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Biodegradation efficiency of functionally important populations selected for bioaugmentation in phenol- and oil-polluted area

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Abstract

Denaturing gradient gel electrophoresis of amplified fragments of genes coding for 16S rRNA and for the largest subunit of multicomponent phenol hydroxylase (LmPH) was used to monitor the behaviour and relative abundance of mixed phenol-degrading bacterial populations (*Pseudomonas mendocina* PC1, *P. fluorescens* strains PC18, PC20 and PC24) during degradation of phenolic compounds in phenolic leachate- and oil-amended microcosms. The analysis indicated that specific bacterial populations were selected in each microcosm. The naphthalene-degrading strain PC20 was the dominant degrader in oil-amended microcosms and strain PC1 in phenolic leachate microcosms. Strain PC20 was not detectable after cultivation in phenolic leachate microcosms. Mixed bacterial populations in oil-amended microcosms aggregated and formed clumps, whereas the same bacteria had a planktonic mode of growth in phenolic leachate microcosms. Colony hybridisation data with catabolic gene specific probes indicated that, in leachate microcosms, the relative proportions of bacteria having *meta* (PC1) and *ortho* (PC24) pathways for degradation of phenol and *p*-cresol changed alternately. The shifts in the composition of mixed population indicated that different pathways of metabolism of aromatic compounds dominated and that this process is an optimised response to the contaminants present in microcosms.

Keywords: Biodegradation; Mixed cultures; Phenol hydroxylase; PAHs; PCR-DGGE

1. Introduction

Intrinsic biodegradation as well as bioremediation by selected biodegradative microorganisms are the key factors in restoration of polluted environments [1]. Bioavailability of pollutants and survival and catabolic activity of introduced microorganisms play important roles in bioremediation technologies. Therefore, before applying bioaugmentation it is necessary to isolate, identify and characterise pollutant-degrading indigenous bacterial strains and analyse their activity in situ. Laboratory experiments with pure cultures are also essential for detailed study of the physiology and genetics of microorganisms.

Catabolic pathways operating in natural communities reflect interactions between microbial species under mixed culture conditions where extensive sharing of nutritional resources is common [2] and interaction of two or several strains is often a prerequisite for growth and biodegradation [3]. It has already been shown that a mixed culture of strains with different catabolic types overcomes incompatibilities in degradation of divergent substrate mixtures [4]. Among multitude of microbial

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species in the environment, few populations that can use specific substrates become dominant, while others exist as minorities [5]. One caveat of microbial remediation systems is the fact that a high concentration of phenolic and other pollutants may be toxic to the microbes within the biological treatment compartment [6]. Therefore, a rapid method is needed to monitor the bacterial community during the treatment process.

Traditional microbiological methods, including plating onto selective minimal agar to enumerate viable bacteria, are known to underestimate the actual population values by one to two orders of magnitude [7]. Direct microscopic counts of bacteria often suffer from a high level of background staining and are very tedious due to accumulation of microorganisms at interfaces (development of a biofilm). On the other hand, molecular techniques have been successfully used to assess microbial activity of different strains and to detect specific functional genes [8,9]. The gene probes have been developed for particular microbial groups biodegrading common phenolic contaminants, including naphthalene [10].

The initial conversion of phenol carried out by either a monocomponent [11,12] or a multicomponent phenol hydroxylase [13,14] leads to central intermediates that are further degraded by either *ortho*- or *meta*-cleavage. The fragments of genes responsible for the monohydroxylation of the *ortho* position of the aromatic ring have been PCR-amplified from environmental DNA. The gene encoding the largest subunit of a multicomponent phenol hydroxylase (LmPH) has been used for the determination of genetic variability among the isolates of phenol-degrading bacteria [8].

The genes of ring-cleavage dioxygenases are good targets for monitoring of biodegrading populations involved in ring-cleavage of aromatics and in early steps of degradation of some polycyclic aromatic hydrocarbons (PAHs), which are very common in crude oil [15–17]. Aerobic biodegradation of PAHs with three or less rings (phenanthrene, anthracene, naphthalene) proceeds through a dioxygenase attack on an aromatic ring resulting in formation of a *cis*-dihydrodiol [18]. Salicylate formed after rearomatisation reactions is further catabolised via catechol or gentisate by dioxygenases. Catechol 2,3-dioxygenase (C23O) genes are among the most informative genetic markers in evaluation of the efficacy of bioremediation [10,19–23].

Our previous work demonstrated a multiplicity of catabolic types of degradation of phenol and *p*-cresol and the existence of characteristic assemblages of species and specific genotypes among the strains isolated from river water continuously polluted with phenolic compounds of oil shale ash leachate [24]. In the present investigation we have concentrated on functional analyses of these bacteria in removing phenols and PAHs from phenolic leachate and crude oil. For optimisation of bioremediation in the field, the dynamics of functionally dominant bacterial populations in laboratory microcosms was studied.

2. Materials and methods

2.1. Bacterial strains and their characterisation

Species identification according to analysis of BIOLOG GN data, rep-PCR genomic fingerprints and sequences of 16S rRNA genes revealed that bacterial strains used in this study belong to *Pseudomonas mendocina* PC1, *P. fluorescens* biotype G PC18, *P. fluorescens* biotype A PC20 and *P. fluorescens* biotype C PC24 [24,25]. Genomic DNA from following *P. putida* reference strains: mt-2 [26], CF600 [27] and EST1026 [28] was extracted and used as positive control for PCR detection of genes coding for C23O, LmPH and phenol monooxygenase (*pheA*), respectively. Approximately 1500-bp sequences of the 16S rRNA genes of strains PC1, PC18, PC20 and PC24 have been deposited in the GenBank data library under accession numbers AF232713, AF228366, AY538264 and AF228367, respectively.

