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ORIGINAL ARTICLE

Application of the knowledge-based approach to strain selection for a bioaugmentation process of phenanthrene- and Cr(VI)-contaminated soil

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Abstract

Aims: The objective of this study was to apply the knowledge-based approach to the selection of an inoculum to be used in bioaugmentation processes to facilitate phenanthrene degradation in phenanthrene- and Cr(VI)-co-contaminated soils.

Methods and Results: The bacterial community composition of phenanthrene and phenanthrene- and Cr(VI)-co-contaminated microcosms, determined by denaturing gradient gel electrophoresis analysis, showed that members of the *Sphingomonadaceae* family were the predominant micro-organisms. However, the Cr(VI) contamination produced a selective change of predominant *Sphingomonas* species, and in co-contaminated soil microcosms, a population closely related to *Sphingomonas paucimobilis* was naturally selected. The bioaugmentation process was carried out using the phenanthrene-degrading strain *S. paucimobilis* 20006FA, isolated and characterized in our laboratory. Although the strain showed a low Cr(VI) resistance (0·250 mmol l^{-1}); in liquid culture, it was capable of reducing chromate and degrading phenanthrene simultaneously.

Conclusion: The inoculation of this strain managed to moderate the effect of the presence of Cr(VI), increasing the biological activity and phenanthrene degradation rate in co-contaminated microcosm.

Significance and Impact of the Study: In this study, we have applied a novel approach to the selection of the adequate inoculum to enhance the phenanthrene degradation in phenanthrene- and Cr(VI)-co-contaminated soils.

Introduction

Soils contaminated with both metals and organic pollutants are considered difficult to remediate. In co-contaminated sites, metal toxicity inhibits the activity of organic-degrading micro-organisms (Said and Lewis 1991). Numerous studies have shown the acute effects of heavy metals on microbial communities. Results from these studies indicated that heavy metal amendments have a negative impact, resulting in severe reduction of metabolic activity, microbial biomass and bacterial diversity (Sandrin and Maier 2003; Ibarrolaza *et al.* 2009). Therefore, bioaugmentation has been considered an alternative treatment for metal-contaminated soil (Roane *et al.* 2001).

Several studies have explored the possibility of obtaining metal-resistant organic-degrading micro-organisms. In view of the fact that such microbes are difficult to recover by enrichment techniques (Fernandes *et al.* 2008), dual bioaugmentation (co-inoculation of a metal-resistant microbial population with an organic-degrading population) (Roane *et al.* 2001; Fernandes *et al.* 2008) and the use of genetically engineered micro-organisms (Brim *et al.* 2006) have been considered in bioaugmentation strategies to enhance the bioremediation process of co-contaminated soils. In all these approaches, the strain selection has been

based on a single criterion: degradation ability, with little or no consideration given to other essential features that are required to be functionally active or persistent in target habitats (Thompson *et al.* 2005).

Thompson *et al.* (2005) suggested an alternative approach called knowledge-based approach to improve microbial selection. This approach bases the initial selection step on *a priori* knowledge of the ubiquity, population dynamics and spatial and temporal distribution of microbial community in the habitats sampled. Once abundant populations have been identified, the second phase of the selection procedure should then be to identify strains that can degrade the target contaminants. Currently, with the technical advances in molecular methods for undertaking more thorough assessment of microbial communities, this knowledge-based approach is becoming increasingly more feasible (Thompson *et al.* 2005).

In a previous work, we demonstrated that in soil contaminated with 2000 mg kg^{-1} of phenanthrene the presence of Cr(VI) at concentrations of 25, 50 and 100 mg kg^{-1} had a negative effect on the phenanthrene elimination rate (Ibarrolaza *et al.* 2009). The objective of this study was to apply the knowledge-based approach to polycyclic aromatic hydrocarbon (PAH)-degrading Cr(VI)-resistant strain selection and to determine the efficacy of this strain in a bioaugmentation process to facilitate phenanthrene degradation in a phenanthrene- and Cr(VI)-co-contaminated soil microcosm.

Materials and methods

Selection of the bacterial inocula

The soil selected for the study was uncontaminated soil from an area near La Plata City, BA, Argentina. The soil showed the following physicochemical properties: the texture was a clay loam, a pH of 5·8, 50 mg kg^{-1} hydrocarbons (GC-FID) and a Cr(VI) concentration of <0·7 mg kg^{-1}.

Soil microcosms consisted of 2 kg of sieved soil (2 mm mesh) in a glass container with a capacity of 5 kg. Three treatments were carried out: 'C' uncontaminated soil, 'F' contaminated with 2000 mg of phenanthrene per kg of dry soil and 'FCr' contaminated with 2000 mg of phenanthrene and 100 mg of Cr(VI) per kg of dry soil. One treatment contaminated with phenanthrene (2000 mg kg^{-1}) and Cr(VI) (100 mg kg^{-1}) was amended with $HgCl_2$ (1·5% w/w) for the determination of abiotic processes (abiotic control). Two microcosms of each treatment were prepared. Cr(VI) in solution as K_2CrO_4 [10 mg of Cr(VI) per ml] was evenly dispersed in soil microcosms, and the phenanthrene was delivered in an acetone solution. The soil was manually mixed with a spatula. The microcosms were incubated at 24 ± 2°C (regional weather conditions) in darkness for 123 days. The microcosms were aerated every week by manual mixing, and the moisture content of the soil was corrected when necessary to 20 ± 2% by the addition of distilled water.

