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Comparative bioaugmentation with a consortium and a single strain in a phenanthrene-contaminated soil: Impact on the bacterial community and biodegradation

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ABSTRACT

The efficiency of two inoculation strategies, using a consortium (CON) or an isolated strain (AM), on phenanthrene-contaminated soil was determined with special concern on the study of the bacterial community composition by PCR-DGGE and pyrosequencing of 16S rRNA gene fragments.

Both strategies stimulated the phenanthrene degradation, increasing the cultivable heterotrophic bacteria number and biological activity. At the end of the treatments, the microcosms inoculated with AM reached the lowest values of phenanthrene but also the lowest dehydrogenase activity.

In DGGE patterns a reduction in number of bands in the contaminated and inoculated microcosms was observed, being the most significant differences attributed to inoculation with AM.

The pyrosequencing technique yielded results that correlated with the fingerprint, showing that the bacterial community composition based on relative abundance was significantly modified by treatments.

Sphingomonadales and Burkholderiales were highly stimulated by phenanthrene contamination and inoculation. In the Phe microcosm, the higher increase in Actinomycetales (mainly *Arthrobacter*) was observed.

Effectively, the use of the strain AM as inoculant became the best strategy to remediate the soil mainly based on the degradation efficiency, however it caused more drastic changes in microbial community than inoculation with CON, what can be compromising the ulterior functionality of the soil.

1. Introduction

Polycyclic aromatic hydrocarbons (PAH) are amongst the most widespread organic contaminants in soils, water, and wastewater (Puglisi et al., 2007). The presence of PAH in contaminated soils and sediments poses a serious risk to human environments, since they have ecotoxic, mutagenic, and, in some cases, carcinogenic effects (ATSDR, 2005).

Although biological processes have received much scientific attention, the development of efficient in situ bioremediation processes for decontamination of hydrocarbon-contaminated soils still remains a challenging task (Szulc et al., 2014). The most commonly used type of eco-friendly strategies holding the promise of epitomizing in situ bioremediation are, bioaugmentation by inoculation with PAH degrading strains and biostimulation by supplementation with nutrients to stimulate indigenous microbial activity during bioremediation (Tyagi et al., 2011; Simarro et al., 2013).

Bioaugmentation efficiency is a function of the ability of the inoculated microbial degraders to remain active in the natural environment (Alexander, 1999). In most cases, failure was related to poor survivability and adaptability of the introduced microorganisms due to improper strain selection (Szulc et al., 2014). In previous works, we studied the bioaugmentation with a single degrading strain, *Sphingomonas paucimobilis* 20006FA. The study revealed a reduction in genetic and functional diversity of soil, which could have caused an accumulation of toxic phenanthrene metabolites reducing efficiency in phenanthrene degradation during the inoculation period (Coppotelli et al., 2008).

Although several PAH degrading bacterial species have been isolated (Samanta et al., 2002), it is not expected that a single

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isolate would exhibit the ability to degrade completely all PAH. As has been established, bioaugmentation requires different species of introduced PAH-degrading microorganisms, which can compete with the indigenous microbial community in PAH-contaminated soil, especially if they are to participate in the main carbon and energy flux processes and enhance PAH removal (Dejonghe et al., 2001). The benefits of using consortia for bioaugmentation have been extendedly discussed, since they can share biochemical steps in order to completely mineralize recalcitrant and/or toxic substrates (Mrozik and Piotrowska-Seget, 2010) and they can better overcome the barriers present in the new ecological and physicochemical environments. Many studies indicated that the use of consortia of aromatic-degrading bacteria has been more effective in removing pollutants as compared with selected single strains (Ghazali et al., 2004; Jacques et al., 2008).

The ultimate goal of any remediation process must be not only to remove the contaminant(s) from the polluted soil but also, most importantly, to restore the capacity of the soil to function according to its potential (Epelde et al., 2009). The measurement of microbiological parameters such as microbial biomass, enzyme activities and the diversity of soil microbial communities may serve as a good index of the impact of pollution on soil health (Labud et al., 2007).

The use of culture-independent methods to study microbial diversity has expanded our view of the microbial world and allowed access to extreme and difficult environments to study (Amann et al., 1995). Fingerprinting molecular biology techniques, like PCR-DGGE were employed to investigate the soil community structure; however, it only allows an assessment of the dominant members of communities (de Araujo and Schneider, 2008). Recently, pyrosequencing has emerged as the most powerful tool to analyze complex microbial communities in different ecosystems (Hervé et al., 2014), allowing inferring the biological function(s) of organisms and their contribution to the functional status of the sampled environment (Sun et al., 2013).

