 60% of the cumulative percentage of averaged similarity grouped by time (0, 33 and 101 days), consortium (C2PL05, BOS08) and temperature (high, 15–25 °C; low, 5–15 °C) range; average similarity of the groups was determined by the SIMPER method

		Time (days)			Consortium		Temperature	
Band	Clone ID	0	33	101	C2PL05	BOS08	High	Low
22	DUB-25RS	28.55	27.89			25.81		
20	DUB-24RS		29.93		25.21	17.97	23.66	
36	Unidentified*			35.46			10.29	21.05
4	Unidentified*	28.55		11.20	23.62	17.55	23.15	17.53
27	Unidentified*							13.90
2	Unidentified*				11.98			
24	DUB-26RS			9.29				
Cumulative similarity (%)		57.10	57.81	55.95	60.81	61.34	57.10	52.49
Average similarity (%)		44.33	40.70	53.27	35.43	46.60	44.33	40.57

*Unidentified bands from DGGE after several cloning attempts.

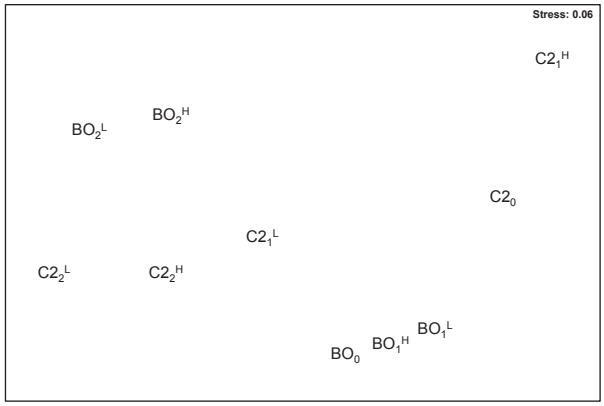

Fig. 6. MDS plot showing the similarity between consortia BOS08 (BO) and C2PL05 (C2) incubated at low (superscript L) and high (superscript H) temperature on day 0, 33 and 101 (subscripts 0, 1 and 2, respectively).

for that similarity. The lowest similarity (35.43%) was observed within the consortium C2PL05 (Table 3), due to the high abundance of band 20 (DUB-24RS, *Pseudomonas* sp.) and the lack of band 22 (DUB-25R, *Microbacterium* sp.). In conclusion, bands 20 (DUB-24RS, *Pseudomonas* sp.), 22 (DUB-25R, *Microbacterium* sp.), 24 (DUB-26RS, *Acinetobacter* sp.), 36 and 4 (both unidentified) were responsible for the most for the similarity or dissimilarity between bacterial communities of the different treatments. Another band showing a lower contribution to these percentages, but not yet cloned, was band 12, from which two genotypes were identified (DUB-22RS and DUB-23RS) as representing *Pseudomonas* sp. Regarding the identification of DIC strains (Fig. 5) *Rhodococcus* sp. was exclusive to the C2PL05 consortium and *Ralstonia* sp. and *Bacillus* sp. were only found in the BOS08 consortium.

Discussion

PAH degradation capability of bacterial consortia

In contrast to previous works (Spain & VanVeld, 1983; Johnsen & Karlson, 2005), consortium BOS08 did not require pre-exposure to PAHs to induce microbial adaptation and consequently degradation of those pollutants. In agreement with Margesin & Schinner (2001), our results showed that addition of PAHs to the forest consortium BOS08 was rapidly followed by an initial increase in PAH-degrading bacteria. Given the origin of consortium BOS08, extracted from a soil rich in organic matter and decaying wood, it is possible that the biodegradation process may be associated with wood-degrading bacteria and fungi. However, our results confirmed that fungi were not present in the initial conditions when PAH concentration was high. Fungi appeared only at the end of the biodegradation process (101 and 137 days) and only at high temperature when PAH concentration was depleted and toxicity was low. These results therefore confirm that the biodegradation process was carried out mainly by bacteria when PAH concentration and toxicity were high.

The two consortia showed a lower depletion rate for pyrene than for perylene, which was opposite to that expected from their physicochemical properties, as pyrene has a lower molecular size and hydrophobicity and a much higher aqueous solubility and thus bioavailability. A possible explanation for these results could lie in the extensively reported high toxicity and phototoxicity of pyrene under visible and UV radiation for a wide range of organisms (Ferreira, 2001; Petersen & Dahllöf, 2007).