2.2. Microcosm experiments, growth media and cell enumeration

Inocula for microcosms were pregrown in $1 \times$ M9 medium [24] containing 2.5 mM phenol. Cells of individual strains in the late logarithmic phase of growth were harvested by centrifugation at 12,000*g* for 10 min at 4 °C, washed and suspended in sterile $1 \times$ M9 medium and used to inoculate microcosms at final concentration of cells of approximately $1–1.5 \times 10^7$ colony forming units (CFU) ml^{-1}. Mixed cultures contained approximately equal numbers of cells of each strain added.

Microcosm experiments with oil were carried out in 250 ml Erlenmeyer flasks containing 60 mg of crude oil in 100 ml of mineral medium. M9 mineral medium was sterilised before addition of crude oil as the sole source of carbon and energy. Microcosm experiments with phenolic leachate were performed in 250 ml Erlenmeyer flasks containing 50 ml filter-sterilised (0.22 μm pore size Millipore filter) oil shale leachate. The pH was adjusted to 7.5 with HCl and $20 \times$ M9. Uninoculated flasks were included as reference for abiotic losses. Both microcosms were incubated for 30 days at 25 °C with rotary shaking (120 rpm) and analysed periodically. Bacterial populations were enumerated on phenol agar plates using the spread-plate technique. After incubation at 30 °C for 4 days the number of phenol-degrading bacterial strains (CFU ml^{-1}) was determined. The plates were prepared in triplicate and mean values and standard deviations were calculated.

2.3. Chemical analyses of leachate samples

After inoculation of microcosms, samples for substrate analysis were periodically withdrawn from abiotic control variant and leachate microcosms, filtered through a 0.22 μm Millipore filter and stored frozen until the analysis with HPLC [24] or GC-MS. In the latter case phenolic compounds were extracted by vigorous shaking with 1 ml of diethylether. Phenols and PAHs were identified using a GC-MS system consisting of Varian Star 3400 Gas Chromatograph (Varian Inc., USA) and the Finnigan MAT Magnum™ Mass spectrometer (Finnigan MAT Inc., USA) operated in electron impact ionisation mode. The capillary column Econo-Cap EC-5, 30 m in length with inside diameter of 0.25 mm and a stationary-phase film thickness of 0.25 μm (Alltech, USA) was used for the separation of compounds. Helium was used as carrier gas. The injection volume was 1 μl, injector temperature and split ratio were 270 °C and 1:10, respectively. The total run time was 20 min and the following temperature program was used: 100–200 °C (8 °C min^{-1}), 200–280 °C (30 °C min^{-1}), 280 °C for 5 min. Substrates were quantified using calibration graphs with internal standard diphenylmethane, which was added to the extracts at the end of the extraction. All results are given as recovery-corrected. The recoveries of the studied compounds varied from 49% (SD% 4.3) to 90% (SD% 4.6%) for benzoate and naphthalene, respectively. The recoveries of the phenolic compounds were in the range 70–90% (except for 2,3-dimethylphenol: 52%). Combined standard uncertainty of the results is uncertainty of the result that takes into account all significant uncertainty sources and is expressed as a standard deviation, i.e. at approximately 68% confidence level [29]. Combined standard uncertainties of the results were between 6% and 10%. The uncertainties include contributions from sample preparation, instrumental measurement (both samples and calibration standards) and peak integration.

2.4. Chemical analyses of oil-amended microcosm samples

Samples were collected from oil-amended microcosms 30 days after inoculation with single strain PC20 and a mixture of strains and compared with abiotic controls. All samples for chemical analyses were stored at −20 °C until use. Hydrocarbons in oil-amended microcosms were extracted and analysed by GC-MS. For extraction from oil-amended microcosms, samples were transferred to the separation funnel. The sample bottle was washed with 3 ml of carbon tetrachloride (CCl_4), which was thereafter transferred directly to the extracts bottle. The sample bottle was then washed with an additional 3 ml of CCl_4, and transferred to the separation funnel, which was shaken vigorously for 1 min with periodic venting to release the excess pressure. The organic layer was allowed to separate from the water layer for 10 min and the process repeated four more times. All solvent extracts were combined.

2.5. DNA extraction

Template DNA was isolated from leachate and oil-amended microcosms inoculated with mixed microbial populations at different time points. DNA was extracted from samples (2 ml) using UltraClean microbial DNA isolation kits (Mo Bio Laboratories, Solana Beach, CA, USA) according to manufacturer's instructions. Three serial 10-fold dilutions of samples (10^0–10^{-2}) were made in sterile water and used for DNA extraction. The extracted DNA was stored at −20 °C. DNA was quantified spectrophotometrically and samples were diluted to give final concentration of approximately 15 ng μl^{-1}.