In this study the efficiency of a bioaugmentation strategy in phenanthrene-contaminated soil microcosms was determined using a phenanthrene-degrading consortium, enriched from a chronically contaminated petrochemical soil and previously characterized by Festa (Festa et al., 2013); and it was compared with the effect of bioaugmentation with a phenanthrene-degrading bacterium isolated from the consortium. We emphasize the study of the changes in soil bacterial community composition caused by phenanthrene contamination and bioaugmentation, using PCR-DGGE and pyrosequencing of PCR-amplified bacterial 16S rRNA gene fragments.

2. Materials and methods

2.1. Bacterial strains and growth conditions

A phenanthrene-degrading consortium (CON) was previously isolated from a chronically hydrocarbon-contaminated soil near La Plata city, Argentina. It was characterized in terms of structure, diversity and functionality, showing the capacity of degrading the 58% of the supplied phenanthrene in liquid mineral medium (LMM), in the first 7 days of incubation (Festa et al., 2013). One strain identified as *Sphingobium* sp. (AM), which was isolated from the CON, showed a degradation of the 75% of the supplied phenanthrene (Festa et al., 2013).

2.2. Soil characteristics and preparation

The soil selected for the study was an uncontaminated soil from an area near La Plata city, Argentina. It was analyzed in the Laboratory of Soil Science at the University of La Plata and showed the following physicochemical properties: a texture containing clay loam with a pH of 5.8–5.9, 3.60% organic carbon, 6.21% soil organic matter, 0.296% total nitrogen, 0.00042% available phosphorus, and 0.05 g kg^{-1} hydrocarbons. The soil was sieved in a 2-mm mesh and stored for 24 h at room temperature until use.

2.3. Preparation of cultures used as inocula

The CON inoculum was cultivated in LMM (Vecchioli et al., 1990) supplemented with 2000 mg l^{-1} of phenanthrene for 7 days at 28 °C and 150 rpm, then filtered (to eliminate phenanthrene crystals), centrifuged, washed and resuspended in 0.85% NaCl. The AM inoculum was cultured in R2 (Reasoner and Geldreich, 1985) for 24 h at 28 °C and 150 rpm, then centrifuged and washed to eliminate carbon sources and resuspended in 0.85% NaCl.

2.4. Preparation of soil microcosms

Soil microcosms consisted of 0.5 kg of sieved soil in a glass container with 1 kg capacity. They were artificially contaminated with 2000 mg of phenanthrene (Carlo Erba, Milan, Italy, >99.5% purity) per kilogram of dry soil. The phenanthrene was delivered in an acetone solution (150 mg/mL) and mixed into the soil manually with a spatula.

Three treatments were carried out in triplicates trays: (1) Phe: a contaminated and non-inoculated microcosm was used as a control of natural attenuation; (2) Phe + CON: microcosm was inoculated with 1×10^8 cells of CON per gram of dry soil one day after phenanthrene was added to the soil; (3) Phe + AM: microcosm was inoculated with 1×10^8 cells of AM strain per gram of dry soil one day after phenanthrene was added to the soil. In the two inoculated treatments a specific volume of the obtained cells suspension was added to the respective microcosms to achieve the desired inoculum density. A non-inoculated and non-contaminated microcosm (made in triplicate) was used as a control (Control). The same volume of bidistilled water was added to Phe and Control microcosms to standardize the moisture content. All microcosms were incubated at 24 ± 2 °C in the dark for 63 days and were mixed weekly for aeration. The moisture content of the soil was corrected when necessary to $20 \pm 2\%$ by adding distilled water. The 20% of water content correspond to a 65% of soil Water Holding Capacity.

2.5. Chemical analysis

A soil sample (25 g) was mixed with anhydrous sodium sulfate (25 g) and hydrocarbons were extracted for 6 h with ethyl acetate in a Soxhlet apparatus. The phenanthrene concentration in the ethyl acetate extracts was determined by HPLC (Coppotelli et al., 2010). The residual phenanthrene concentration was determined 2 days after the artificial contamination and every 7 days for the first 28 days and later on after 63 days. The statistical analysis of the phenanthrene degradation data was performed by parametric one-way ANOVA test followed by Tukey's honestly significant difference (HSD) post-hoc test, using XLStat-Pro statistical package version 7.5.2 (Addinsoft SARL, France).

2.6. Microbial enumeration and biological activity

Bacterial enumeration by CFU: In order to determine total heterotrophic cultivable bacteria and PAH-degraders, 10 g (wet weight) of soil sample were suspended in 100 ml of 0.85% NaCl, homogenized for 30 min on a rotary shaker (250 rpm) and decanted for 5–10 min. The total heterotrophic cultivable bacteria were quantified in duplicates by a successive 1/10 dilutions. These suspensions were spread on R2A medium plates (Reasoner and Geldreich, 1985) and after 7 days of incubation at 20 ± 2 °C colonies

were counted. The PAH-degraders were quantified in sterile polypropylene microplates according to Wrenn and Venosa (Wrenn and Venosa, 1996). The wells contained LMM and a PAH-mix in pentane as a substrate. The pentane was evaporated and the wells were inoculated with an aliquot of serial dilutions of the sample and incubated at 20 ± 2 °C for 21 days. Most Probable Number (MNP) method was used to enumerate PAH degraders. The microbial enumeration was carried out in triplicates.