Chemical analysis

Two soil subsamples (25 g) of each microcosm were collected at 3, 12, 25, 38, 50, 75 and 120 days of treatment. They were mixed with anhydrous sodium sulfate (25 g), and hydrocarbons were extracted for 6 h with n-hexane in a Soxhlet apparatus; n-hexadecane (Merck, Schuchardt, Germany) was added as the internal standard. The phenanthrene concentration in the soil samples was quantified by GC-FID (Vecchioli *et al.* 1997).

Three soil subsamples of 1·5 g of each Cr(VI)-contaminated microcosm were extracted by shaking for 16 h with distilled water (10 ml) to measure the water exchangeable Cr(VI) fraction (WEF) (Ibarrolaza *et al.* 2009). Soluble Cr(VI) was determined at 2, 4, 8, 9, 11, 15, 18, 21, 35, 58 and 62 days of treatment. The extracts were centrifuged and filtered. The amount of soluble Cr(VI) was determined spectrophotometrically using the diphenylcarbazide (DPC) assay (Clesceri *et al.* 1998).

Microbial enumeration and biological activity

The most probable number of PAH-degrading bacteria was determined in 96-well microtiter plates according to Wrenn and Venosa (1996), after 0, 7, 15, 21, 31,50, 75, 110 and 125 days of treatment. A mixture of four PAH was used as carbon source (10 g phenanthrene, 1 g anthracene, 1 g fluorene and 1 g dibenzothiophene per l). The plates were incubated at 24 ± 1°C for 3 weeks.

Dehydrogenase activity (reduction of 2,3,5-triphenyl-2H-tetrazoliumtrichloride, TTC, to triphenyl formazan, TPF), usually related to the cell density of viable microorganisms and their oxidative capability, was determined from six soil subsamples of each treatment, as described by Thalman (1968), at 6, 13, 20, 27, 34, 66 and 125 days of treatment.

Genetic diversity

To investigate the changes in the genetic diversity of the soil microbial communities, denaturing gradient gel electrophoresis (DGGE) was performed for the C, F and FCr soil microcosms at different treatment times. The total DNA was extracted from four soil subsamples of 1 g of each soil microcosm at 12, 38, 75 and 123 days of treatment as described by Kuske *et al.* (1997). The DNA pellets obtained were suspended in 250 μl of TE buffer, with humic acid

contaminants removed using Genomic-tips (20/G) by Qiagen Inc. (Chatsworth, CA, USA). Genetic diversity analysis of the soil microcosm bacterial community was performed at every sampling point by PCR amplification of bacterial 16S rDNA fragments followed by DGGE. The 16S rDNA was amplified using eubacteria primers GC-341F (5′-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCCTCCTACGGGAGGCAGCAG-3′) and 907R (5′-CCGTCAATTCCTTTGAGTTT-3′) (Muyzer *et al.* 1998). The PCRs contained 1 μl of DNA, 1 U of AmpliTaq, the manufacturers' recommended buffer as supplied with the polymerase enzyme, 200 ng μl^{-1} of BSA, 0·2 mmol l^{-1} dNTPs and 0·5 μmol l^{-1} of each primer in a total reaction volume of 50 μl. Amplification was performed on a Mastercycler® Eppendorf thermocycler (Eppendorf, Wesseling-Berzdorf, Germany) using a step-down PCR. The programme included an initial denaturation step at 95°C for 4 min, the first cycle step at 94°C for 30 s, 62°C for 45 s, at 72°C for 1 min (10 cycles), followed by a step down at 94°C for 30 s, 57°C for 45 s and 72°C for 1 min (25 cycles). The final extension was carried out at 72°C for 10 min. The PCR products were analysed by agarose gel electrophoresis and purified using a QIAquick PCR Purification kit (Qiagen Inc.). DGGE was performed on a DGGE-2401 apparatus (C.B.S Scientific Co., Del mar, CA, USA). The purified PCR amplicons were resolved in 6% (w/v) polyacrylamide gels (acrylamide-*N*,N_0-methylenbisacrylamide, 37·5 : 1). The amounts of PCR products loaded in the DGGE gels were standardized (5–7 μg per well). The gel contained a linear gradient of 40–70% denaturant [100% denaturant corresponds to 7 mol l^{-1} urea and 40% (v/v) formamide]. Electrophoresis was performed in TAE buffer (pH 8·1) (40 mmol l^{-1} Tris, 20 mmol l^{-1} acetic acid, 1 mmol l^{-1} Na_2EDTA) at a temperature of 60°C. A prerun at 50 V for 30 min was followed by the main run at a constant voltage of 100 V for 16 h. The postelectrophoresis gel was stained for 30 min with Sybr Gold and documented with a Universal Hood II gel documentation system (Bio-Rad, Hercules, CA, USA).

Nucleotide sequence determination

Dominant DGGE bands were excised under UV illumination using a sterile scalpel blade, and the excised bands were briefly washed in 0·5 ml sterile Millipore H_2O. The gel sliver was then crushed with a pipette tip in 0·1 ml fresh sterile buffer and incubated at 4°C for 48 h to allow passive diffusion of the band DNA. The released DNA was then re-amplified as above with primers 341-F and 907-R. The PCR products were analysed by agarose gel electrophoresis and purified using a QIAquick PCR Purification kit. The purified amplicons were cloned into the pGEM-T Easy cloning vector (Promega, Madison, WI, USA). For each excised band, single colonies containing inserts were selected at random, and these inserts were sequenced. Nucleotide sequences were determined by sequencing service, Macrogen, Seoul, Korea. Nucleotide sequences were compared with those in the National Center for Biotechnology Information GenBank database using BLAST program. Phylogenetic tree was performed using the Molecular Evolutionary Genetics Analysis package (MEGA ver. 4.0; Tamura *et al.* 2007). The sequences were aligned with the CLUSTALW function, and neighbour-joining phylogenetic tree was constructed with the Jukes–Cantor algorithm. The robustness of the phylogeny was tested by bootstrap analysis with 500 iterations.