2.6. Primers and PCR conditions

The primers and PCR conditions used for the amplification of fragments of genes coding for C23O, the LmPH and phenol monooxygenase (*pheA*), are listed in Table 1. Time-dependent changes in mixed populations were detected by amplification of 16S rRNA gene

Table 1
Characteristics of DNA probes in the study

Probe, primer		Primer sequence (5′ → 3′)	Size bp	References
16S rDNA	PRBA338f	ACTCCTACGGGAGGCAGCAG	236	[30]
	PRUN518r	ATTACCGCGGCTGCTGG		[31]
C23O	E3F	GGTATGGCGGCTGTGCGTTTCGACCA	356	[25]
	E2R	AGAACACTTCGTTGCGGTTACC		
PheA	pheA1	CAGGATCGAATATCGGTGGCCTCG	947	[24]
	pheA2	CTTCACGCTGGCGTAACCAATCGC		
LmPH	PheGC	CRATYGACGARCTGCGYCA	209	This study
	Phe212	GTTGGTCAGCACGTACTCGAAGG AGAA		[8]
GC[a] clamp		CGCCCGCCGCGCGCGGCGGGCGGGGC GGGGGCACGGGGGG		[31]

[a] The GC clamp was attached to the end of the 5′ end of the PRBA338f and PheGC primers.

products using universal bacterial primers targeting the V3 variable region (Table 1). The GC-clamp (40 bp) was added to the F338GC primer to enable denaturing gradient gel electrophoresis analysis (DGGE). PCR amplification was performed in a total volume of 50 μl. DNA (15 ng) of extracted from samples was added as a template to a 50 μl reaction mixture. The PCR mixture included 1 × PCR buffer provided in the kit (with $(NH_4)_2SO_4$), 200 μM concentrations of each deoxynucleoside triphosphate, 2.5 mM $MgCl_2$, 20 pmol of each primer and 0.5 U of *Taq* DNA polymerase (Fermentas). Step cycles for probe reactions were as follows: the V3 region of 16S rRNA genes were amplified at 95 °C for 1 min, 53 °C for 1 min, 72 °C for 2 min; C23O genes were amplified at 94 °C for 45 s, 58 °C for 45 s and 72 °C for 20 s; *pheA* genes were amplified at 94 °C for 1 min, 61 °C 45 s and 72 °C for 1 min; LmPH genes were amplified at 94 °C for 1 min, 65 °C for 1 min, 72 °C for 2 min. All amplifications were performed for 32 cycles, and 72 °C for 10 min was employed as a single final extension step.

2.7. Analysis of PCR products

PCR was carried out as described above to assess fluctuations in catabolic potential with time. PCR mixes (5 μl) were subjected to agarose gel (2%) electrophoresis for approximately 15 min at 100 V in 1 × TAE buffer, pH 8.3. DNA fragments were stained for 20 min in 1 × TAE buffer with ethidium bromide (final concentration, 0.5 $\mu g l^{-1}$) and de-stained twice in MilliQ water for 20 min prior to UV transillumination. A molecular weight marker (100 bp DNA ladder, Fermentas) was included at both sides of each gel and PCR products were quantified by comparison with a standard using E.A.S.Y Win32 Software (Herolab GmbH, Germany).

2.8. DGGE analysis

Population dynamics were determined in microcosms for 30 days by DGGE analysis of PCR-amplified 16S rRNA and LmPHs genes. Genes encoding the LmPH of strains PC1 and PC18 were used as templates to characterise the functionally dominant population in leachate and oil-amended microcosms. The phenol-hydroxylating activity followed by C23O activity has been shown earlier in these bacteria [24]. *Pseudomonas* sp. CF600 was used as a positive reference strain possessing the LmPH and the strains PC20 and PC24, which lack LmPH and thus did not give a positive amplification reaction.

Approximately 500 ng of a PCR product was applied for DGGE analysis, using the method of Muyzer et al. [31]. The DCode DGGE system (Bio-Rad Laboratories, Hercules, CA) was used for electrophoresis as recommended by the manufacturer. A 10% (w/v) polyacrylamide (37.5:1 acrylamide:bis-acrylamide in 1 × TAE buffer) gels with a gradient of DNA-denaturant agent (100% denaturant agent is 7 M urea and 40% deionised formamide) was performed. The linear gradients of the denaturant used for separation were 35–70% and 40–60% for the 16S rRNA gene and LmPH gene fragments, respectively. To ensure well-polymerised slots, a 3 ml top gel containing no denaturant was added. Wells were loaded with equal amounts of DNA, and electrophoresis was performed in 1 × TAE buffer for 13 h at a constant temperature of 60 °C and a voltage of 100 V. Gels were stained in MilliQ water containing 0.5 $\mu g l^{-1}$ ethidium bromide and de-stained twice in MilliQ water prior to UV transillumination.

2.9. Colony hybridisation analysis

Samples for colony hybridisation were taken from leachate microcosms inoculated with strains PC1, PC18 and PC24. Colonies of both strains of PC1 and PC18 harbouring the C23O gene were distinguishable by different colony morphology and the colonies of strain PC24 were detected by a *pheA* gene probe. Cells were enumerated by standard plating and the relative proportions of each strain were compared by hybridisation of colonies with specific gene markers. Cells from colonies grown on phenol media were hybridised with radioactively labelled gene probes as described previously [32]. Probes for the *pheA* and C23O genes were generated by PCR amplification followed by purification from 2% agarose gels using the QIAquick gel extraction kit (Qiagen Inc.) [25]. The amplified products were subsequently random-prime labelled with [α-^{32}P]dCTP (Amersham Pharmacia Biotech, Inc.) using a DecaLabel™ kit, MBI Fermentas. Autoradiograms were generated using PhosphorImager (Molecular Dynamics).