Dehydrogenase activity: The dehydrogenase activity was measured by the colorimetric reaction (546 nm) of the reduction of 2,3,5-triphenyl-2H-tetrazoliumtrichloride [TTC] to triphenyl formazan [TPF] (Andreoni et al., 2004).

These determinations were performed in triplicates two days after the artificial contamination and every 7 days for the first 28 days and later on after 63 days. The statistical analysis of dehydrogenase activity and counts data were performed by parametric one-way ANOVA test, followed by Tukey's honestly significant difference (HSD) post-hoc test, using XLStat-Pro statistical package v7.5.2 (Addinsoft SARL, France).

2.7. DNA extraction from soil and bacteria

Total DNA of each microcosm was extracted from 1 g of soil sample at 2, 7, 14, 21, 28 and 63 days of incubation time with E.Z.N. A.® Soil DNA Kit (Omega Bio-Tek, Inc., Norcross, GA, USA) following manufacturer's instructions. The same kit was used to extract the genomic DNA of AM strain and CON and the DNA quality was checked in 1% agarose gel electrophoresis using Ethidium bromide. The DNA was stored at −20 °C prior to amplification.

2.8. PCR and DGGE

The set of primers used were GC-341F (5′-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCC GCCCCCTCCTACGGGAGGCAGCAG-3′) and 907R (5′-CCGTCAATTCCTTTGAGTTT-3′) (Muyzer et al., 1998). PCR amplification was performed in 30 μl reaction volume with 1 μl of DNA, 1U of GoTaq, the manufacturers' recommended buffer as supplied with the polymerase enzyme, 200 mM of BSA, 0.2 mM of dNTPs and 5 μM of each primer. Amplification was carried out on an Eppendorf® Mastercycler® thermocycler (Eppendorf, Hamburg, Germany) and PCR program consisted of an initial denaturation step for 4 min at 94 °C, followed at first by 10 cycles of 94 °C for 30 s; 62 °C for 45 s; and 1 min at 72 °C and then by 25 cycles of 30 s at 94 °C, 45 s at 57 °C; and 72 °C for 1 min. The final extension was carried out at 72 °C for 10 min. PCR products were analyzed by agarose gel electrophoresis and the positive amplicons were purified using NucleoSpin® Extract II kit (Macherey-Nagel GmbH & Co. KG, Germany).

DGGE was performed in a DGGE-2000 System (CBS Scientific Company). The purified PCR products were directly loaded in a (6% w vol^{-1}) polyacrylamide gel (acrylamide-*N*,*N*′-methylenbisacrylamide, 37.5:1) containing a linear gradient of 45–70% denaturant (100% denaturant corresponds to 7 M urea and 40% vol vol^{-1} formamide). The electrophoretic run was executed at a temperature of 60 °C at a constant voltage of 100 V for 16 h.

2.9. Statistical analyses

The post-electrophoresis gel was stained for 30 min with SYBR®Gold (1X dye concentration from the original stock solution) and analyzed on GelCompar II software package (Applied Maths, Kortrijk, Belgium). The optical densities of the lanes were determined. These density profiles served as a base for calculating a similarity matrix using Pearson's product moment correlations (Pearson, 1926). The similarity matrix obtained using GelCompar II was further analyzed by metric MDS (Multidimensional Scaling) analysis with the statistical package Primer 6 (version 6.1.13; Primer-E Ltd., United Kingdom). MDS is an ordination method that can reduce complex DGGE patterns to points into a 2-dimensional scale (Fromin et al., 2002). The higher the distance between points, the higher the differences in community composition. The Shannon index was calculated using the DIVERSE procedure available in Primer 6 (version 6.1.13; Primer-E Ltd., United Kingdom).

2.10. Nucleotide sequence determination

Dominant DGGE bands were excised under UV illumination using a sterile scalpel blade, and briefly crushed with a pipette tip in 0.1 ml sterile H_2O and incubated at −80 °C for 30 mins and then at 4 °C for 24 h. The partial length of 16S rDNA was amplified with 341F (5′-CCTACGGGAGGCAGCAG-3′) and 907R (5′-CCGTCAATTCCTTTGAGTTT-3′) primers (Muyzer et al., 1998) and then cloned into the pGEM®-T vector according to the manufacturer's instructions. For each excised band, clones containing recombinant vector were randomly selected and checked for correct insert size via PCR and agarose gel electrophoresis and sequenced by sequencing service, Macrogen, Korea. Nucleotide sequences were compared to those in the National Center for Biotechnology Information GenBank database by using BLAST program. The nucleotide sequences obtained in this study have been deposited in the GenBank database under accession numbers KM213518 and KM213519.

