Biodegradation (2009) 20:95–107
DOI 10.1007/s10532-008-9203-5

ORIGINAL PAPER

Dynamics of microbial community during bioremediation of phenanthrene and chromium(VI)-contaminated soil microcosms

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Received: 14 September 2007 / Accepted: 11 June 2008 / Published online: 5 July 2008

Abstract The combined effect of phenanthrene and Cr(VI) on soil microbial activity, community composition and on the efficiency of bioremediation processes has been studied. Biometer flask systems and soil microcosm systems contaminated with 2,000 mg of phenanthrene per kg of dry soil and different Cr(VI) concentrations were investigated. Temperature, soil moisture and oxygen availability were controlled to support bioremediation. Cr(VI) inhibited the phenanthrene mineralization (CO_2 production) and cultivable PAH degrading bacteria at levels of 500–2,600 mg kg^{-1}. In the bioremediation experiments in soil microcosms the degradation of phenanthrene, the dehydrogenase activity and the increase in PAH degrading bacteria counts were retarded by the presence of Cr(VI) at all studied concentrations (25, 50 and 100 mg kg^{-1}). These negative effects did not show a correlation with Cr(VI) concentration. Whereas the presence of Cr(VI) had a negative effect on the phenanthrene elimination rate, co-contamination with phenanthrene reduced the residual Cr(VI) concentration in the water exchangeable Cr(VI) fraction (WEF) in comparison with the soil microcosm contaminated only with Cr(VI). Clear differences were found between the denaturing gradient gel electrophoresis (DGGE) patterns of each soil microcosm, showing that the presence of different Cr(VI) concentrations did modulate the community response to phenanthrene and caused perdurable changes in the structure of the microbial soil community.

Keywords Bioremediation · Phenanthrene · Chromium · Microbial community

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Introduction

Large quantities of organic and inorganic compounds are released into the environment every year as a result of human activities (Viñas et al. 2005). Bioremediation of contaminated sites relies on the immense metabolic capacities of the microbial world for the transformation of pollutants into essentially harmless or at least less dangerous compounds (El Fantroussi and Agathos 2005).

Soil contamination is a typical side-effect of industrial activity (Viñas et al. 2005). Soils are complex, multi-component systems with a range of different types of contaminants co-existing in different physical and chemical forms. Despite this complexity, organic contaminants and toxic metals are frequently studied separately and their interactions, as well as the effects that remediation procedures may have on both type of contaminants, have been neglected (Amezcua-Allieri et al. 2005).

Polycyclic aromatic hydrocarbons (PAH) are pollutants that are widely distributed in the ecosphere as a result of fossil fuel combustion and as by-products of industrial activities (Déziél et al. 1996). These ubiquitous organic pollutants exhibit strong carcinogenic and toxic properties (Maliszewska-Kordybach and Smreczak 2003). It is estimated that more than 90% of the total burden of PAH resides are in the surface soils, where most of these compounds accumulate (Wild and Jones 1995). The microbial degradation of PAH has received a great deal of attention as a possible strategy for the bioremediation of PAH-contaminated soils (Johnsen et al. 2002). However, soils contaminated with PAH often contain high levels of other pollutants such as heavy metals, which are often derived from the same sources as PAH (Maliszewska-Kordybach and Smreczak 2003).

Among the heavy metals, chromium is a priority pollutant due to the toxicity and carcinogenicity of its hexavalent form [Cr(VI)] (Kouretev et al. 2006). Cr(VI) is also toxic to the microbial community present in the soil and it could inhibit the biodegradation of organic pollutants in co-contaminated sites (Said and Lewis 1991). Cr(VI) in soils can be reduced chemically or through the activity of soil microorganisms (Kamaludeen et al. 2003). The microbial reduction of hexavalent chromium to relatively less toxic and less soluble trivalent forms seems to be a potential method for the remediation of Cr(VI)-contaminated soils (Krishna and Philip 2005).

In the study described here our objectives were to examine the combined effect of phenanthrene and Cr(VI) on soil microbial activity and soil microbial community composition, and to assess the influence of each pollutant on the bioremediation processes of the other component. Soil microcosms contaminated with 2,000 mg of phenanthrene per kg of dry soil and different Cr(VI) concentrations were studied.

Materials and methods

Soil

The soil selected for the study was uncontaminated soil from an area near La Plata City, Argentina. The soil was analyzed in the Laboratory of Soil Science at the University of La Plata and showed the following physicochemical properties: the texture is a clay loam, a pH of 5.8, 3.60% organic carbon, 6.21% soil organic matter, 2,960 mg kg^{-1} total nitrogen, 4.2 mg kg^{-1} available phosphorus, 50 mg kg^{-1} hydrocarbons and a Cr(VI) concentration of less than 0.7 mg kg^{-1}.

Biometers

The effects of the Cr(VI) concentration on phenanthrene-induced mineralization and cultivable bacterial populations were preliminary determined in biometer flasks (Bartha and Pramer 1965). Six systems contaminated with different Cr(VI) concentrations (incorporated as K_2CrO_4) (0, 25, 50, 500, 1,300 and 2,600 mg kg^{-1} of dry soil) were prepared. Three replicates of each system were placed into the biometer flasks (50 g of dry soil). The flasks were incubated at 24 ± 2°C. After one week all biometer systems were contaminated with 2,000 mg of phenanthrene (Carlo Erba, Milano, Italy, >99.5% purity) per kg of dry soil.

The CO_2 production during the course of 60 days of treatment was determined. The CO_2 produced was trapped in 10 ml of 0.6 M KOH. Periodically the KOH was replaced by fresh KOH solution. The removed KOH was titrated to the phenolphthalein endpoint with standard HCl.

The mean and standard deviation of triplicate independent experiments were calculated. The mean values were compared by parametric one way ANOVA test at the level of $P \leq 0.05$. All statistical analysis were performed using the software 5.0 Systat® under Windows® (Evanston. IL, USA).