2.10. Microscopy

Bacterial aggregates were stained with L-7007 LIVE/DEAD *Bac* Light stain (Molecular Probes Inc.) and examined using an epifluorescent microscope (Olympus U-RFL-T, Japan). Prior to imaging, samples (1 ml) were filtered through a sterile 0.2 μm Nucleopore polycarbonate black filters (Costar; Cambridge, MA) and washed with MilliQ water. Distilled water (1 ml) and mixed *Bac* Light dyes (3 μl) were poured onto a membrane filter and incubated for 20 min in the dark prior to filtration. After filtration, without any washing, the filter membrane was immersed in *Bac* Light mounting oil on a microscopic glass slide and covered with a cover slip. Using this staining method, bacteria with intact cell membranes show green fluorescence whereas bacteria with damaged membranes have red fluorescence.

3. Results

3.1. The population dynamics in leachate and oil-amended microcosms studied by using 16S rRNA gene analysis

In leachate microcosms the DGGE profiles of 16S rRNA gene products demonstrated that the most drastic changes in population structure occurred already during the first day of incubation (Fig. 1(a)). The band representing the population of strain PC20 was not detectable even after the first day of incubation, indicating that this strain was not important in decomposition of leachate substrates. At the same time, *P. mendocina* PC1 was the predominant species in leachate microcosms and its relative abundance increased during the treatment period. The shifts in microbial composition of phenolic leachate microcosms indicated that populations of strains PC18 and PC24 were present throughout the experiment. DGGE profiles revealed an increase in abundance of strains PC24 and PC18 on the third and the fifth or on the third day, respectively. In contrast, in oil-amended microcosms all added strains were present throughout the experiment and showed only a slight decrease in abundance after treatment for 20 days (Fig. 1(b)). Comparisons of DNA band intensities revealed that strain PC24 was predominant in decomposition of substrates in oil-amended microcosm.

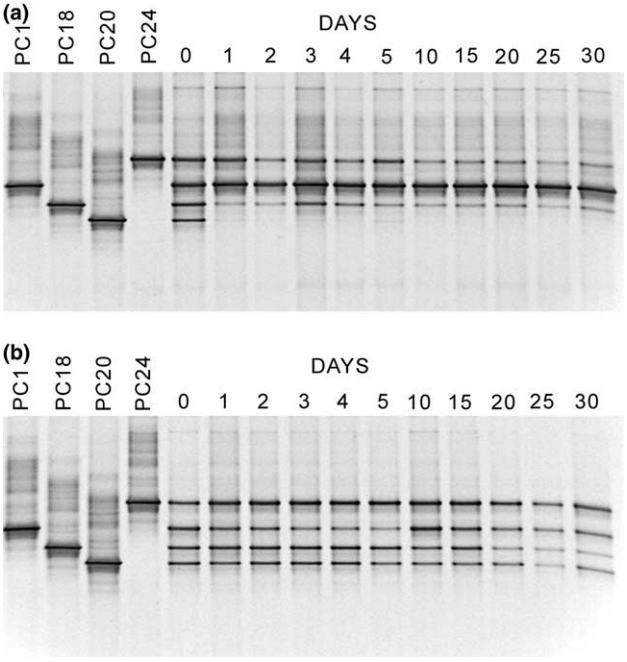

Fig. 1. Negative image of ethidium bromide-stained DGGE gel containing PCR-amplified fragments of 16S rRNA genes from phenolic leachate- (a) and oil-amended (b) microcosms after inoculation with a mixed culture of strains PC1, PC18, PC20 and PC24. Equal quantities (approximately 1×10^7 CFU ml^{-1}) of cell suspensions of each strain were added. Numbers above lanes indicate the days of incubation.

3.2. Monitoring of bacterial populations in leachate- and oil-amended microcosms using LmPH genes as a marker

More precise characterisation of the role of different phenol-degrading bacterial strains in different substrate mixtures was achieved by PCR amplification of phenol hydroxylase (LmPH) genes and DGGE analysis. As expected, comparison of intensities of the PCR amplification products of LmPH DNA bands between strains PC1 and PC18 showed that the dominant population in leachate microcosm throughout the experiment was strain PC1 (Fig. 2(a), see also Fig. 1(a)). In oil-amended microcosms the population of PC1 decreased and a slight increase was detected only between days 10 and 15 (Fig. 2(b)). Conversely, the population of strain PC18 was predominant in oil-amended microcosms. Relative abundance of both populations increased between 5 and 15 days in oil-amended microcosms.

Thus, DGGE analysis of 16S rRNA and LmPH genes indicated that in each microcosm specific populations were selected, and the roles of particular biodegradative bacterial strains may be coordinated in time by substrates available in the mixture.

3.3. Decomposition of phenolic compounds and PAHs in microcosms

Decomposition of phenols, benzoate and PAHs was determined using GC-MS. Fig. 3 shows the biodegradation efficiency of phenol, cresols and benzoic acid in phenolic leachate microcosms. Phenol and benzoic acid were completely removed during the first day and cresols after 10 days. The absence of significant biodegradation of dimethylphenols (3,5-; 2,3-; 2,5-; 2,4- and 2,6-DMP) was observed, with the exception of 3,4-dimethylphenol, which was degraded completely in two days (data not shown).

Only lighter PAHs – naphthalene, anthracene and phenanthrene – were chosen for study. Cresols and naphthalene were totally removed from oil-amended microcosms by mixed populations during 30 days, while degradation of phenanthrene and anthracene was negligible (Fig. 4). A single culture of naphthalene-degrading strain PC20 was less effective in biodegradation of phenols than in removal of PAHs (Fig. 4). This is a good indication for the importance of this strain in decomposition of oil products.