The nucleotide sequences obtained in this study have been deposited in the GenBank database under accession no. HM047771–HM047787.

Characterization of the selected strain

Sphingomonas paucimobilis strain 20006FA was isolated and identified previously in our laboratory from a soil microcosm contaminated with phenanthrene (Coppotelli *et al.* 2008). Its phenanthrene-degrading activity has been widely studied (Coppotelli *et al.* 2010).

Minimal inhibitory concentration (MIC) of chromate

Cells pre-exposed and not pre-exposed to Cr(VI) were evaluated. Flasks, containing 10 ml of R3 medium (Reasoner and Geldreich 1985) supplemented with different concentrations of Cr(VI) (as K_2CrO_4) (0; 0·4; 0·8; 1·6; 3·2; 6·5; 13; 26 and 52 mg l^{-1}) and pH adjusted to 7·0, were inoculated with 50 μl of an 0·5 McFarland Scale strain suspension prepared from a fresh overnight culture grown in R3 medium. Flasks without metal were used as controls. All flasks were incubated at 28°C, and the bacterial growth was monitored by optical density analysis at 600 nm for 48 h. The MIC was determined as the one in which no growth occurred at 72 h of incubation.

Growth studies in presence of Cr(VI)

Sphingomonas paucimobilis 20006FA was cultivated aerobically at 28°C in R3 medium (pH 7·0) or in R3 medium amended with 6·5 mg l^{-1} of Cr(VI) (as K_2CrO_4) by shaking (350 rev min^{-1}) for 72 h. An aliquot of this starter culture was used to inoculate fresh R3 broth containing either 0 or 6·5 mg l^{-1} of Cr(VI), giving an initial optical density at 600 nm (OD_{600}) of 0·05. The cells were grown for 12 h. These cultures were used to inoculate new fresh R3 medium and R3 medium amended with 6·5 mg l^{-1} of

Cr(VI). A R3 medium amended with 6·5 mg l^{-1} of Cr(VI) and noninoculated was used as abiotic Cr(VI) reduction control. All cultures were carried out in triplicate. The amount of residual Cr(VI) in the medium was quantified spectrophotometrically at a wavelength of 540 nm using the 1, 5-DPC method at different times for 24 h. The cells were previously separated by centrifugation and filtration. Additionally, the residual total Cr concentration in the medium was determined by atomic absorption spectrophotometry (Shimazdu AA6650F; Shimadzu Corporation, Kyoto, Japan).

The capacity of the strain *S. paucimobilis* 20006FA to degrade phenanthrene and to reduce Cr(VI) simultaneously was determined in liquid mineral medium (MML) (Vecchioli *et al.* 1990) supplemented with 200 mg l^{-1} of phenanthrene, as the sole source of carbon and energy, in the presence of different Cr(VI) concentration (0; 0·4; 1·6; 3·2 and 6·5 mg l^{-1}). The media were inoculated with a Cr(VI)-pre-exposed culture of *S. paucimobilis* 20006FA, as described earlier. All cultures were carried out in triplicate. After 96 h of incubation, the residual amount of Cr(VI) in the supernatant was determined spectrophotometrically using the DPC assay, and the residual concentration of phenanthrene was quantified by reversed-phase high-pressure liquid chromatography (HPLC) using a Waters® chromatograph with a Symmetry Waters® C18 column (15 cm × 4·6 mm i.d., bead size 5 μm, pore size 100 Å, Waters Corp., Milford, MA). A linear gradient of 15 mmol l^{-1} phosphoric acid in nanopure water solution and methanol (20 : 80 to 15 : 95, v/v) over 15 min and a flow rate of 1 ml min^{-1} was used.

Bioaugmentation process

An inoculated microcosm (I-FCr) was carried out in duplicate trays. The I-FCr was contaminated with 2000 mg of phenanthrene and 100 mg of Cr(VI) per kg of dry soil and inoculated with $1{\cdot}4 \times 10^8$ CFU of *S. paucimobilis* strain 20006FA per gram of dry soil at days 3, 10 and 17. The inoculum was cultured in R3 medium at 28°C for 48 h. Cells were harvested by centrifugation, and the pellet was washed twice in 0·85% NaCl solution (SF). The pellet was re-suspended in SF, and optical density at 600 nm was adjusted to 5. The cell density of the inoculum was measured by dilution plating on R2A. A volume of this suspension was added to inoculated microcosm to obtain the desired inoculum density.

The microcosm was incubated and monitored by chemical analysis, microbial enumeration, biological activity and genetic diversity, as described earlier.

Results

Selection of the bacterial inocula

To investigate the changes in the genetic diversity and dynamics of the microbial communities under the influence of phenanthrene and phenanthrene and Cr(VI), DGGE was performed for the C, F and FCr soil microcosms at different times of treatment (Fig. 1).