PAH degradation ability is a general characteristic of some microbial communities when they are exposed to

PAHs (Macleod & Semple, 2002; Johnsen & Karlson, 2005; Tian *et al.*, 2008). Microbial consortia were obtained under very different levels of contamination. However, although large differences were observed at the initial microbial composition of both consortia, they shared some strains (*Microbacterium* sp. and *Acinetobacter* sp.). The lower diversity found within the C2PL05 consortium (more details are given by Molina *et al.*, 2009) obtained from a chronically and heavily polluted area with petroleum hydrocarbons was typical of aged soils exposed to PAHs. These pollutants drive the selection of specific bacteria that are able to degrade them (Viñas *et al.*, 2005).

Most of the species identified using DGGE (culture-independent rRNA approaches) in this work were *Gammaproteobacteria* (*Pseudomonas* and *Acinetobacter*) except DUB-26RS, which were 98% similar to *Microbacterium* sp. (phylum *Actinobacteria*). In agreement with Harayama *et al.* (2004), identifications determined by culture-dependent methods showed some differences from those identified by culture-independent rRNA approaches. DIC identified by culturable techniques belonged to a greater extent to the *Proteobacteria*, *Gammaproteobacteria* (*Pseudomonas*, *Pshycrobacter*, *Acinetobacter*) and *Betaproteobacteria* (*Ralstonia*). Only two cultivable strains, DIC-46RS and DIC-47RS, identified as representing *Rhodococcus* sp. and *B. psychrodurans*, belonged to the phyla *Actinobacteria* and *Firmicutes*, respectively. Genera such as *Bacillus*, *Pseudomonas* and *Ralstonia* were identified within consortium BOS08, obtained from decaying wood in a pristine forest. These genera are typical of decomposing wood systems and have been previously mentioned as important aerobic cellulose-degrading bacteria, such as *Bacillus* sp. (Lynd *et al.*, 2002), or degraders of the highly oxidized oxalate (*Pseudomonas* sp., *Ralstonia* sp.) released by white-rot fungi during degradation of lignocellulose (Dutton & Evans, 1996). Lignin is one of the most slowly degraded components of dead plants, and makes a major contribution to the formation of humus as it decomposes. The breakdown of lignin is mediated by extracellular enzymes, such as laccase, lignin peroxidise and manganese peroxidase (Hatakka, 1994, 2001). The lack of specificity and the high oxidant activity of these enzymes make them able to degrade different PAHs (e.g. Pickard *et al.*, 1999). For this reason, *Bacillus*, *Pseudomonas* and, in particular, *Ralstonia* identified within consortium BOS08 and typical of decomposing wood systems have also been previously identified as degraders of aromatic compounds (Zhuang *et al.*, 2002; Chauhan *et al.*, 2008; Luo *et al.*, 2009). Although many eukaryotic laccases have been identified and studied, laccase activity has been reported in relatively few bacteria, including some strains identified in our decomposing wood consortium BOS08, such as *Ralstonia* sp. and *Bacillus* sp., and others such as *Azospirillum lipoferum*, *Marinomonas mediterranea*, *Amycolatopsis* sp., *Streptomyces coelicolor*, *Arthrobacter cholorophenolicus* and *Rhodococcus opacus* (McMahon *et al.*, 2007; Dawkar *et al.*, 2009; Brown *et al.*, 2011).

HMW-PAH degradation at low temperatures

In agreement with Eriksson *et al.* (2001), PAH biodegradation rates were significantly higher at moderate temperatures (15–25 °C) because metabolic activity, diffusion and mass transfer were each facilitated (Leahy & Colwell, 1990; Haritash & Kaushik, 2009). However, there are also microorganisms with the capacity to degrade HMW-PAHs efficiently even at lower temperatures (Margesin *et al.*, 2002); these microorganisms were present in both consortia (BOS08 and C2PL05).