3.4. Development of bacterial aggregates in microcosms

Enumeration and monitoring of mixed bacterial populations in microcosms using CFU data from spread-plate analyses showed that the number of bacteria was the highest two days after the inoculation in phenol leachate microcosms. In oil-amended microcosms, total CFUs of mixed populations started to decrease from

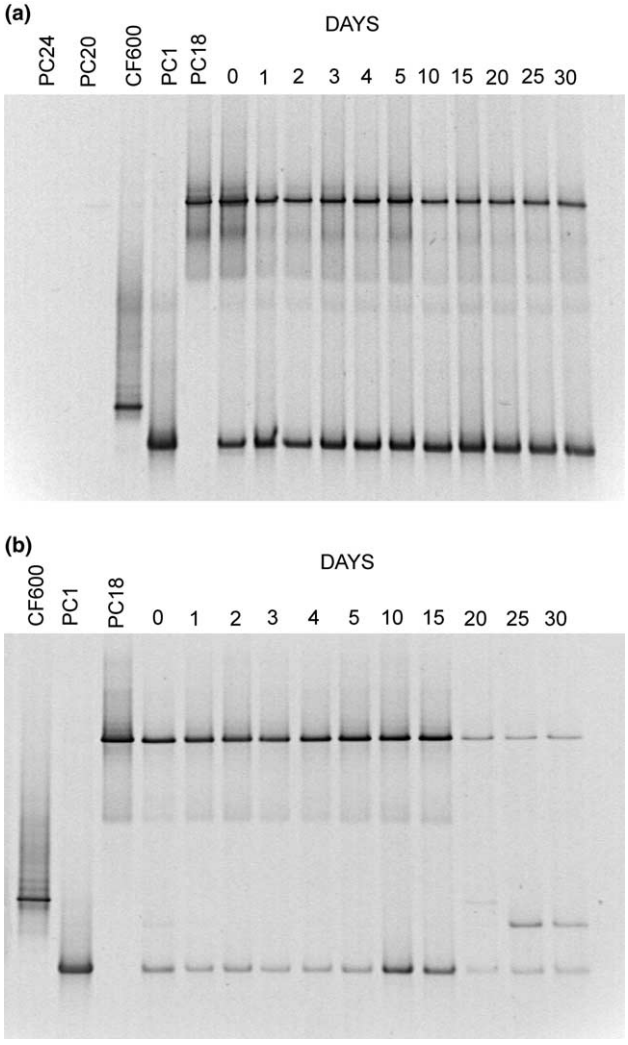

Fig. 2. Negative images of DGGE gels containing PCR-amplified fragments of LmPH genes from strains PC1 and PC18 during incubation for 30 days in phenolic leachate- (a) and oil-amended (b) microcosms. The DNA probes were taken from the same microcosms used in experiments presented in the Fig. 1. The DNA of strains PC20 and PC24 served as negative controls and DNA of *Pseudomonas* sp. CF600 was used as a positive control. Numbers above the lanes indicate the days of incubation.

the beginning of the experiment and showed a slight increase only 5–10 days after inoculation (Fig. 5). CFU data most probably do not reflect the real abundance of bacteria and differences between molecular data and standard plate counts can be explained by clumping of bacteria and formation of microbial aggregates.

Development of bacterial aggregates of single strains and mixed cultures in oil-amended microcosms (Fig. 6(a)–(e)) was followed using the *Bac* Light LIVE/DEAD viability method. Three forms of bacteria were detected: viable, dead and lysed showing green, red and no fluorescence, respectively, among both aggregated and non-aggregated cells. In oil-amended microcosms, aggregated cells of single strain PC24 were not detected one day after the inoculation (Fig. 6(d)), whereas the

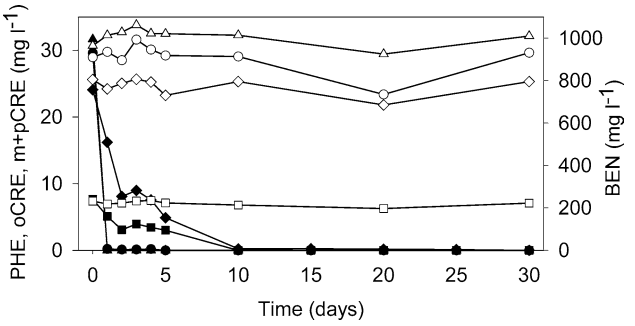

Fig. 3. Removal of phenolic compounds and benzoate by mixed culture of strains PC1, PC18, PC20 and PC24 during incubation for 30 days in leachate microcosms. The concentration of phenol (PHE, ○ control, ● culture), *o*-cresol (*o*CRE, □ control, ■ culture), *m*- and *p*-cresol (*m* + *p*CRE, ◇ control, ◆ culture), benzoate (BEN, △ control, ▲ culture) were determined. Values are the averages of triplicate determinations. The combined standard uncertainties [29] of the results were between 6% and 10%.