Soil microcosms

Soil microcosms consisted of 2 kg of sieved soil (2 mm mesh) in a glass container with a capacity of 5 kg. The Cr(VI) concentrations for the preparation of the microcosms were chosen according to the results obtained in the biometer systems. Concentrations that did not produce marked inhibitory effects on phenanthrene-induced mineralization and on the counts of cultivable bacterial populations were chosen. Six soil microcosms were carried out in duplicate trays: "C" uncontaminated soil; "F" contaminated with 2,000 mg of phenanthrene per kg of dry soil; "Cr100": contaminated with 100 mg of Cr(VI) per kg of dry soil; "F-Cr25", "F-Cr50" and "F-Cr100"

contaminated with phenanthrene (2,000 mg kg^{-1}) and 25, 50 and 100 mg kg^{-1} of Cr(VI), respectively. In addition, one soil microcosm contaminated with phenanthrene (2,000 mg kg^{-1}) and Cr(VI) (100 mg kg^{-1}) was amended with $HgCl_2$ (1.5% wt wt^{-1}) (Song et al. 1990) for the determination of abiotic processes (abiotic control).

Cr(VI) in solution as K_2CrO_4 (10 mg of Cr(VI) per ml) was evenly dispersed in soil microcosms. The phenanthrene was delivered in an acetone solution and mixed into the soil manually with a spatula. The microcosms were incubated at 24 ± 2°C (regional climate conditions) in the dark for 130 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

A soil sample (25 g) was 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. During the first month of the experiment the determinations were performed weekly and thereafter at 50, 75 and 120 days. Three samples (25 g) from each soil microcosm were analyzed at each sampling time.

The phenanthrene concentration in the soil samples was quantified by GC-FID. A Perkin-Elmer autosystem gas chromatograph equipped with a flame ionization detector was used with nitrogen as the carrier gas. The injection port was maintained at a temperature of 280°C and the detector at a temperature of 300°C. The oven was set at 50°C (initial time 4 min), then raised to 150°C (rate 4°C min^{-1}) and to 280°C at a rate of 10 °C min^{-1} (final time 15 min). A fused-silica capillary column PVMS/54 (50 m × 0.25 mm i.d.) was used. Data acquisition and handling was computer-assisted (PE NELSON Model 1022) (Vecchioli et al. 1997)

The amount of soluble Cr(VI) was determined spectrophotometrically using the diphenylcarbazide assay (Clesceri et al. 1998). Samples of 1.5 g of each soil microcosm contaminated with Cr(VI) were extracted by shaking for 16 h with distilled water (10 ml) to measure the water exchangeable Cr(VI) fraction (WEF).

Microbial enumeration and biological activity

Cultivable bacterial counts were determined after 4, 18, 38 and 120 days of treatment from soil microcosms and at the end of experiment (60 days) from biometer systems.

For this purpose, 10 g (wet mass) of soil sample suspended in 100 ml of 0.85% NaCl was homogenized for 30 min on a rotary shaker (250 rpm). Samples (0.1 ml) of 10-fold dilutions were spread on plates containing R2A-agar medium (Reasoner and Geldreich 1985) in order to count cultivable heterotrophic bacteria, and on R2A-agar supplemented with 500 mg l^{-1} of Cr(VI) as K_2CrO_4 to count cultivable Cr(VI)-resistant heterotrophic bacteria (Bader et al. 1999). The agar plates were incubated at 24 ± 2°C for 10 days. The MPN of PAH-degrading bacteria was determined in 96-well microtiter plates according to Wrenn and Venosa (1996). 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 for 3 weeks at 20 ± 2°C.

Dehydrogenase activity (reduction of 2,3,5-triphenyl-2*H*-tetrazoliumtrichloride, TTC, to triphenyl formazan, TPF), usually related to the cell density of viable microorganisms and their oxidative capability, was determined from the soil microcosm systems as described by Thalman (1968). The determinations were performed weekly during the first 34 days and thereafter at 66 and 125 days. Three samples (5 g) from each microcosm were analyzed at each sampling time. The influence of the added phenanthrene and/or Cr(VI) on the enzymatic activity was determined by subtracting the TPF formation obtained in the control microcosm from the TPF formation obtained in the contaminated microcosms.

Genetic diversity

In order to investigate the changes in the genetic diversity of the soil microbial community caused by phenanthrene contamination, Cr(VI) contamination and co-contamination with phenanthrene and Cr(VI), DGGE was performed for the C, Cr100, F, F-Cr25, F-Cr50 and F-Cr100 soil microcosms at different treatment times.

The total DNA was extracted from 2 g soil aliquots from each soil microcosm after 38, 75 and

123 days of treatment, as described by Kuske et al. (1997). The DNA pellets obtained were suspended in 100 µl of TE buffer, with humic acid contaminants removed using Genomic-tips (20/G) from Qiagen (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 (denaturing gradient gel electrophoresis).

The 16S rDNA was amplified using eubacteria primers GC-341F (5′-CGCCCGCCGCGCCCCGC GCCCGGCCCGCCGCCCCCGCCCCCTCCTACGG GAGGCAGCAG-3′) and 907R (5′-CCGTCAATTCC TTTGAGTTT-3′) (Muyzer et al. 1998)

The PCR reactions contained 1 µl of DNA, 1 U of AmpliTaq, the manufacturers' recommended buffer as supplied with the polymerase enzyme, 200 mM of BSA, 0.2 mM dNTPs and 20 pM of each primer in a total reaction volume of 50 µl. Amplification was performed on a Progene FPROGO5Y thermocycler (Techne, Burlington, NY, USA) using a step-down PCR. The programme included an initial denaturation step for 4 min at 95°C, the first cycle step at 94°C for 30 s; 62°C for 40 s; and 1 min at 72°C (10 cycles), followed by a step down of 30 s at 94°C, 40 s at 57°C; and 72°C for 1 min (25 cycles). The final extension was carried out at 72°C for 10 min. The PCR products were analyzed by agarose gel electrophoresis and purified using a QIAquick PCR Purification Kit (Qiagen Inc., Chatsworth, CA, USA).