Clear differences were found between the DGGE patterns of C, F and FCr microcosms. The DGGE patterns of the F microcosm indicated a remarkable dominance of

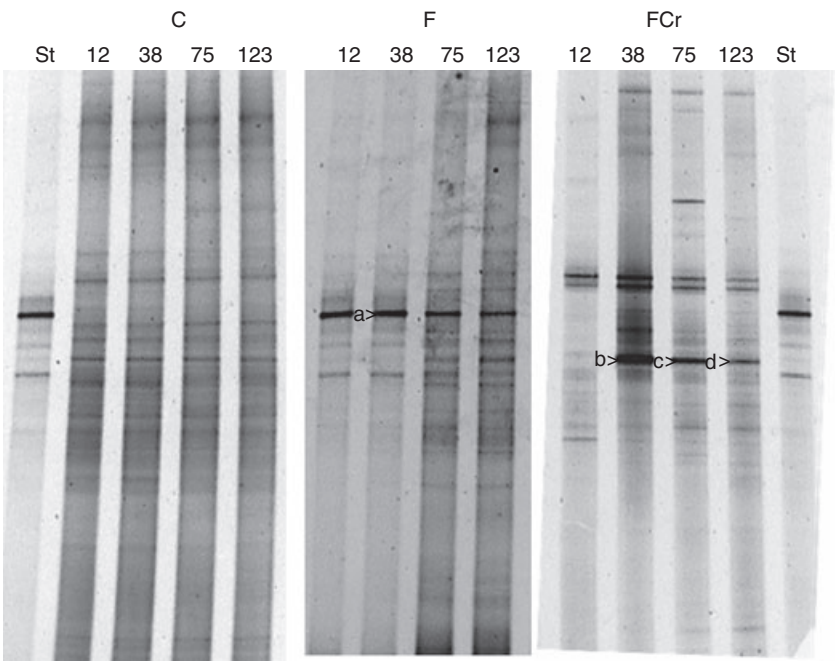

Figure 1 PCR-DGGE analysis of bacterial populations in C, F and FCr soil microcosm samples at 12, 38, 75 and 123 days of treatment. DGGE, denaturing gradient gel electrophoresis.

Table 1 Percentage of phenanthrene elimination of F and FCr microcosms during bioremediation process. Results are means of four determinations in two independent experiments

	% of phenanthrene elimination	
Day	F microcosm	FCr microcosm
12	46	8
38	91	76
75	98	95

one band (a) (Fig. 1) from the early stage of the experiment (day 12), when the 46% of the incorporated phenanthrene had been eliminated (Table 1), until 123 days of treatment.

In the FCr microcosm, although the DGGE patterns at day 12 of treatment showed the appearance of some intense bands, this microbial community may not be related to phenanthrene degradation because only the 8% of the incorporated phenanthrene had been eliminated before that time. After 38 days of treatment, and when the FCr microcosm showed a high phenanthrene-degrading activity (Table 1), the DGGE profile showed the appearance of a very intense band (b) (Fig. 1), which remained until the end of the treatment (bands c and d) (Fig. 1).

The dominant bands in the DGGE profiles of F and FCr microcosms, at different times, were excised, re-amplified and cloned, and partial 16S rRNA gene sequences were determined. Phylogenetic analysis revealed that in both microcosms, F and FCr, the predominant bands showed a phylogenetic relationship with the *Sphingomonadaceae* family. However, DGGE profile results showed that the addition of Cr(VI) produced a selective change of predominant *Sphingomonas* species (Fig. 2), whereas the sequences of the dominant bands present in FCr microcosm (b, c and d) matched closely with that of *S. paucimobilis*, in F microcosm, dominant band (a) sequence matched closely with that of *Novosphingobium* sp.

In our laboratory, a strain of *S. paucimobilis*, labelled 20006FA, had been isolated from a soil microcosm contaminated with phenanthrene and selected for its ability to grow in different PAHs (anthracene, phenanthrene and dibenzothiophene) as sole carbon and energy source. Its phenanthrene-degrading activity, the cell ability to promote bioavailability of phenanthrene (by biosurfactant excretion and adhesion to the phenanthrene crystals) and the capacity of the strain to establish itself in phenanthrene-contaminated soil were previously described (Coppotelli *et al.* 2008, 2010). However, the chromate resistance and chromate reduction capacity of *Sphingomonas* sp. have not been well documented as yet.

Characterization of *Sphingomonas paucimobilis* 20006FA

Sphingomonas paucimobilis 20006FA showed a MIC of chromate of 13 mg l^{-1} of Cr(VI) (72 h of incubation). However, when the strain was previously exposed to