In accordance with previous studies that considered some strains of these genera (*Acinetobacter* and *Pseudomonas*), such as those that are cold-tolerant (Margesin *et al.*, 2003; Ma *et al.*, 2006), our results showed that they are able to grow and efficiently degrade HMW-PAHs at a low temperature range (5–15 °C), but at significantly lower rates than at higher temperature. In addition, although time was a significant factor in the distribution of bacterial communities, temperature affected only the C2PL05 consortium. It is probable that these results can be explained on the basis of temperature within the original environment where the consortia were extracted. Whereas the bacterial communities of BOS08 were adapted to temperatures below 20 °C throughout the year, the C2PL05 consortium was adapted to an environment reaching maximum temperatures above 20 °C for several months. Hence, although the C2PL05 consortium contained cold-tolerant species that were able to degrade at low temperatures, these are in lower proportions than for the BOS08 consortium, resulting in significant differences between percentages of PAH depletion and dynamics of the bacterial community at different temperature ranges. Therefore, the cold-adapted microorganisms are important for the *in-situ* biodegradation in cold environments. These results are important, as bacteria with PAH-degrading capacity are present in areas not previously exposed to these compounds, and may therefore respond efficiently to a contamination event. Therefore, the application of an efficient bioremediation process such as biostimulation could prove efficient in those systems where the autochthonous microbial communities may develop PAH-degrading capacity. On the other hand, bioaugmentation with natural microbial consortia, such as BOS08, may be important when autochthonous microbial populations are not degrading PAHs, especially at low temperatures. In addition, the study and isolation of

enzymes from the BOS08 consortium involved in HMW-PAH degradation metabolism at low temperatures is an important line of research for future bioremediation applications.

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References

Atlas RM & Bartha R (1972) Biodegradation of petroleum in seawater at low temperatures. *Can J Microbiol* **18**: 1851–1855.

Bautista LF, Sanz R, Molina MC, González N & Sánchez D (2009) Effect of different non-ionic surfactants on the biodegradation of PAH by diverse aerobic bacteria. *Int Biodeterior Biodegradation* **63**: 913–922.

Bence AE, Kvenvolden KA & Kennicutt MC (1996) Organic geochemistry applied to environmental assessments of Prince William Sound, Alaska, after Exxon Valdez oil spill – a review. *Org Geochem* **24**: 7–42.

Brakstad OG & Bonaunet K (2006) Biodegradation of petroleum hydrocarbons in seawater at low temperatures (0–5°C) and bacterial communities associated with degradation. *Biodegradation* **17**: 71–82.

Brown ME, Walker MC, Nakashige TG, Iavarone AT & Chang M (2011) Discovery and characterization of heme enzymes from unsequenced bacteria: application to microbial lignin degradation. *J Am Chem Soc* **45**: 18006–18009.

Canet R, Birnstingl JG, Malcolm DG, Lopez-Real JM & Beck AJ (2001) Biodegradation of polycyclic aromatic hydrocarbons (PAH) by native microflora and combinations of white-rot fungi in a coal-tar contaminated soil. *Bioresour Technol* **76**: 113–117.

Chauhan A, Fazlurrahman, Oakeshot JG & Jain RK (2008) Bacterial metabolism of polycyclic aromatic hydrocarbons: strategies for bioremediation. *Indian J Microbiol* **48**: 95–113.

Clarke KR (1993) Non-parametric multivariate analyses of changes in community structure. *Aust J Ecol* **18**: 117–143.

Couling NR, Towel MG & Semple KT (2010) Biodegradation of PAH in soil: influence of chemical structure, concentration and multiple amendments. *Environ Pollut* **158**: 3411–3420.

Dawkar VV, Jadhav UU, Telke AA & Govindwar SP (2009) Peroxidase from *Bacillus* sp. VUS and its role in the decolorization of textile dyes. *Biotechnol Bioprocess Eng* **14**: 361–368.

Dutton MV & Evans CS (1996) Oxalate production by fungi: its role in pathogenicity and ecology in the soil environment. *Can J Microbiol* **42**: 881–895.

Eriksson M, Jong-Ok K & Mohn WW (2001) Effects of low temperature and freeze–thaw cycles on hydrocarbon biodegradation in Arctic Tundra soil. *Appl Environ Microbiol* **67**: 5107–5112.

Ferreira MMC (2001) Polycyclic aromatic hydrocarbons: a QSPR study. *Chemosphere* **44**: 125–146.

Harayama S, Kasai Y & Hara A (2004) Microbial communities in oil-contaminated seawater. *Curr Opin Biotechnol* **15**: 205–214.

Haritash AK & Kaushik CP (2009) Biodegradation aspects of polycyclic aromatic hydrocarbons (PAH): a review. *J Hazard Mater* **169**: 1–15.