DGGE was performed on a BioRad D GENE System (BioRad, Munich, Germany). The purified PCR-amplicons were directly applied onto 6% (wt vol-1) polyacrylamide gels (acrylamide-N,N′-methylenbisacrylamide, 37.5:1). The gel contained a linear gradient of 30–65% denaturant (100% denaturant corresponds to 7 M urea and 40% (vol vol^{-1}) formamide). Electrophoresis was performed in 1 × TAE buffer (40 mM Tris (pH 8.1), 20 mM acetic acid, 1 mM Na_2EDTA) at a temperature of 60°C. A pre-run at 50 V for 30 min was followed by the main run at a constant voltage of 100 V for 16 h. The post-electrophoresis gel was stained for 1 h with ethidium bromide and analyzed using a GelComparII software package (Applied Maths, Kortrijk, Belgium). Similarity matrixes of the banding patterns were made with the Dice equation and the dendrogram was calculated by the Unweighted Pair Group Method with Arithmetic mean (UPGMA) (Sokal and Michener 1958).

Results

Effects of Cr(VI) concentration on phenanthrene-induced mineralization and cultivable bacterial populations

During the first 7 days of incubation, when only Cr(VI) solution had been added to biometer systems (B-FCr), all systems showed an increase in the CO_2 production rate and this was followed by a decrease (Fig. 1). The cumulative CO_2 evolved during this period was significantly higher ($P < 0.05$) in all Cr(VI)-contaminated systems compared with the reference system (B-F). The initial rates of CO_2 production were undistinguishable regardless of the level of Cr(VI) contamination (Fig. 1). Only the highest Cr(VI) concentration (B-FCr2600 system) showed different dynamics, with a lag phase of 4 days before the increase in the CO_2 production rate.

After 7 days of treatment, when systems were spiked with phenanthrene, the B-FCr25, B-FCr50 and B-FCr500 had reached a basal respiration rate and the CO_2 production rate in B-FCr1300 and B-FCr2600 had started to decrease. Phenanthrene mineralization was detected in B-F, B-FCr25 and B-FCr50 systems 18 days after phenanthrene incorporation and the respiration rate of B-FCr25 and B-FCr50 systems was not different from that of the B-F system. In the B-FCr500 system the respiration rate did not increase until 35 days after the addition of phenanthrene and a significant reduction in phenanthrene-induced mineralization was observed. In the B-FCr1300 and B-FCr2600 systems phenanthrene mineralization was not detected at any time during the experiment.

In accordance with the inhibitory effect on phenanthrene-induced mineralization, the numbers of cultivable bacteria populations in the B-FCr500, B-FCr1300 and B-FCr2600 systems were significantly lower ($P < 0.05$) than those in B-F, B-FCr25 and B-FCr50 (Table 1). In particular, the number of PAH-degrading bacteria in the systems contaminated with the highest Cr(VI) concentration was below the detection limits of the method employed.

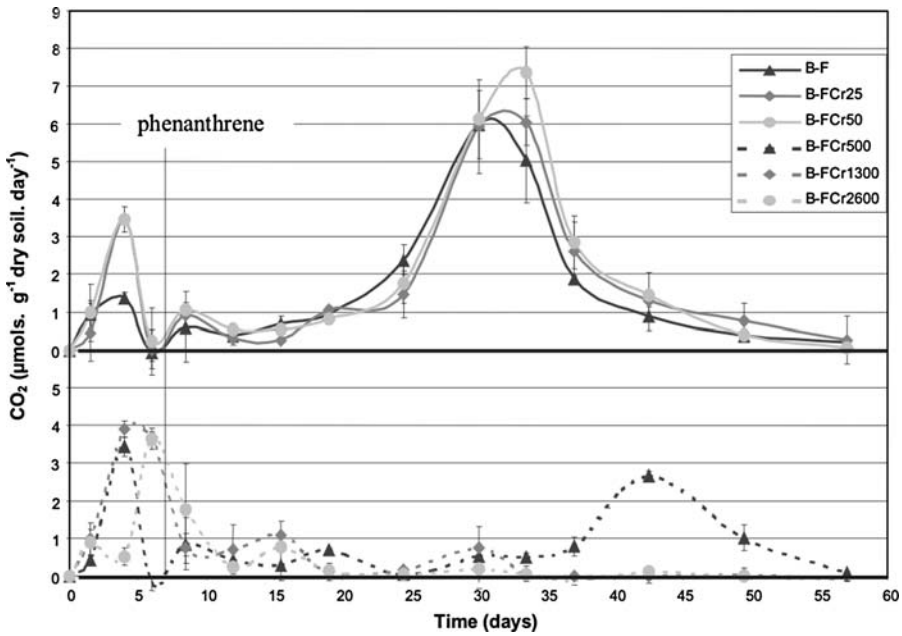

Fig. 1 CO_2 production rate of F, F-Cr25, F-Cr50, F-Cr500, F-Cr1300 and F-Cr2600 biometers flask systems during 60 days of incubation. Results are means of triplicate independent experiments. Bars represent standard deviations. The time of phenanthrene incorporation are indicating

Table 1 Counts of different populations of cultivable bacteria of B-F, B-FCr25, B-FCr50, B-FCr500, B-FCr1300 and B-FCr2600 biometers flask systems after 60 days of incubation