To study the phylogenetic relationship between the 16S rDNA sequences from the DGGE bands, strain AM and selected sequences available in GenBank (http://www.ncbi.nlm.nih.gov/Genbank), a distance-based evolutionary tree was constructed using Neighbor-Joining method and p-distance algorithm with the Molecular Evolutionary Genetics Analysis package (MEGA version 4.0) (Tamura et al., 2007). The robustness of the phylogeny was tested by bootstrap analysis with 500 iterations.

2.11. PCR and pyrosequencing

Samples of day 14 of incubation of each microcosm were use for PCR amplification using the 16S rRNA universal bacterial primers, 341Fbac (CCTACGGGAGGCAGCAG) (Muyzer et al., 1993) and 909R (CCCCGYCAATTCMTTTRAGT) (Tamaki et al., 2011) to amplify a 568-bp fragment of the 16S rRNA gene flanking the V3 and V4 regions. PCR was performed in duplicate in a volume of 20 μl. A single-step 30 cycle PCR using HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA) was used under the following conditions: 94 °C for 3 mins, followed by 28 cycles of 94 °C for 30 s; 53 °C for 40 s and 72 °C for 1 min; after which a final elongation step was performed at 72 °C for 5 mins. Following PCR, amplicon products from different samples were mixed in equal concentrations and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA). Samples were sequenced utilizing Roche 454 FLX titanium instruments and reagents, following manufacturer's guidelines. This process was performed at the Molecular Research laboratory (MR DNA; Shallowater, TX) based upon established and validated protocols (http://www.mrdnalab.com/). These sequence data are available at the NCBI Short-Read Archive under accession number SRP061331.

2.12. Analysis of the pyrosequencing data set

The sequence data derived from the high-throughput sequencing process were analyzed employing a pipeline developed at Molecular Research LP (www.mrdnalab.com). Sequences were first depleted of barcodes and primers, then short sequences (<200 bp), sequences with ambiguous base calls, and sequences with homopolymer runs

exceeding 6 bp were all removed. Sequences were de-noised and chimeras were removed using custom software (Dowd et al., 2008b) and the Black Box Chimera Check software B2C2 (freely available at http://www.researchandtesting.com/B2C2.html). The rest were checked for high quality based on criteria utilized by RDP version 9 (Cole et al., 2009). Sequence data were then clustered into Operational Taxonomic Units (OTUs) with 3% divergence using uClust version v1.2.22. OTUs were then taxonomically classified using BLASTn.NET algorithm (Dowd et al., 2005) against a database of high quality bacterial 16S rRNA sequences derived from GreenGenes (10-2011 version). (Edgar, 2010). The outputs were compiled and validated using taxonomic distance methods (Dowd et al., 2008a,b).

Taxonomy was defined based on the following percentages: >97%, species; between 97% and 95%, unclassified genus; between 95% and 90%, unclassified family; between 90% and 85%, unclassified order; between 85% and 80%, unclassified class; between 80% and 77%, unclassified phylum; <77%, unclassified.

For statistical analysis of the data, Hill's numbers (species richness [^{0}D], the exponential of Shannon entropy [^{1}D] and the inverse Simpson index [^{2}D]) (Hill, 1973) were used as diversity measures in accordance with current consensus (Jost, 2006; Chao et al., 2012). Hill's numbers are defined to the order of q (^{q}D), where parameter q indicates the weight given toward rare or common species. ^{0}D (species richness) is insensitive to relative frequencies, and is therefore weighted toward rare species. ^{1}D (exponential of Shannon entropy) is weighted toward common species, and ^{2}D (inverse Simpson concentration) is weighted toward abundant species. Species richness (^{0}D) was estimated using Chao1-bc, a bias-corrected form of Chao1 (Chao, 2005). Rarefaction curves, diversity and richness measures were calculated using EstimateS (Version 9) (Colwell, 2013). Since these measures are influenced by sequencing depth, normalization was performed through re-sampling and diversity estimated was computed from 905 sequences (corresponding to the number of reads in the shallowest sampled community) that were randomly drawn from each sample. Venn diagram was implemented by R packages of VennDiagram and ColorBrewer Palettes (Chen and Boutros, 2011; Neuwirth, 2014), R version 3.1.2.

3. Results

3.1. Composition of the PAH-degrading consortium

The PAH-degrading consortium (CON) used as inoculant in this study was previously isolated from a chronically contaminated soil and characterized in terms of structure, diversity and functionality, showing the capacity of degrading the 58% of the supplied phenanthrene in liquid medium in the first 7 days of incubation (Festa et al., 2013).