Hatakka A (1994) Lignin-modifying enzymes from selected white rot fungi: production and role in lignin degradation. *FEMS Microbiol Rev* **13**: 125–135.

Hatakka A (2001) Biodegradation of lignin. *Biopolymers*, Vol. 1, *Lignin, Humic Substances and Coal* (Hofrichter M & Steinbuchel A, eds), pp. 129–180. Wiley-VCH, Weinheim.

Johnsen A & Karlson U (2005) PAH degradation capacity of soil microbial communities – does it depend on PAH exposure? *Microb Ecol* **50**: 488–495.

Jørgensen KS, Järvinen O, Sainio P, Salminen J & Suortti AM (2005) Quantification of soil contamination. *Manual of Soil Analysis: Monitoring and Assessing Soil Bioremediation* (Margesin R & Schinner F, eds), pp. 97–119. Springer, Berlin.

Jukes TH & Cantor CR (1969) Evolution of protein molecules. *Mammalian Protein Metabolism* (Munro HN, ed), pp. 21–132. Academic Press, New York.

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

Kanaly RA & Harayama S (2010) Advances in the field of high-molecular-weight polycyclic aromatic hydrocarbon biodegradation by bacteria. *Microb Biotechnol* **3**: 136–164.

Katoh K & Toh H (2008a) Recent developments in the MAFFT multiple sequence alignment program. *Brief Bioinform* **9**: 286–298.

Katoh K & Toh H (2008b) Improved accuracy of multiple ncRNA alignment by incorporating structural information into a MAFFT-based framework. *BMC Bioinformatics* **9**: 212.

Lafortune I, Juteau P, Déziel E, Lépine F, Beaudet R & Villemur R (2009) Bacterial diversity of a consortium degrading high-molecular-weight polycyclic aromatic hydrocarbons in a two-liquid phase biosystem. *Microb Ecol* **57**: 455–468.

Lane DJ (1991) 16S/23S rRNA sequencing. *Nucleic Acid Techniques in Bacterial Systematics* (Stackebrandt E & Goodfellow M, eds), pp. 115–175. John Wiley & Sons, Chichester.

Leahy JG & Colwell RR (1990) Microbial degradation of hydrocarbons in the environment. *Microbiol Rev* **54**: 305–315.

Luo YR, Tian Y, Huang X, Yan CL, Hong HS, Lin GH & Zheng TL (2009) Analysis of community structure of a microbial consortium capable of degrading benzo(a)pyrene by DGGE. *Mar Pollut Bull* **58**: 1159–1163.

Lynd LR, Weimer PJ, van Zyl WH & Pretorius IS (2002) Microbial cellulose utilization: fundamentals and biotechnology. *Microbiol Mol Biol Rev* **66**: 506–577.

Ma Y, Wang L & Shao Z (2006) *Pseudomonas*, the dominant polycyclic hydrocarbons-degrading bacteria isolated from Antartic soils and the role of large plasmids in horizontal gene transfer. *Environ Microbiol* **8**: 455–465.

Macleod CJA & Semple KT (2002) The adaptation of two similar soils to pyrene catabolism. *Environ Pollut* **119**: 357–364.

MacNaughton SJ, Stephen JR, Venosa AD, Davis GA, Chang YJ & White DC (1999) Microbial population changes during bioremediation of an experimental oil spill. *Appl Environ Microbiol* **65**: 3566–3574.

Margesin R & Schinner F (2001) Bioremediation (natural attenuation and biostimulation) of diesel-oil contaminated soil in an alpine glacier skiing area. *Appl Environ Microbiol* **67**: 3127–3133.

Margesin R, Zacke G & Schinner F (2002) Characterization of heterotrophic microorganisms in alpine glacier cryoconite. *Arct Antarct Alp Res* **34**: 88–93.

Margesin R, Gander S, Zacke G, Gounot AM & Schinner F (2003) Hydrocarbon degradation and enzyme activities of cold-adapted bacteria and yeasts. *Extremophiles* **7**: 451–458.

McMahon AM, Doyle EM, Brooks S & O'Connor KE (2007) Biochemical characterization of the coexisting tyrosinase and laccase in the soil bacterium *Pseudomonas putida* F6. *Enzyme Microb Technol* **40**: 1435–1441.

Microbics Corporation (1992) *Microtox Manual Vol. III, Condensed Protocols. A Toxicity Tested Handbook*. Microbics Corporation, Carlsbad, CA.