	Heterotrophic bacteria log CFU g^{-1} dray soil	Cr(VI)-resistant heterotrophic bacteria log CFU g^{-1} dray soil (%)*	PAH-degrading bacteria log MPN g^{-1} dray soil
B-F	7.84 ± 0.02	5.44 ± 0.15 (0.40)	6.04 ± 1.51
B-FCr25	7.79 ± 0.10	6.78 ± 0.25 (9.77)	7.14 ± 1.21
B-FCr50	7.13 ± 0.28	6.27 ± 0.20 (9.35)	4.59 ± 1.15
B-FCr500	6.18 ± 0.05	4.64 ± 0.10 (2.89)	<3
B-FCr1300	6.22 ± 0.54	4.35 ± 0.12 (1.35)	<3
B-FCr2600	5.00 ± 0.20	3.10 ± 0.05 (1.26)	<3

*In parenthesis: percentage of cultivable Cr(VI)-resistant heterotrophic bacteria with regard of the cultivable heterotrophic bacteria population

Each value is the mean of triplicate independent experiments, ± standard deviations are shown

Soil microcosms

Chemical analysis

The concentrations of phenanthrene during the treatment in phenanthrene-contaminated microcosms are shown in Fig. 2. After 65 days of treatment the F microcosm showed an important level of phenanthrene elimination, reaching a phenanthrene concentration below the cleanup standards for soil (50 mg kg^{-1}) (Argentinean National law 24051), which corresponds to the removal of about 98% of the initially supplied phenanthrene.

The F-Cr soil microcosms showed elimination curves that were different from that of the F soil microcosm (Fig. 2). In F-Cr soil microcosms an initial phase with a low elimination rate (lag phase) occurred, but this lag phase was not correlated with the initial Cr(VI) concentration. As a result, the F-Cr100 soil microcosm reached a phenanthrene concentration below the cleanup standards for soil after 85 days of treatment, whereas in the case of microcosms F-Cr25 and F-Cr50 it was necessary over 100 days of treatment to reach that cleanup level. The abiotic control showed an elimination level of 15.5 ± 2.7 % (data not shown), indicating that the phenanthrene was

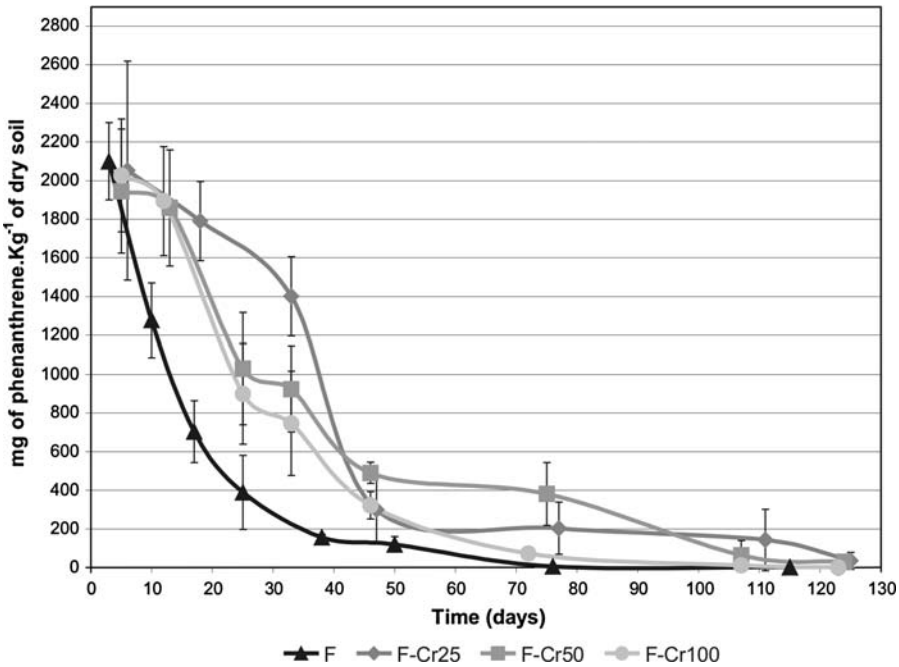

Fig. 2 Concentration of phenanthrene in the contaminated microcosms F, F-Cr25, F-Cr50 and F-Cr100 during bioremediation process. Results are means of duplicate independent experiments. Bars represent standard deviations

removed mainly by microbial degradation even in the presence of Cr.

The Cr(VI) concentration in soil (WEF) readily decreased in all Cr(VI)-contaminated soil microcosms (Fig. 3). After 18 days of treatment, when the Cr(VI) concentration did not show significant changes, residual Cr(VI) concentration in the WEF was 10.08 ± 0.06 mg of Cr(VI) kg^{-1} for the F-Cr100 soil microcosm, 1.14 ± 0.32 mg of Cr(VI) kg^{-1} for the F-Cr50 soil microcosm, and less than 0.73 ± 0.07 mg of Cr(VI) for F-Cr25 soil microcosm kg^{-1}, corresponding to a decrease in the WEF of 89.92 ± 0.06%, 97.71 ± 0.64%, and more than 97.09 ± 0.29% respectively.

The Cr100 soil microcosm curve showed initial Cr(VI) removal behavior that was not different to that of the F-Cr100 soil microcosm (Fig. 3). However, after 18 days of treatment, the percentage of Cr(VI) elimination in WEF of the Cr100 soil microcosm was lower (84.6 ± 2.0%) than that observed for the F-Cr100 soil microcosm, corresponding to residual concentration of 15.4 ± 2.0 mg of Cr(VI) kg^{-1}

The presence of Cr(VI) caused a negative effect on phenanthrene elimination whereas the co-contamination with phenanthrene reduced the residual Cr(VI) concentration in the WEF.

Enumeration of cultivable bacterial populations

The addition of phenanthrene to the soil led to an early increase in the density of cultivable heterotrophic and PAH-degrading bacteria in the F soil microcosm (Fig. 4a and b) and these populations remained higher than in the control soil microcosm until the end of treatment, when more than 98% of the phenanthrene had been eliminated.

The contamination with 100 mg kg^{-1} of Cr(VI) produced an extremely small initial decrease in the number of cultivable heterotrophic bacteria (Fig. 4a). Furthermore, selective enrichment in cultivable Cr(VI)-resistant heterotrophic bacteria was not observed throughout the whole experiment (Fig. 4c), representing only 0.1–5% of the total cultivable heterotrophic bacteria population. Similar percentages were found in the C soil microcosm (0.2–9%).