Using culture dependent methods, there have been isolated five strains from the CON. These strains were identified as *Sphingobium* sp. (AM), *Enterobacter* sp. (B and B1) and *Pseudomonas* sp. (T and Bc), but only the single strain AM showed the ability to degrade the 75% of phenanthrene after 7 days of incubation (Festa et al., 2013).

In this study, the bacterial composition of the consortium was profiled using pyrosequencing of PCR-amplified bacterial 16S rRNA gene fragments. This procedure generated an average of 996 quality-filtered sequences; they were grouped into a total of 21 OTUs using 97% minimum similarity. All representative sequences of each OTU were classified into the domain Bacteria, phylum Proteobacteria (Alpha, Beta and Gamma with 89.4%, 2.9% and 7.7% of the reads respectively). Relative phylotype frequency at the genus level revealed the presence of *Sphingobium* 87.4%, *Enterobacter* 6.6%, *Achromobacter* 1.9%, *Inquilinus* 1.1%, *Bordetella* 1.0%, *Luteibacter* 0.6%, *Sphingomonas* 0.5%, *Pseudomonas* 0.4%, and *Bradyrhizobium* 0,3% (Fig. S1 in Supplemental material).

Since the consortium, CON, and the single strain, AM, were efficient in degrading phenanthrene in liquid medium, their degradation capacities were compared in bioaugmentation studies.

3.2. Bioaugmentation studies

3.2.1. Chemical analysis

The concentrations of phenanthrene during the treatment in Phe, Phe + CON and Phe + AM microcosms are shown in Fig. 1.

Whereas the phenanthrene elimination in Phe microcosm began after day 7 of the incubation time, in the bioaugmented microcosms phenanthrene elimination was observed since the first day, and during the first period (21 days) the elimination rate was higher than in Phe. When compared the two inoculated microcosms, the Phe + AM microcosm showed the lowest ($P < 0.05$) phenanthrene concentration from day 14 and during the whole treatment.

At the end of incubation period (day 63) all microcosms showed significant hydrocarbon elimination (more than 95% of that initially supplied) however in inoculated microcosms the residual concentration of phenanthrene was significantly lower ($P < 0.05$) than that of Phe (73.0 mg/Kg of dry soil). The phenanthrene concentration was 5.8 mg/Kg of dry soil for Phe + AM and 23.6 mg/

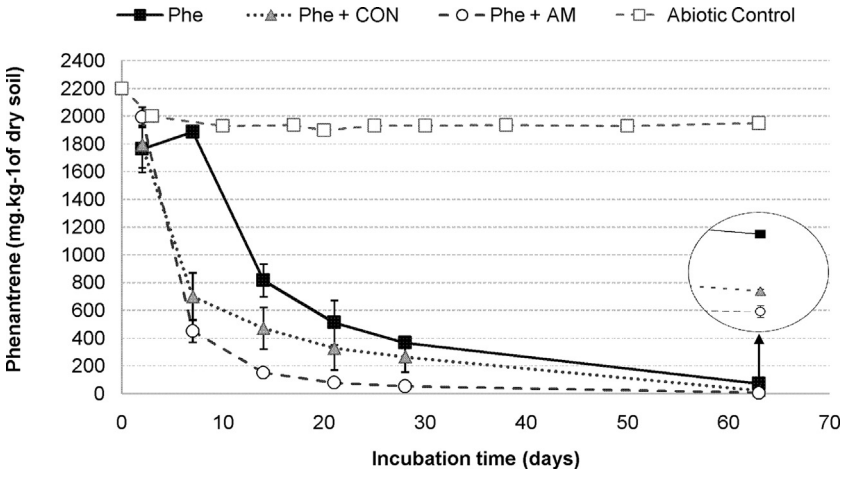

Fig. 1. Concentration of phenanthrene in Phe, Phe + CON and Phe + AM microcosms during bioremediation process. Results are means of triplicate independent microcosms. Bars represent standard deviations.