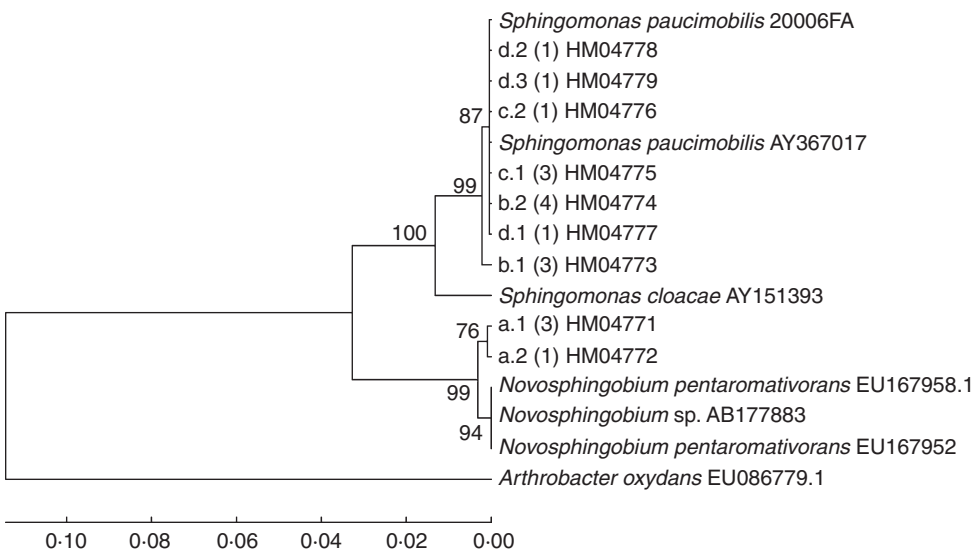

Figure 2 Neighbour-joining tree based on a distance matrix analysis of 16S rRNA gene sequences of denaturing gradient gel electrophoresis bands. *Arthrobacter oxydans* was used as outgroup. Sequences were aligned with ClustalW. The scale indicates the Jukes–cantor distances. The numbers at each node correspond to the bootstrap per cent values. The numbers of clones with identical sequence are indicated in parentheses. The GenBank accession numbers are indicated.

Cr(VI), MIC was 26 mg l^{-1} of Cr(VI), indicating that the level of resistance can be increased by metal exposure.

The total Cr and Cr(VI) concentrations in the supernatant of chromate-pre-exposed culture of *S. paucimobilis* 20006FA in R3 broth containing 24 mg l^{-1} of K_2CrO_4 [6·5 mg l^{-1} of Cr(VI)] were determined. After 24 h of incubation, the remaining total Cr and Cr(VI) concentrations were 5·413 ± 0·109 and 1·336 ± 0·120 mg l^{-1}, respectively, corresponding to 62·74 ± 1·79% of Cr(VI) reduction. The total Cr and Cr(VI) concentrations determined in the control were 6·422 ± 0·276 and 6·120 ± 0·057 mg l^{-1}, respectively, showing an abiotic Cr(VI) reduction of 4·63 ± 3·83%.

The cultures of *S. paucimobilis* 20006FA growing in MML supplemented with phenanthrene and Cr(VI) showed that, after 96 h of incubation, the Cr(VI) was completely reduced, in all assayed concentrations, reaching residual concentration below the detection limit (0·063 mg l^{-1}), with the concomitant degradation of phenanthrene. The degradation of phenanthrene was complete (final concentration <3 mg l^{-1}) with added Cr(VI) in concentration below 3·2 mg l^{-1}. Higher Cr(VI) concentration inhibited significantly the phenanthrene degradation, which reached only the 19% of supplemented phenanthrene, with added Cr(VI) in concentration of 6·5 mg l^{-1}.

As a result of the clear predominance in FCr soil microcosms of a band whose sequence showed the highest similarity to 16S rRNA gene sequence of *S. paucimobilis* 20006FA strain (Fig. 1) and to phenanthrene degrading (Coppotelli *et al.* 2010) and Cr(VI) reduction capacities found in this strain, *S. paucimobilis* 20006FA was selected for a bioaugmentation process in soil microcosms cocontaminated with phenanthrene and Cr(VI).

Bioaugmentation process

Chemical analysis

The concentrations of phenanthrene (Phe) during the treatment in contaminated microcosms are shown in Fig. 3a. The abiotic control showed a final phenanthrene concentration of 1850 mg kg^{-1}, indicating that the phenanthrene was removed mainly by microbial degradation. Phenanthrene biodegradation was modelled using a three-parameter logistic function described by the following equation: $(\mathrm{Phe} = \mathrm{Phe}_0/(1 + (t/t_{1/2})^b)$, where t is the treatment time, Phe is the phenanthrene concentration, b is the slope parameter and $t_{1/2}$ is the phenanthrene half-life (Table 2). The adjustment of the equation was obtained by nonlinear regression (SigmaPlot for Windows; Systat software, Inc., San Jose, CA). The rate of phenanthrene elimination (dPhe/dt *vs* t) was estimated by differentiation of the previous equation (Fig. 3b).

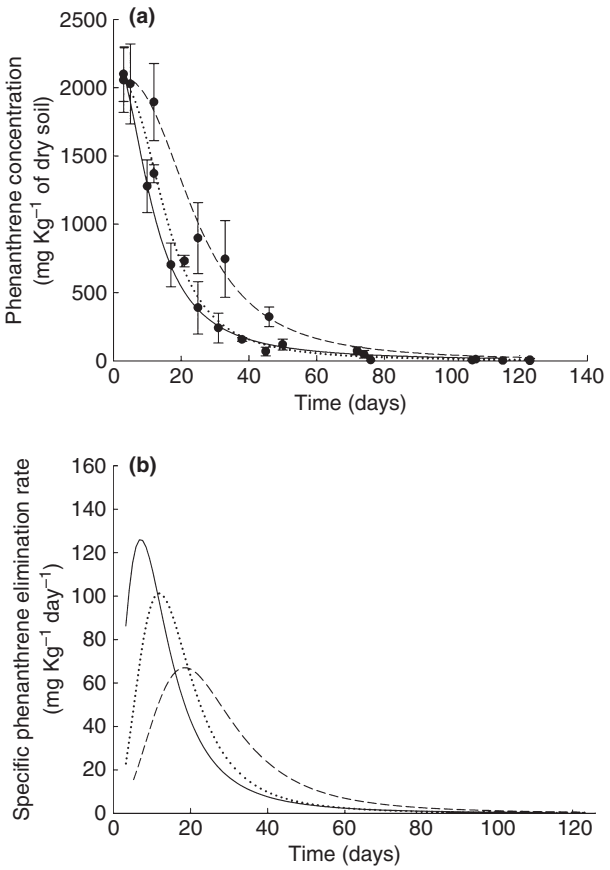

Figure 3 (a) Concentration of phenanthrene in the F (●), FCr (▲) and I-FCr (■) microcosms during bioremediation process. Results are means of four determinations in two independent experiments. (b) Phenanthrene degradation rates of F (-), FCr (- - - -) and I-FCr (· · · ·) microcosms were calculated by differentiation of the phenanthrene depletion curves.