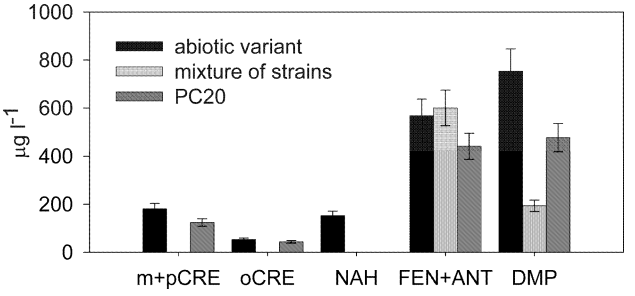

Fig. 4. Comparison of decomposition of aromatic compounds and PAHs in an abiotic control, in a mixture of strains PC1, PC18, PC20 and PC24 and in a single culture of strain PC20 after incubation for 30 days in oil-amended microcosms. Values are the averages of triplicate determinations. Error bars indicate the combined standard uncertainties [29] of the results. Abbreviations: *m* + *p*CRE, *m*- and *p*-cresol; *o*CRE, *o*-cresol; NAH, naphthalene; FEN + ANT, phenanthrene and anthracene; DMP, dimethylphenols.

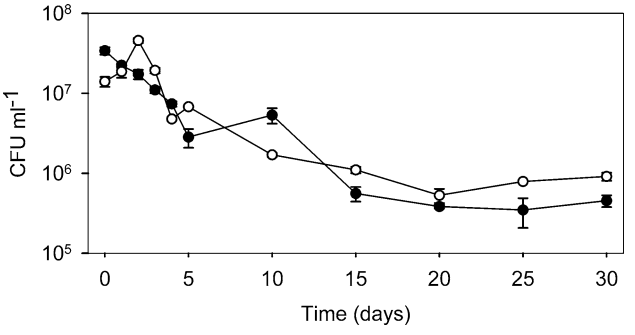

Fig. 5. Enumeration of bacteria in mixed populations from phenolic leachate- (○) and oil-amended microcosms (●) during incubation for 30 days. Values are the averages of triplicate determinations. Error bars indicate standard deviations.

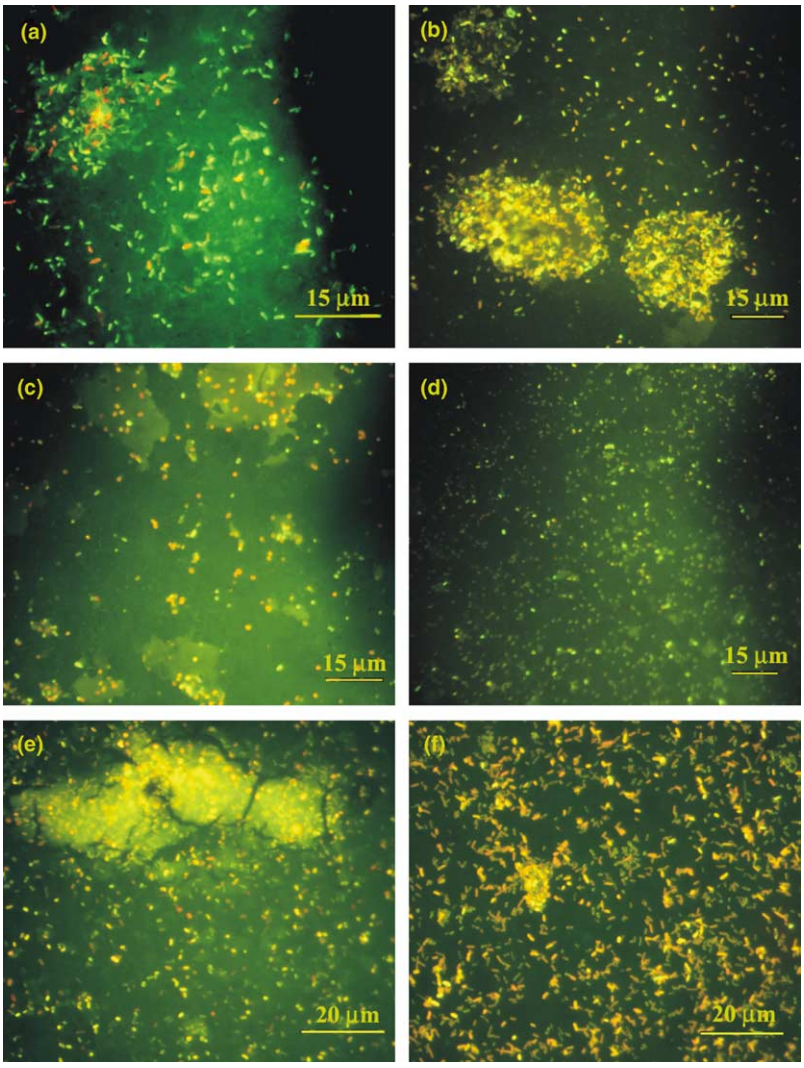

Fig. 6. Formation of bacterial aggregates by single strains in oil-amended microcosms (a – PC1; b – PC18; c – PC20 and d – PC24) and by mixed cultures in oil-amended (e) and leachate microcosms (f). Cells are visualised using the *Bac* Light LIVE/DEAD viability stain. Viable cells are green, whereas dead cells show red fluorescence due to compromised cell membrane. Images were obtained 24 h after cells were inoculated. Magnification 1000×.

strains PC1, PC18 and PC20 grew predominantly as clumps (Fig. 6(a)–(c)). Mixed culture of strains grew in aggregated forms in an oil-amended microcosm and in planktonic mode of growth in phenolic leachate microcosms (Fig. 6(e) and (f)). The cell aggregates were revealed during incubation of oil-amended microcosms. This may explain the artificial data showing decrease in the numbers of CFU during the decomposition of PAH in oil-amended microcosm.