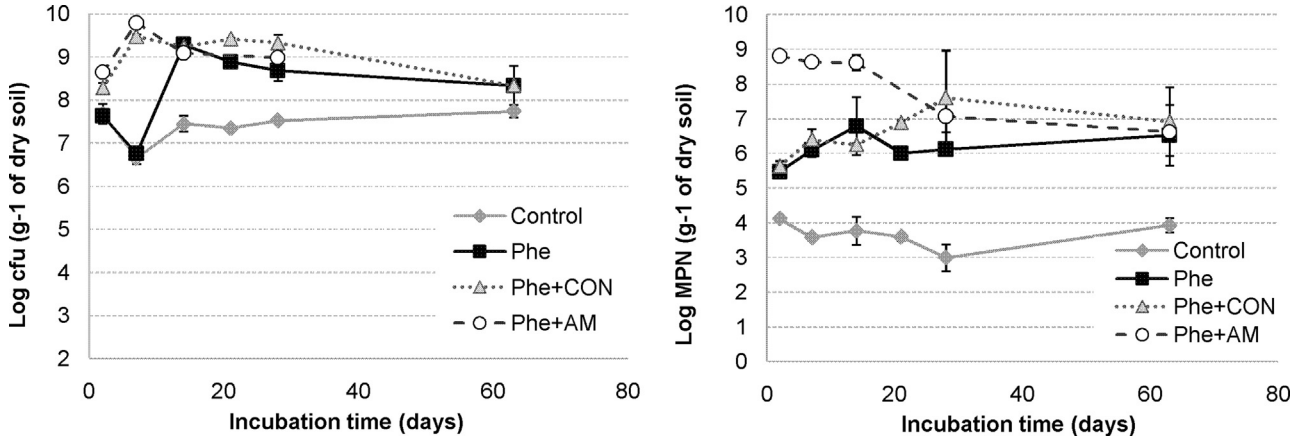

Fig. 2. Cultivable bacterial populations in the Control, Phe, Phe + CON and Phe + AM microcosms during bioremediation process. (a) Heterotrophic cultivable bacteria (log cfu g^{-1} of dry soil). (b) PAH-degrading bacteria (log MNP g^{-1} of dry soil). Results are means of triplicate independent microcosms. Bars represent standard deviations.

Kg of dry soil for Phe + CON, showing a significant higher degradation ($P < 0.05$) in Phe + AM. Additionally, only both inoculated microcosms reached a phenanthrene concentration below the cleanup standards for soil in Argentina (below 50 mg kg^{-1}, Argentinian National Law 24.051) (Poder Ejecutivo Nacional 24051, 1992).

3.2.2. Enumeration of cultivable bacterial populations

The addition of phenanthrene into the soil produced an increase in the density of heterotrophic and PAH-degrading bacteria in the Phe microcosm on day 14 of incubation (Fig. 2a and b). The addition of the different inocula to the microcosms produced an additional increase of the cultivable heterotrophic bacteria after the first day of treatment.

Regarding PAH-degrading bacteria, while contamination with phenanthrene produced an increase on day 14 of incubation and then remained constant, the inoculated microcosms behave differently. The Phe + AM microcosm showed the highest counts of PAH-degrading bacteria during the first 15 days of incubation and then a significant decrease was observed, while in Phe + CON microcosm PAH-degrading bacteria increased significantly until day 28 and then began to decrease. It is noteworthy that the microcosm Phe + AM, with higher counts of degrading bacteria during the first 28 days of incubation, showed the greatest degradation.

After 63 days of treatment, no significant differences were observed among viable counts of heterotrophic bacteria in the contaminated microcosms ($P < 0.05$) and the Control microcosm. By this time, more than 95% of phenanthrene had been eliminated. Nevertheless, the number of cultivable PAH-degrading population in the contaminated microcosms remained significantly higher ($P < 0.05$) than in the Control microcosm throughout the whole experiment.

3.2.3. Biological activity

The dehydrogenase activity determined by analyzing the reduction of TTC to TPF in the microcosms is shown in Fig. 3. The phenanthrene incorporation (Phe microcosms) caused an inhibitory effect on dehydrogenase activity and therefore remained significantly lower ($P < 0.05$) in comparison to the Control microcosm during the first 7 days of treatment. Subsequently a stimulatory effect was observed until the day 20 of treatment, indicating an active degradation of phenanthrene by indigenous microorganisms. Both inoculations caused a marked stimulatory effect on dehydrogenase activity in Phe + CON and Phe + AM microcosms, which remained significantly higher than the Phe microcosm during the first 7 days of incubation ($P < 0.05$); this was coincident with the period of degradation of phenanthrene. At the end of treatment, a significantly low dehydrogenase

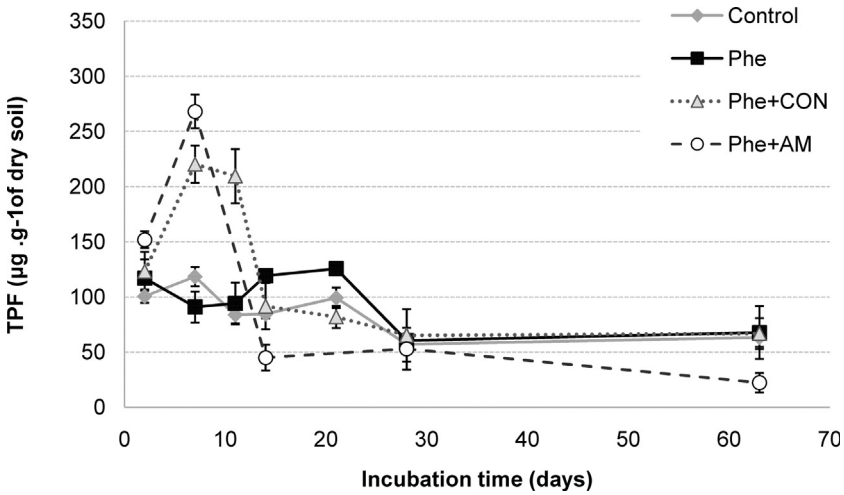

Fig. 3. Dehydrogenase activity (µg of TPF per g of dry soil) of Control, Phe, Phe + CON and Phe + AM microcosms during bioremediation process. Results are means of triplicate independent microcosms. Bars represent standard deviations.