The F-Cr soil microcosms showed a delayed response in terms of the cultivable heterotrophic and PAH-degrading populations with respect to the F soil microcosm (Fig. 4a and b). However, and in agreement with the phenanthrene elimination results, the response of the measured cultivable bacterial populations did not show a correlation with the initial Cr(VI) concentration. Despite the

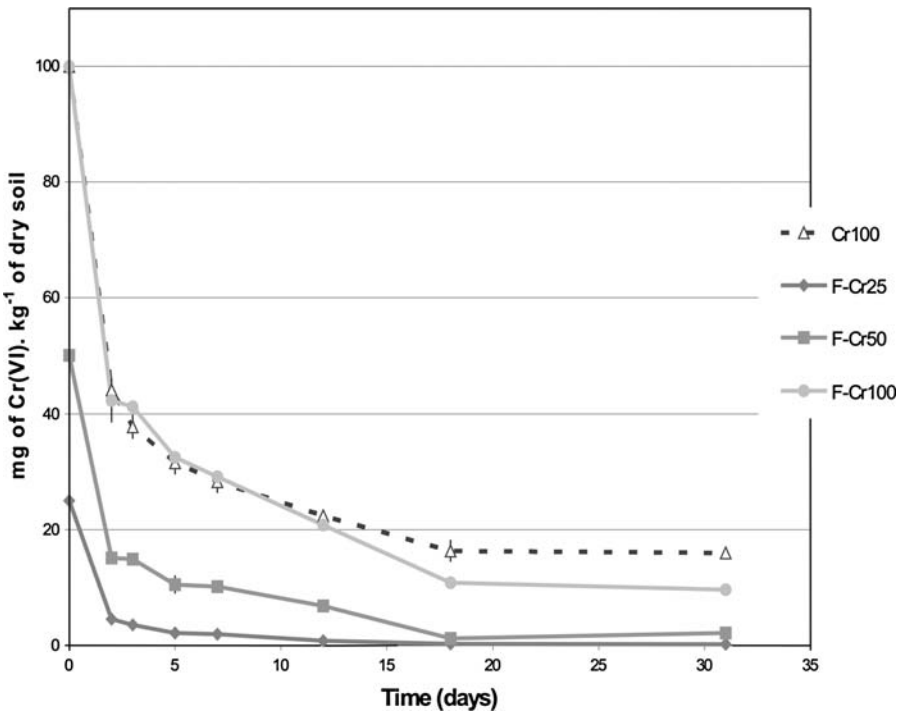

Fig. 3 Concentration of Cr(VI) in the water soluble fraction of Cr-100, F-Cr25, F-Cr50 and F-Cr100 microcosms, during the first 30 days of treatment. Results are means of duplicate independent experiments. Bars represent standard deviations

fact that soil microcosm F-Cr100 showed an initial inhibitory effect on the cultivable heterotrophic population, after 18 days of treatment showed a number of cultivable heterotrophic bacteria (Fig. 4a) and PAH-degrading bacteria (Fig. 4b) significantly higher than in the F-Cr25 and F-Cr50 soil microcosms. In addition, the number of cultivable PAH-degrading bacteria for the F-Cr100 soil microcosm remained higher than that in the F-Cr25 and F-Cr50 soil microcosms throughout the whole experiment.

In the same way as the Cr100 soil microcosm, the F-Cr25 and F-Cr50 soil microcosms did not show a selective enrichment in the cultivable Cr(VI)-resistant heterotrophic population, except for F-Cr25 soil microcosm after 120 days of treatment. The F-Cr100 soil microcosm showed a progressive selection of Cr(VI)-resistant heterotrophic bacteria during the first 38 days of treatment (Fig. 4c).

Dehydrogenase activity

The incorporation of phenanthrene in the F soil microcosm produced an initial inhibitory effect, with levels below the control values during the first 10 days of treatment (Fig. 5). A subsequent stimulatory effect was observed, with the highest enzymatic activity found after 20 days of treatment. The F soil microcosm clearly presented the highest dehydrogenase activity values.

In contrast, the contamination with Cr(VI) of the Cr100 soil microcosm caused a marked inhibitory effect on soil dehydrogenase activity throughout the whole experiment, except for a short period (about 20 days of treatment) when the system showed a relative recovery of its enzymatic activity.

The F-Cr soil microcosms showed similar initial behavior to the F soil microcosm, but with an extended initial inhibitory phase and with lower dehydrogenase activity values into the stimulatory period (Fig. 5). Interestingly, once again the response of the soil microbial community did not show a correlation with the initial Cr(VI) concentration, and the F-Cr100 soil microcosm presented higher dehydrogenase activity values than the F-Cr25 and F-Cr50 soil microcosms.

At the end of the experiment the F soil microcosm reached dehydrogenase activity values that were not significantly different to the control soil, whereas a persistent inhibitory effect on dehydrogenase activity was observed in all Cr(VI)-contaminated soil microcosms.