3.5. Dynamics of mixed bacterial cultures in leachate microcosm determined by colony hybridisation

As in leachate microcosms, bacteria grow predominantly planktonically and colony hybridisation was used to compare cell numbers of different strains. Because of the rapid disappearance of strain PC20 the leachate microcosm (see Fig. 1(a)), a mixed culture of three strains (PC1, PC18 and PC24) was studied. Initially, the percentage of PC1 cells increased and those of PC18 and PC24 decreased (Fig. 7). The behaviour of the PC24 population indicated that this strain was not active in degradation of phenol, cresols and dimethylphenols during the first 10 days of incubation. Between days 10 and 15 PC1 abundance decreased and that of PC24 increased (Fig. 7). The third peak in abundance of strain PC1 and the respective decrease in abundance of PC24 was observed on the 25th day. These changes show that relative proportions of bacterial strains having *meta* (PC1) and *ortho* (PC24) pathways and were co-ordinated in a microcosm consortium throughout the experiment. The shifts in the composition of mixed population in leachate microcosms indicated that different pathways of metabolism of aromatics dominated in different stages of decomposition of pollutants.

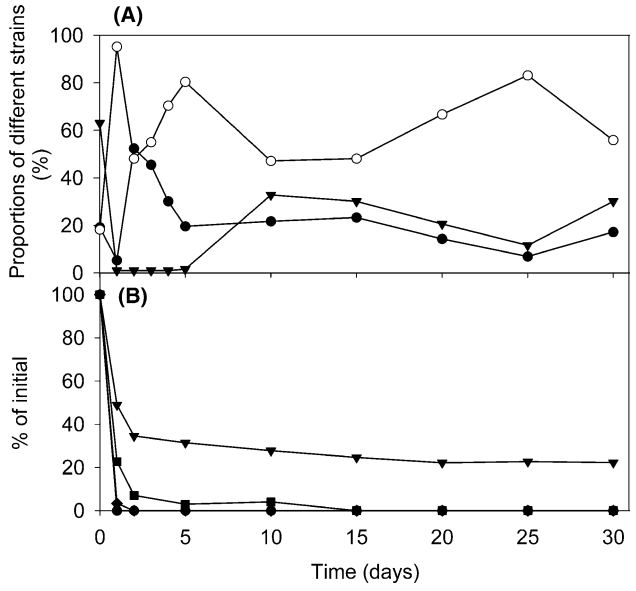

Fig. 7. Relative percentage of each population (○, PC1; ●, PC18 and ▼, PC24) in mixed culture during incubation for 30 days incubation (panel A) and biodegradation curves for phenolic compounds: phenol (●), *m*- and *p*-cresol (◆), *o*-cresol (■) and dimethylphenols (▼) in leachate microcosm (panel B). For strains PC1 and PC18, a C23O gene probe, and in case of the strain PC24, a *pheA* gene probe were used for hybridisation of colonies. At each time point at least 200 colonies were studied. Experiments were repeated with three microcosm cultures and results from a typical experiment from one culture are presented.

4. Discussion

Bacterial growth on pollutant mixtures is an important aspect of bioremediation. In our previous work, interactions between phenol- and *p*-cresol-degrading bacteria in mixtures undergoing biodegradation were described [25]. As reported by Reardon et al. [33] interactions between bacterial species in mixed cultures may be substrate-dependent and cannot be predicted by simple competition models. Phenol- and oil-degrading mixed cultures used in this study comprised four strains (PC1, PC18, PC20, PC24) with known complementary degradative capabilities (defined consortia).

It has been shown that the phenol-degrading strains used have physiological and genetic differences [24]. All of these strains assimilated phenol and *p*-cresol but not *m*-toluate [24]. In addition, strain PC18 is able to degrade salicylate and strain PC20 salicylate and naphthalene. Strains PC1 and PC18 use *meta* cleavage of catechol during growth on phenol. In strain PC20 both *meta* and *ortho* cleavage are induced with phenol and in strain PC24 phenol induces only *ortho* pathway, whereas both strains contain the *pheBA* operon. In strains PC1 and PC20 *p*-cresol is degraded via *meta* cleavage of catechol and in strains PC18 and PC24 via protocatechuate using the *ortho* pathway [24]. To understand interactions within a pollutant-degrading bacterial consortium in situ, functionally dominant populations were identified using analysis of the DNA isolated from laboratory microcosm samples. Molecular techniques are increasingly used in bioremediation studies and Watanabe and coworkers [8] used a combination of molecular and microbiological methods to detect and characterise dominant phenol-degrading bacteria in activated sludge. TGGE and DGGE analyses of 16S rRNA- and LmPH-encoding genes have been used to study functional activity and structural fluctuations of bacterial consortia in a microcosm [9].

In this study, DGGE analysis of PCR products of 16S rRNA genes and of the gene encoding the LmPH showed a shift in composition of bacterial populations during incubation for 30 days on phenolic leachate and crude oil. DGGE analysis of PCR products of 16S rRNA genes showed that *P. mendocina* PC1 became dominant and *P. fluorescens* PC20 disappeared after incubation of a mixed population in leachate microcosms for 30 days (Fig. 1(a) and (b)). Disappearance of a particular population (PC20) from the leachate microcosm and maintenance of the same population in the naphthalene-contaminated oil microcosms throughout the entire experiment indicates that changes in bacterial consortia largely depend on substrate properties. In contrast, no dominant population was detected in oil-amended microcosms by DGGE. Phenol-degrading strain PC20 used in this study is able to metabolise naphthalene. This strain is interesting because it has two large plasmid replicons, a conjugative naphthalene plasmid and a smaller plasmid encoding the degradative *ortho* pathway with *pheBA* operon (data not shown). We believe that the presence of the naphthalene plasmid in the strain PC20 can cause persistence of this strain in oil-amended microcosms. It is likely that carbon sources other than PAHs contributed to microbial growth in phenolic leachate microcosm.