activity was observed in all microcosms, being the activity in Phe + AM microcosm significantly the lowest ($P < 0.05$).

3.3. Analysis of bacterial community

3.3.1. Community structure

The temporal dynamics of bacterial communities produced by phenanthrene contamination and inoculation treatments were studied by DGGE at different time during incubation. Three independent replicates per treatment and per sampling time were analyzed, as there was no difference in DGGE profiles among them only the analysis of one set of results is presented.

DNA patterns obtained from the PCR amplicons of the total community DNA are presented in Fig. 4, dynamic in patterns indicates the changes in the predominant soil microbial community provoked by the phenanthrene contamination and the inoculations.

The comparison of DGGE patterns of the Phe microcosm showed important changes in bacterial community structures since the day 14 of treatment with respect to the Control, with a notable reduction in the number of bands and an increment in the intensity of three predominant bands (bands 1–3). The predominant band (band 2) was excised, reamplified and cloned. The phylogenetic analysis revealed that clones were phylogenetically related (Fig. S2 in Supplemental material) and had 99% sequence similarity (analyzed with BLAST) with *Novosphingobium* sp. (GenBank accession number KM213518).

Clear differences were found between the DGGE patterns of Phe and inoculated microcosms. In all cases, the inoculated microcosms showed a significant reduction in the number of bands, being this reduction more dramatic in the case of Phe + AM microcosm. The Shannon indexes from DGGE analysis on day 14 were 4.54 for Control, 4.51 for Phe, 4.47 for Phe + CON and 3.76 for Phe + AM.

The DGGE patterns of the Phe + CON microcosm indicated a remarkable dominance of the band 4, belonging to the CON, from the beginning of the experiment (day 2) until day 63 of treatment. This band was excised, re-amplified and cloned. The phylogenetic analysis revealed that clones were phylogenetically related (Fig. S2 in Supplemental material) and had 99% sequence similarity (analyzed with BLAST) with *Sphingobium* sp. (GenBank accession number KM213519). Band 4 migrated at the same position than the band 8 from CON, analyzed in a previous work (Festa et al., 2013), which showed a high similarity with the Sphingomonadaceae family.

Similarly, in patterns of Phe + AM microcosms, the band migrating at the same position of the strain AM was predominant during the whole experiment (band 5).

To compare broad-scale differences between bacterial community profiles, a multidimensional scaling approach (MDS) was employed. An MDS ordination plot of unconstrained data (Fig. 5) revealed that profiles from soil samples containing inocula were differentiated from those containing only Phe and Control, at 22% of similarity level, with an MDS stress value of 0.1 (stress values below 0.2 indicate that an MDS ordination plot is a good spatial representation of differences in data) (Muckian et al., 2009).

Although the phenanthrene contamination caused effects on community structure (Phe microcosms), the samples containing the inocula AM or CON were the most spatially distant from Phe and Control, being the most marked differences attributed to the