Table 2 Model parameters of phenanthrene degradation in F, FCr and I-FCr microcosms

Microcosms	r	$t_{1/2}$ (day)	b (mg kg^{-1} day^{-1})
F	0·9996	11·66 ± 0·39	2·08 ± 0·12
FCr	0·9965	24·46 ± 2·25	2·75 ± 0·62
I-FCr	0·9986	15·85 ± 0·94	2·70 ± 0·37

r, Correlation coefficient; b, slope parameter; $t_{1/2}$, phenanthrene half-life.

When F and FCr results are compared, it can be seen that the presence of Cr(VI) caused a significant effect on phenanthrene biodegradation parameters, an increase in $t_{1/2}$ for phenanthrene biodegradation (Table 2) and a decrease in maximum rate of phenanthrene degradation (Fig. 3b).

The addition of *S. paucimobilis* 20006FA to the I-FCr microcosm managed to moderate partially the effect of

the presence of Cr(VI) on $t_{1/2}$ for phenanthrene biodegradation (Table 2) and maximum rate of phenanthrene degradation (Fig. 3b). In spite of the fact that the strain was inoculated from the third day of treatment, the maximum degradation rate in the I-FCr microcosm was reached after 14 days of treatment (after the second inoculation) and later than in F microcosm (Fig. 3b).

The Cr(VI) concentration in soil (WEF) quickly decreased, and consequently, after 14 days of treatment, it was below 10 mg kg^{-1} in all Cr(VI)-contaminated soil microcosms (Fig. 4); the inoculation with *S. paucimobilis* 20006FA did not produce a significant change in the Cr(VI) elimination pattern.

Enumeration of PAH-degrading bacteria

Figure 5 shows that the FCr microcosm showed a delayed response in the PAH-degrading bacteria with respect to the F microcosm. The inoculation with the strain *S. paucimobilis* 20006FA resulted in a clear increase in the number of PAH-degrading bacteria in the I-FCr microcosm in comparison with the F and FCr soil microcosms, during inoculation period (21 days) (Fig. 5).

Dehydrogenase activity

Figure 6 shows the dehydrogenase activity determined by analysing the reduction of TTC to TPF in the C, F, FCr and I-FCr microcosms. In contaminated microcosms, the addition of phenanthrene as the co-contamination with phenanthrene and Cr(VI) produced an initial inhibitory effect, with dehydrogenase levels below the control values.

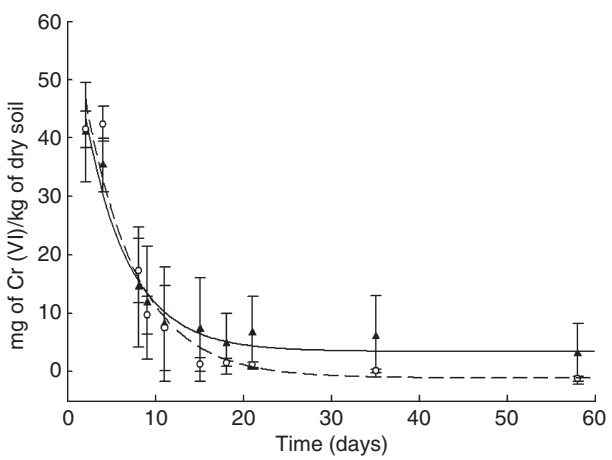

Figure 4 Concentration of Cr(VI) in the water-soluble fraction of FCr and I-FCr microcosms. Results are means of six determinations in two independent experiments. Bars represent standard deviations. (–▲–, ——) FCr; (–○–, - - -) I-FCr.

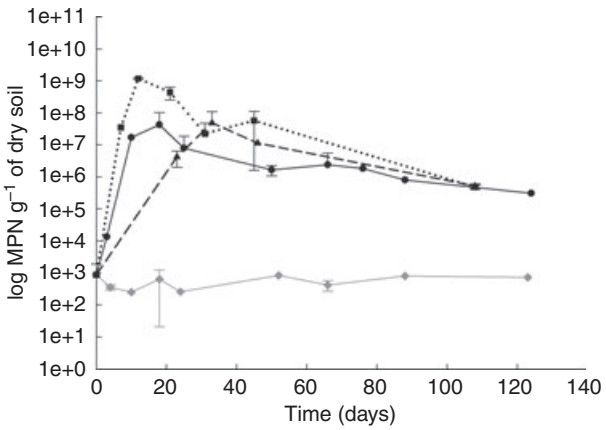

Figure 5 PAH-degrading bacteria of C (◇), F (●), FCr (▲) and I-FCr (■) microcosms. Results are means of four determinations in two independent experiments. Bars represent standard deviations.