Mohn WW & Stewart GR (2000) Limiting factors for hydrocarbon biodegradation at low temperature in Artic soils. *Soil Biol Biochem* **32**: 1161–1172.

Molina MC, González N, Bautista LF, Sanz R, Simarro R, Sánchez I & Sanz JL (2009) Isolation and genetic identification of PAH degrading bacteria from a microbial consortium. *Biodegradation* **20**: 789–800.

Petersen DG & Dahllöf I (2007) Combined effects of pyrene and UV-light on algae and bacteria in arctic sediment. *Ecotoxicology* **16**: 371–377.

Pickard MA, Roman R, Tinoco R & Vazquez-Duhalt R (1999) Polycyclic aromatic hydrocarbon metabolism by white rot fungi and oxidation by *Coriolopsis gallica* UAMH 8260 laccase. *Appl Environ Microbiol* **65**: 3805–3809.

Simarro R, Gonzalez N, Bautista LF, Sanz R & Molina MC (2010) Optimization of key abiotic factors of PAH (naphthalene, phenanthrene and anthracene) biodegradation process by a bacterial consortium. *Water Air Soil Pollut* **217**: 365–374.

Spain JC & VanVeld PA (1983) Adaptation of natural microbial communities to degradation of xenobiotic compounds-effects of concentration, exposure time, inoculum and chemical structure. *Appl Microbiol* **45**: 428–435.

Surridge AKJ, Wehner FC & Cloete TE (2009) Bioremediation of polluted soil. *Adv Appl Biorem* **17**: 103–121.

Sutherland JB, Rafii F, Khan AA & Cerniglia CE (1995) Mechanisms of polycyclic aromatic hydrocarbon degradation. *Microbial Transformation and Degradation of Toxic Organic Chemicals* (Young LY & Cerniglia CE, eds), pp. 296–306. Wiley-Liss, New York.

Swofford DL (2003) *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods), Version 4.0b 10*. Sinauer Associates, Sunderland, MA.

Taketani RG, Franco NO, Rosado AS & van Elsas JD (2010) Microbial community response to a simulated hydrocarbon spill in mangrove sediments. *J Microbiol* **48**: 7–15.

Tian Y, Luo Y, Zheng T, Cai L, Cao X & Yan C (2008) Contamination and potential biodegradation of polycyclic aromatic hydrocarbons in mangrove sediments of Xiamen, China. *Mar Poll Bull* **56**: 1184–1191.

Viejo RM (2009) Resilience in intertidal rocky shore assemblages across the stress gradient created by emersion times. *Mar Ecol Prog Ser* **390**: 55–65.

Viñas M, Sabaté J, Espuny MJ & Solanas AM (2005) Bacterial community dynamics and polycyclic aromatic hydrocarbon degradation during bioremediation of heavily creosote-contaminated soil. *Appl Environ Microbiol* **71**: 7008–7018.

Wilke BM (2005) Determination of chemical and physical soil properties. *Manual of Soil Analysis: Monitoring and Assessing Soil Bioremediation* (Margesin R & Schinner F, eds), pp. 47–95. Springer, Berlin.

Wong WSD (2009) Structure and action of ligninolytic enzymes. *Appl Biochem Biotechnol* **157**: 174–209.

Wrenn BA & Venosa AD (1996) Selective enumeration of aromatic and aliphatic hydrocarbon-degrading bacterial by most probable number (MPN). *Can J Microbiol* **42**: 252–258.

Yakimov MM, Giuliano L, Gentile G, Crisafi E, Chernikova TN, Abraham W-R, Lünsdorf H, Timmis KN & Golyshin PN (2003) *Oleispira antarctica* gen. nov., sp. nov., a novel hydrocarbonoclastic marine bacterium isolated from Antarctic coastal sea water. *Int J Syst Evol Microbiol* **53**: 779–785.

Zhang Z, Schwartz S, Wagner L & Miller W (2000) A greedy algorithm for aligning DNA sequences. *J Comput Biol* **7**: 203–214.

Zhuang W-Q, Tay J-H, Maszenan AM & Tay STL (2002) *Bacillus naphthovorans* sp.nov from oil contaminated tropical marine sediments and its role in naphthalene biodegradation. *Appl Microbiol Biotechnol* **58**: 547–553.

Zimmermann W (1990) Degradation of lignin by bacteria. *J Biotechnol* **13**: 119–130.