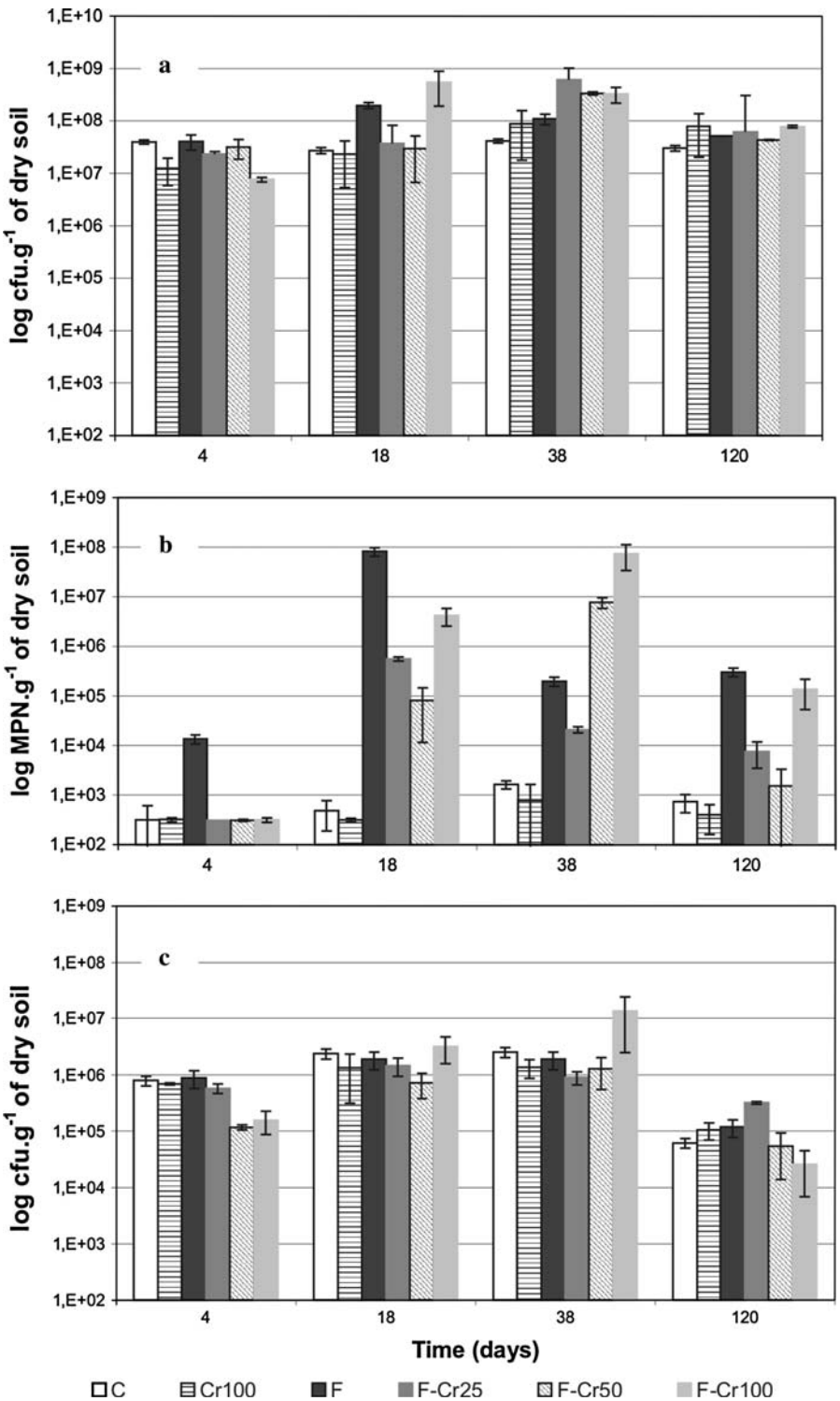

Fig. 4 Culturable bacterial populations in the control and contaminated microcosms at 4, 18, 38 and 120 days of treatment. (**a**) Heterotrophic culturable bacteria (log cfu g^{-1} of dry soil). (**b**) PAH degrading bacteria (log MPN g^{-1} of dry soil). (**c**) Cr(VI)-resistant heterotrophic bacteria (log cfu g^{-1} of dry soil). Results are means of duplicate independent experiments. Bars represent standard deviations

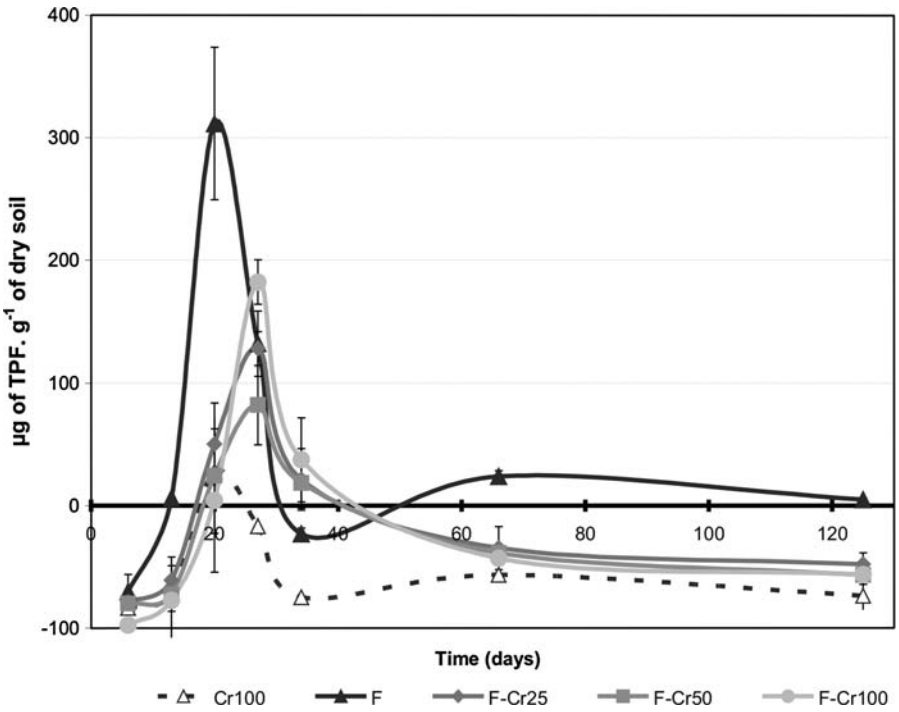

Fig. 5 Changes in net dehydrogenase activity of contaminated microcosms during the bioremediation process. The data are calculated as follows: µg of TPF (triphenyl formazan) per g of dry contaminated soil minus μf of TPF per g of dry control soil. Results are means of duplicate independent experiments. Bars represent standard deviations

Genetic diversity

Two replicates per treatment and per sampling time were analyzed for DNA extraction and there were no difference in DGGE profiles between them, it is for that reason that only one set of results are presented.