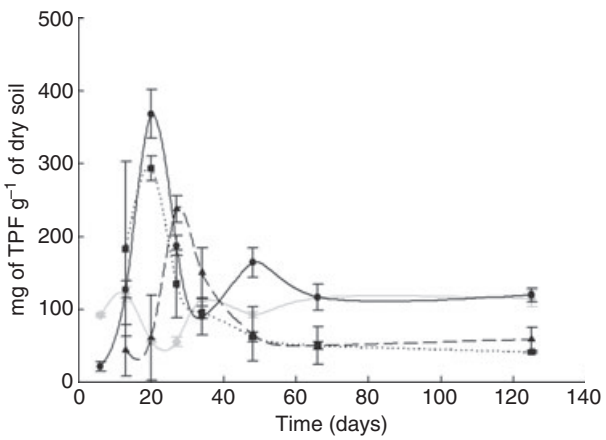

Figure 6 Dehydrogenase activity of C (◇), F (●), FCr (▲) and I-FCr (■) microcosms during the bioremediation process. Results are means of six determinations in two independent experiments. Bars represent standard deviations.

A subsequent stimulatory effect was observed in F, FCr and I-FCr microcosms.

The F soil microcosm presented the highest dehydrogenase activity values, and its maximum level was observed after 20 days of treatment. FCr microcosm had an initial inhibitory phase, and its maximum dehydrogenase activity value occurred after 30 days of treatment. In I-FCr microcosm, the presence of the inoculum notably corrected the inhibitory effects caused by the presence of Cr(VI). As a result, it did not show an inhibitory phase and its maximum dehydrogenase activity value occurred after 20 days, and it was significantly higher than that observed in FCr microcosm. However, the maximum value of I-FCr microcosm was lower than that observed in F microcosm.

At the end of the experiment, the F microcosm reached dehydrogenase activity values that were not different from the control microcosm; in contrast, a persistent inhibitory effect on dehydrogenase activity was observed in all Cr(VI)-contaminated microcosms (FCr and I-FCr).

Genetic diversity

To investigate the changes in the genetic diversity under the influence of the co-contamination with phenanthrene and Cr(VI) and the inoculation with *S. paucimobilis* 20006FA, DGGE was carried out for the I-FCr microcosms at different times of treatment. For a better understanding, Fig. 7 shows the DGGE profiles of FCr and I-FCr microcosms.

Clear differences were found between DGGE patterns of FCr and I-FCr microcosms. The DGGE pattern of I-FCr microcosm during the inoculation period (day 12) showed that the inoculation protocol drastically reduced the number of bands compared with the FCr microcosm (Fig. 7). A recovery in the number of bands of I-FCr microcosm occurred after 38 days of treatment. However, FCr and I-FCr microcosms did not share any bands, except for the predominant one.

Phylogenetic analysis revealed that the predominant band in the I-FCr microcosm from the beginning of the experiment (day 12) until 123 days of treatment clustered with the strain *S. paucimobilis* 20006FA, indicating that the inoculated strain remained as a dominant population during the whole experiment. Clearly, the inoculation protocol used influenced the natural selection process, which took more than 12 days in the FCr microcosm (Fig. 7), producing the early predominant establishment of the phenanthrene-degrading strain *S. paucimobilis* 20006FA.

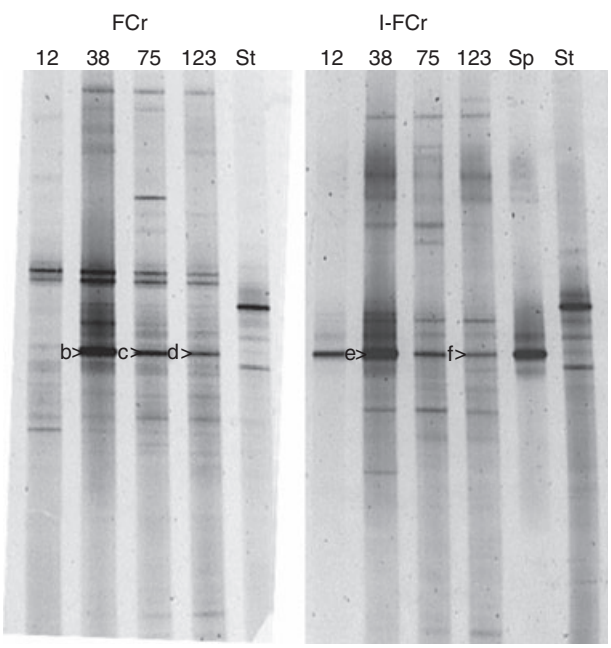

Figure 7 PCR-DGGE analysis of bacterial populations in F, FCr and I-FCr soil microcosm samples at 12, 38, 75 and 123 days of treatment and *Sphingomonas paucimobilis* 20006FA (Sp). DGGE, denaturing gradient gel electrophoresis.

Discussion

Bioaugmentation of contaminated soil might be considered to be a straightforward approach; yet the use of this technology has not become common in part because of their limited success (Pandey *et al.* 2009). Several authors attribute bioaugmentation failure to the classic method, enrichment and isolation used to obtain pollutant-degrading micro-organisms (Bouchez *et al.* 2000; El Frantroussi and Agathos 2005; Singer *et al.* 2005). The ecological background constitutes a major obstacle to obtain a successful bioremediation performance of such an inoculum, and the best way to overcome these obstacles is to look for micro-organisms from the same ecological niche as the polluted area (El Frantroussi and Agathos 2005; Thompson *et al.* 2005).

This study has applied the knowledge-based approach proposed by Thompson *et al.* (2005) to the selection of the adequate inoculum to enhance the phenanthrene degradation in phenanthrene- and Cr(VI)-co-contaminated soils.