Strain PC20 is most likely unable to degrade phenol in a phenolic leachate microcosm because variable phenol concentrations may affect selection of phenol-degrading bacteria with different kinetic properties [34]. Information on the rate of pollutant biodegradation can be obtained by determination of the half saturation constant, K_s. High concentrations of phenol promote expression of phenol degradation activities with high K_s (low affinity), while strains with low K_s (high affinity) are enriched in the case of low phenol concentrations in the medium [35]. Additionally, high concentrations of phenol cause inefficient growth of low-K_s bacteria, which are outcompeted by high-K_s bacteria [36]. Strains used in this study exhibit kinetically different catabolic activities and have different affinities for phenol. Strain PC1 grows rapidly on phenol and its K_s value for phenol is almost one order of magnitude lower than that of the strain PC20 (data not shown).

To understand the catabolic significance of the strains used in bioremediation of pollutant mixtures, bacterial consortium structure was characterised using catabolic genes as markers. A similar approach has been used to analyse the diversity of functionally dominant populations by Watanabe and Hamamura [5]. Although the C23O-specific primers are often used for identification of dominant bacteria in degradation of one- and two-ringed aromatic hydrocarbons, [19,21–23], detection of phenol-degrading genes was used to characterise catabolic activity in microcosms. Different cleavage pathways were determined among the strains studied using primers specific for multicomponent phenol hydroxylase (LmPH) (Fig. 2) and single component phenol monooxygenase (PheA) genes (Fig. 7). It has been previously reported that multicomponent phenol hydroxylases are predominant in bacteria isolated from phenol-polluted area [37]. The multicomponent phenol hydroxylases are classified into two types according to genetic organisation of the operons: *dmp* type [38] and *mop* type [14], linked to genes for C23O or catechol 1,2-dioxygenase, respectively. In strains PC1 and PC18 used in this study initial phenol degradation is performed by multicomponent phenol hydroxylase into catechol, the aromatic ring of which is further cleaved by C23O. In contrast, in strains PC24 and PC20 the *pheBA* operon encoding phenol degradation and *ortho* pathway is induced [24]. Phenol hydroxylase (PH) and C23O genes from strains PC1 and PC18 were constantly present in phenolic leachate microcosms for 30 days. A PH + C23O-positive strain PC18 dominated in oil-amended microcosms, possibly because of the ability of this strain to degrade salicylate, an intermediate in naphthalene degradation. Accumulation of intermediates in a mixed culture may stimulate enrichment of bacteria that can further metabolise these substrates [39]. In oil-amended microcosms, C23O-positive bacterial populations decreased 20 days after the inoculation, possibly due to disappearance of growth-promoting substrates. The observed fluctuations in the composition of microcosm consortia appear to be driven by carbon and energy sources liberated from crude oil. Wikström et al. [19] also detected C23O in various soil types and recorded a relationship between the abundance of this gene and PAHs concentration.

Molecular monitoring of genes coding for catabolic enzymes of pollutant degradation pathways can show which population has major importance in a specific polluted ecosystem. The behaviour of the strains PC1 and PC18, in which *meta* pathway is induced by phenol, is not very similar in phenolic leachate microcosms (Fig. 7). Throughout this study, the *pheBA* operon (*ortho* pathway) containing strain PC24 fluctuates differently, possibly through qualitative and quantitative changes in chemical composition of degrading pollutants. Fluctuations in catabolic activity studied using *pheA* and C23O genes as markers suggest that the activity of each strain in a mixed culture is regulated within a specific time scale. It is well known that *meta* and *ortho* ring fission of phenolic compounds are alternative pathways for a single strain and that pollutants are degraded faster under mixed culture conditions. In this case commensal interactions between different species support degradation.

Intermediates of a catabolic pathway of one strain (e.g., strain PC18) may be further degraded by another strain (e.g., strain PC24) possessing suitable catabolic pathway. Additionally, in strain PC18, catechol *meta* and protocatechuate *ortho* pathways are induced with phenol and *p*-cresol, respectively [25], and this strain can decompose dimethylphenols in the presence of phenol and *p*-cresol [4].

The planktonic growth form in aqueous suspensions represents just one possible survival strategy of microorganisms. A likely advantage of the alternative strategy, formation of biofilms [7], is higher availability of nutrients. Many pollutant-degrading bacteria are known to secrete biosurfactants that increase aqueous concentration of naphthalene, indicating that a microorganism can promote solubilisation of its substrate [40]. Despite this fact the majority of microorganisms live in biofilms, in both nutrient-rich and oligotrophic environments [41]. Among the strains used in this study, PC18 grows mostly in aggregated forms in oil-amended microcosm. The functional activity in oil-amended microcosms and flexibility of using different contaminants show that this strain may be effective in bioremediation. It is important to examine biodegradation of pollutants at their low concentrations, because due to their poor solubility in water, they may often not be degraded.

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