The DGGE patterns of the C, Cr100, F, F-Cr25, F-Cr50 and F-Cr100 soil microcosms after 38 days of treatment, at the end of the rapid elimination phase of phenanthrene in the F soil microcosm, are shown in Fig. 6. Visual inspection of the DGGE gel showed the absence of intense bands in the fingerprint of C and Cr100 soil microcosms, whereas contamination with phenanthrene and co-contamination with phenanthrene and Cr(VI) resulted in dramatic changes in the genetic diversity of soil microbial community, with the appearance of some bands with a very high intensity.

Clear differences were found between the DGGE patterns of the F and F-Cr soil microcosms, showing that the presence of different Cr(VI) concentrations did modulate the community response to phenanthrene. An increase in the Cr(VI) concentration led to the disappearance of the most intense bands found in the fingerprint of the F soil microcosm and the appearance of other intense bands.

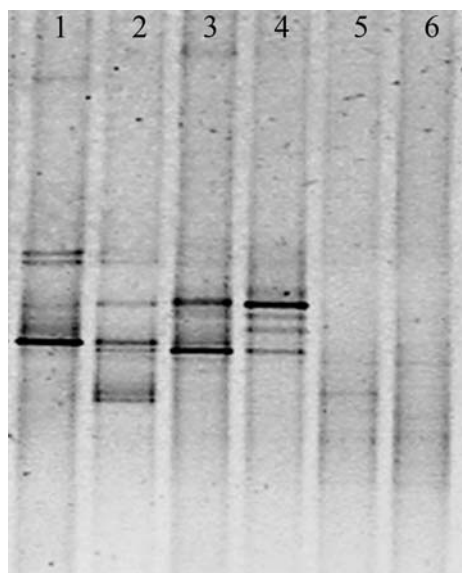

Fig. 6 PCR-DGGE analysis of bacterial populations in soil samples of microcosms after 38 days of treatment. 1: F-Cr100; 2: F-Cr50; 3: F-Cr25, 4: F; 5: Cr100; 6: C

The dendrogram of the UPGMA analysis of the DGGE of different contaminated soil microcosms at different treatment times is represented in Fig. 7. The patterns indicate the changes in microbial community

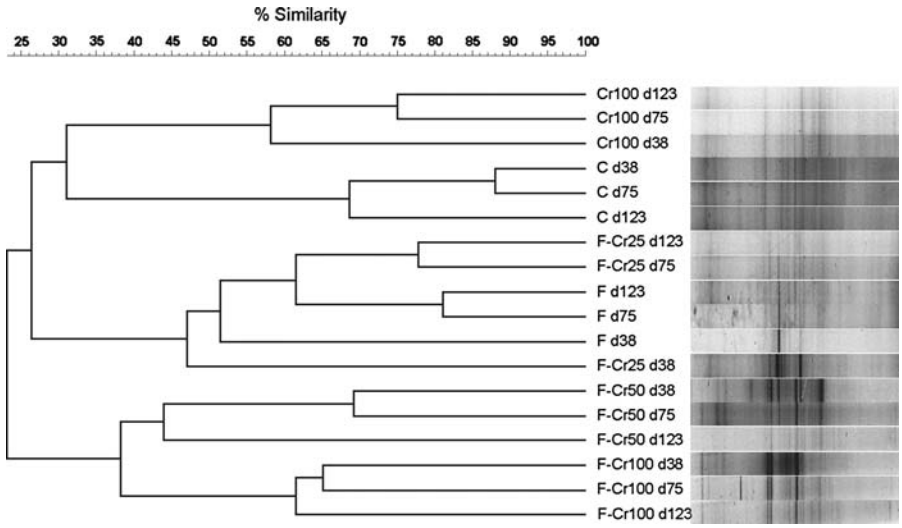

Fig. 7 Drendrogram of the clusters analysis of the DGGE patterns of contaminated microcosms during the bioremediation process, as calculated by using Dice equation and UPGMA. The differences between profiles are indicated by percentage similarity

structure over time. On analyzing the F and F-Cr microcosms, significant and perdurable changes in the microbial community can be seen. In F-Cr50 and F-Cr100 microcosms these changes led to the formation of clusters according to Cr (VI) concentration, regardless of the incubation time. F-Cr50 and F-Cr100 microcosm clusters were clearly differentiated from F microcosm. In contrast, the F-Cr25 microcosm did not different from F microcosm in the same way that the higher Cr(VI) concentrations did. On analyzing the Cr100, F and F-Cr100 microcosms, clusters which were clearly differentiated from each other can be seen.

Discussion

The effects of PAH (Gentry et al. 2003; Castle et al. 2006) and heavy metals (Rasmussen and Sørensen 2001, Dai et al. 2004; Viti and Giovannetti 2005) on composition and activity of soil microbial community have recently been studied. Maliszewska-Kordybach and Smreczak (2003) found that the deleterious influence on soil microorganism activity was more marked in the case of soils contaminated with both groups of these pollutants than in soil amended with heavy metals or PAH alone. However, the impact of the negative effects on the soil microbial community in the degradation of PAH has only recently been emphasized (Sokhn et al. 2001; Wong et al. 2005) and the influence on biological remediation of heavy metals has not been well documented.