In a previous work, we had demonstrated that the presence of Cr(VI) did modulate the community response to phenanthrene (Ibarrolaza *et al.* 2009). This is clearly visible in the DGGE profiles shown in Fig. 1. However, both communities (F and FCr) showed phenanthrene-degrading activity (Table 1), and the only difference between them is the presence of Cr(VI), the DGGE profiles of F and FCr microcosms did not share any clearly predominant band. For the phylogenetic analysis, the intense bands that appeared during the period of phenanthrene-degrading activity, after 12 and 38 days of treatment for F and FCr microcosms, respectively, were selected. Bands of high intensity are commonly found in samples where substantial microbial activity has been detected and represent the populations that are more competitive under the selective conditions used (Nakatsu *et al.* 2005). In both, F and FCr microcosms, the predominant bands showed a phylogenetic relationship with the *Sphingomonadaceae* family. *Sphingomonas* are a major group of PAH-degrading bacteria present at many contaminated sites and can be considered important biocatalysts for soil bioremediation (Leys *et al.* 2004). However, DGGE profile results showed that the addition

of Cr(VI) produced a selective change of predominant *Sphingomonas* species.

The strain *S. paucimobilis* 20006FA is able to grow with phenanthrene as sole source of carbon and energy, showing different mechanisms to enhance phenanthrene bioavailability (Coppotelli *et al.* 2010). The capacity of the strain to establish itself in phenanthrene-contaminated soil was previously described (Coppotelli *et al.* 2008). According to a direct comparison of MIC value of *S. paucimobilis* 20006FA with those of strains reported by other authors (Srinath *et al.* 2001; Viti *et al.* 2003), it might be defined as Cr(VI) sensitive; however, it was capable of reducing chromate. This is in agreement with the data of Ishibashi *et al.* (1990) and Viti *et al.* (2003), who reported chromate-sensitive strains that reduced chromate efficiently.

The inoculation of the strain *S. paucimobilis* 20006FA in a phenanthrene- and Cr(VI)-co-contaminated microcosm (I-FCr) stimulated the phenanthrene biodegradation (Fig. 3), in comparison with the FCr microcosm, reducing the phenanthrene half-life (Table 2). This is in agreement with DGGE profile results that showed how the inoculation protocol caused an early establishment of the strain in the I-FCr microcosms (Fig. 7) and an initial increase in the number of PAH-degrading bacteria (Fig. 5). However, and despite the fact that the inoculation of the strain seemed to rectify the initial inhibitory effects that the presence of Cr(VI) produced on dehydrogenase activity (Fig. 6), the inoculation of the strain did not produce changes either in the Cr(VI) elimination pattern (Fig. 4) or in the late inhibitory effect on dehydrogenase activity observed in all Cr(VI)-contaminated microcosms (Fig. 6). These results would suggest that, independently of the establishment of the *S. paucimobilis* 20006FA strain and its Cr(VI) elimination capacities, other biotic and abiotic factors govern Cr(VI) behaviour. An explanation may be that the energy requirements to maintain concurrent metal reduction and organic degradation are too high, and the introduced organism cannot perform both activities under environmental conditions (Roane *et al.* 2001).

In spite of the fact that the inoculation with *S. paucimobilis* 20006FA managed to moderate the effect of the presence of Cr(VI) on phenanthrene degradation activity, in the I-FCr microcosm, the maximum rate of phenanthrene degradation was reached after 14 days of treatment (Fig. 3b), later than in F microcosms. However, at the beginning of the treatment, the I-FCr microcosms showed higher PAH-degrading bacteria counts than F microcosm (Fig. 5), and the DGGE patterns of I-FCr microcosm showed an early predominant establishment of the strain. The behaviour observed in the I-FCr microcosm could be partially explained by the inhibitory effect of Cr(VI) on phenanthrene-degrading activity of *S. paucimobilis* 20006FA, determined in cultures of the strain in MML supplemented with phenanthrene and different Cr(VI) concentrations. After 10 days of treatment, around 95% of incorporated Cr(VI) was eliminated from the WEF (Fig. 4), the second inoculation was performed, and by this time, the I-FCr microcosm showed the maximum phenanthrene degradation rate, but it never reached the degradation rate values of F microcosm. On the other hand, in the FCr microcosm, the maximum phenanthrene degradation rate was reached after 20 days of treatment (Fig. 3b), when more than 90% of Cr(VI) had been eliminated from the WEF that occurred with the simultaneous increase in the PAH-degrading bacteria counts (Fig. 5).

We emphasize that using the classic approaches for strain selection, the strain *S. paucimobilis* 20006FA would be rejected as inoculant in phenanthrene- and Cr(VI)-contaminated soil, because of its low Cr(VI) resistance. Furthermore, the few reports found in literature about Cr(VI) tolerance of *Sphingomonas* sp. showed a high susceptibility of *S. paucimobilis* KPS01 to Cr(VI) (Tada and Inoue 2000) and the low Cr(VI) reduction capacity of *S. paucimobilis* EPA 505 (Shen *et al.* 1996). However, the knowledge-based approach applied in this study was successful in demonstrating that, in the presence of Cr(VI), the composition of the soil bacterial community during the phenanthrene biodegradation showed the predominance of a population phylogenetically related to *S. paucimobilis*, encouraging the authors to study the Cr(VI) reduction capacity of the strain *S. paucimobilis* 20006FA and to select this strain for the bioaugmentation assays. The inoculation of this strain, with the protocol used in this study, managed to facilitate the degradation of phenanthrene in a phenanthrene- and Cr(VI)-co-contaminated soil.

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