In a preliminary experiment on biometer systems we studied the effect of Cr(VI) contamination on phenanthrene-induced mineralization and cultivable bacterial populations in order the obtain some basis for understanding the dynamics of the community. Cr(VI) inhibited phenanthrene mineralization (Fig. 1) and reduced drastically the number of PAH-degrading bacteria (Table 1) in the range 500 to 2,600 mg kg^{-1}. This response was similar to that observed in other studies, where heavy metals generally suppressed the community responses to carbon addition and led to lower carbon mineralization rates and longer lag phases (Nakatsu et al. 2005). Contamination with low concentrations of Cr(VI) (25 and 50 mg kg^{-1}) did not show significant changes in the microbial community response to phenanthrene (Fig. 1). These results are in contrast to the lower Cr(VI) concentrations (10 and 18 mg kg^{-1}) that were needed to inhibit the catabolism of xylene (Nakatsu et al. 2005). However, it is important to note that in our experiments on biometer systems, the phenanthrene was added after 7 days of Cr(VI) contamination. During this 7 day period a significant increase in microbial activity was observed in all F-Cr biometer systems (Fig. 1). In the same time period the soil microcosm experiments showed a fast decrease of Cr(VI) from WEF (Fig. 3). The effects of metals on soil microorganisms depend upon their availability in soil solution (Shi et al. 2002). Cr in Cr(VI)-contaminated soils is mainly present in the fraction bound to organic matter (Han et al. 2004) and Cr(VI) reduction by soil organic matter and biotic mechanisms has been widely reported (Wittbrodt and

Palmer 1996; Kimbrough et al. 1999, Tokunaga et al. 2003). It could be assumed that phenanthrene was incorporated when a large proportion of Cr(VI) had been eliminated of the WEF.

The increase in mineralization rate observed immediately after Cr(VI) addition (Fig. 1) could be due to biotic and or abiotic process. The heterotrophic Cr(VI)-resistant microorganisms could be active at the expense of leaked nutrients through cell lysis from Cr(VI)-sensitive microorganisms, as proposed by other authors (Rajapaksha et al. 2004; Viti et al. 2006), and/or at the expense of partially oxidized organic products from the oxidation of soil organic matter by Cr(VI). In soil microcosms, an increment in cultivable Cr(VI) resistant population during the first days of experiment was not detected (Fig. 4c), discouraging us to doing substantial inference that an early Cr(VI) resistant population was established.

For bioremediation experiments in soil microcosms, when the phenanthrene and Cr(VI) (25, 50 and 100 mg kg^{-1}) were simultaneously incorporated, the degradation of phenanthrene (Fig. 2), dehydrogenase activity (Fig. 5) and the increase in PAH-degrading bacteria counts (Fig. 4b) were retarded. Interestingly, however, these negative effects did not show a correlation with Cr(VI) concentration and, furthermore, the F-Cr100 soil microcosm presented higher PAH degrading bacteria counts (Fig. 4b) and dehydrogenase activity (Fig. 5) than the F-Cr25 and F-Cr50 soil microcosms. It is also interesting that the residual Cr(VI) concentration in WEF was lower in presence of phenanthrene than when it was incorporated alone (Fig. 3). Organic matter enhances the reduction of chromate in soil by increasing microbial activities, by acting as an electron donor and by lowering the O_2 level of the soil through increased microbial respiration, thus creating reducing conditions (Zayed and Terry 2003).

The genetic diversity results showed that whereas the DGGE profiles of the Cr100 soil microcosm did not show any intense bands (Fig. 6), the DGGE profiles of F and F-Cr microcosms showed the presence of bands with high intensities. 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 presence of phenanthrene as a driving force for community changes, the effect of Cr(VI) led to selective shifts in the structure of the phenanthrene-degrading community (Fig. 7), with the most intense bands replaced by others (Fig. 6) demonstrating the functional redundancy of the soil microorganism related with the phenanthrene catabolism. Regardless of the rapid and significant reduction of the Cr(VI) concentration in the WEF and the phenanthrene elimination, co-contamination with phenanthrene and Cr(VI) caused significant and perdurable changes in the structure of the microbial soil community (Fig. 7). These changes were characteristics and different from those produced on soil microbial community by the presence of only one of these pollutants (Fig. 7).

The structures of microbial communities established in the F and F-Cr soil microcosms after 38 days, i.e., the period of active phenanthrene elimination, demonstrated the influence of Cr concentration, with the most dramatic results obtained from 50 mg kg^{-1}. This influence was also observed in the dynamics of PAH-degrading bacteria (Fig. 4b). The most marked increase in PAH-degrading bacteria was detected in F-Cr50 and F-Cr100 soil microcosms after 38 days of treatment. Moreover, the highest Cr(VI) concentration produced the progressive selection of Cr(VI)-resistant heterotrophic bacteria (Fig. 4c) during the period of active phenanthrene elimination. It could be assumed that a different phenanthrene degrading Cr(VI)—resistant community was established at higher Cr(VI) concentration and that community proved to have a major efficiency on phenanthrene degradation activity. The lowest Cr(VI) concentration (25 mg kg^{-1}) allowed the establishment of a community that was more similar to the F soil microcosm (Figs. 6 and 7) but it had a smaller increase in the PAH-degrading population (Fig. 4b). This latter behavior could be attributable to Cr(VI) inhibitory effect, while a more complex mechanism could be governing the dynamic microbial community at higher Cr(VI) concentrations.

The remarkable difference between the microbial community composition of the different F-Cr soil microcosms might be a consequence of the selective pressure of toxic Cr(VI) and the different environments generated by the complex interaction between soil organic matter, microbial activity, a heavy metal with oxidizer properties and the incorporation of a biodegradable molecule.

Conclusions

To our knowledge, this is the first work that studied the influence of different concentrations of heavy metal on the efficiency of bioremediation processes and the bacterial community composition of soil co-contaminated with heavy metal and PAH. This represents an initial study that allowed to demonstrate that the presence of different Cr(VI) concentrations did modulate the community response to phenanthrene. More studies are needed to elucidate the complex mechanisms that govern the response of microbial community to co-contamination with these pollutants.

Acknowledgements This research was partially support by the Agencia Nacional de Promoción Científica y Tecnológica (PICT2004-25300). Ibarrolaza A. and Coppotelli B. are doctoral fellowships of CONICET (Argentine Research Council). Donati E. is research member of CONICET.

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