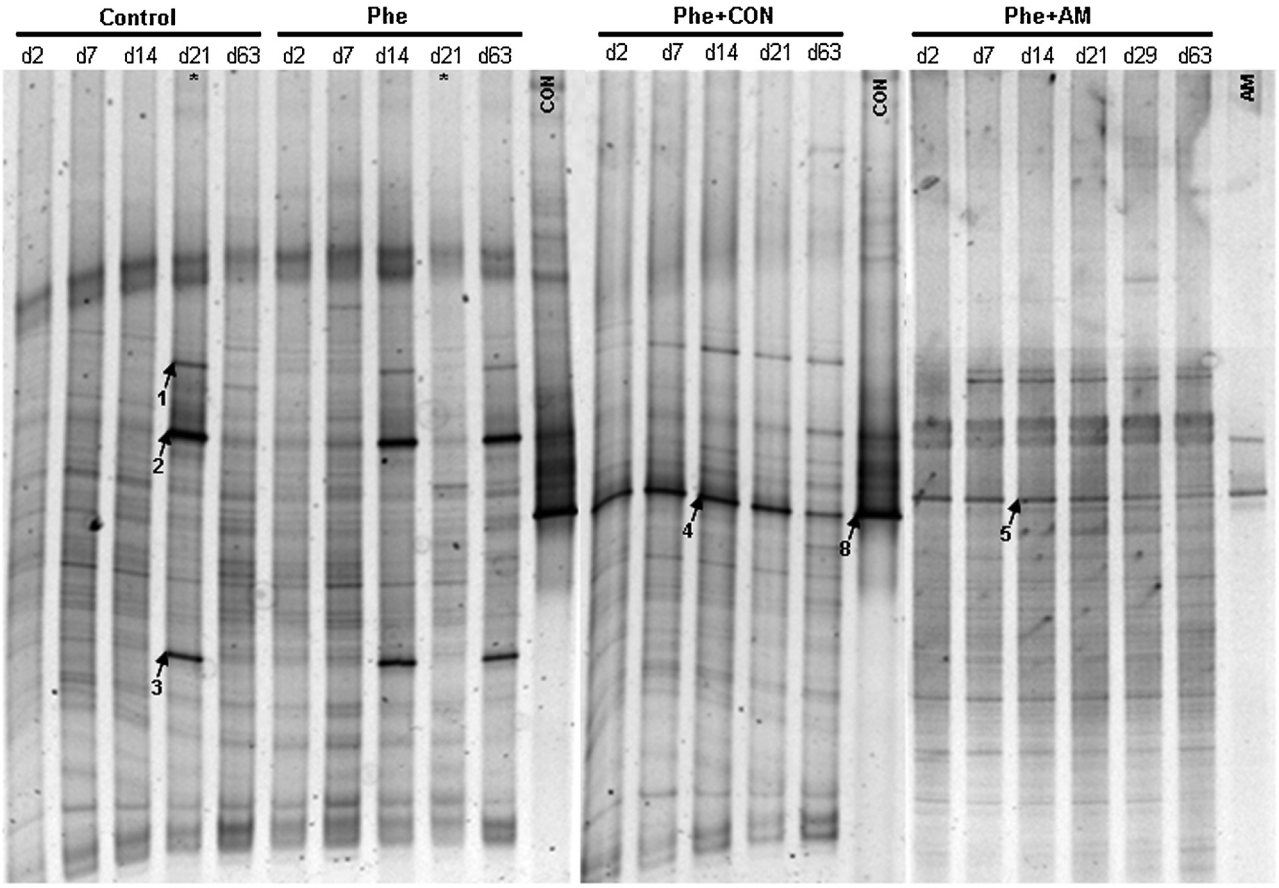

Fig. 4. PCR-DGGE analysis of bacterial populations in soil samples of the four microcosms during the incubation time. Streets with (*) are inverted.

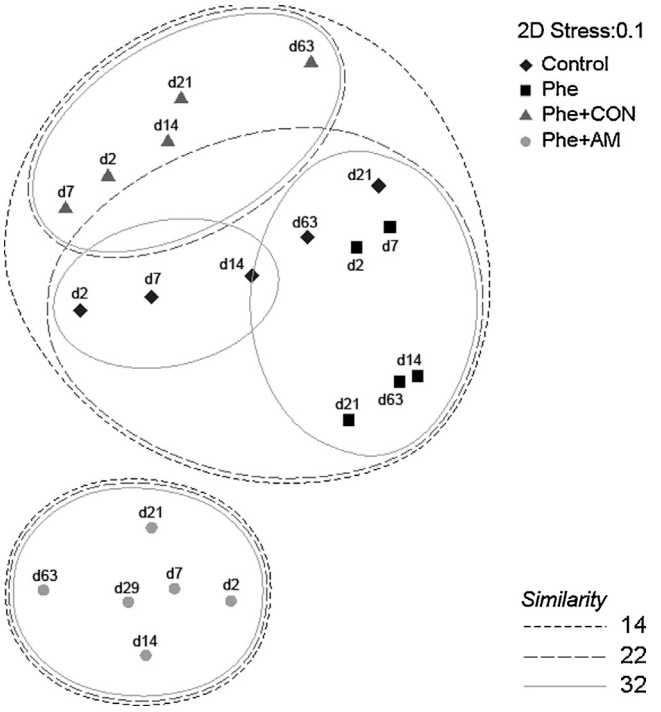

Fig. 5. MDS of DNA-derived DGGE banding patterns obtained with primers 341F-GC and 907R showing changes in bacterial community structure in the three contaminated microcosms and the Control at 2, 7, 14, 21, 28 and 63 days of treatment. Proximity of samples indicates a higher degree of community similarity as indicated by overlap in OTUs.

inoculation with AM, which was still different at lower similarity levels (14%). This analysis indicated that the structure of bacterial community was strongly affected by inoculation and by the nature of the inocula

3.3.2. Bacterial composition: general analysis of the pyrosequencing data set

The taxonomic composition and the diversity of the bacterial communities present in the four soil microcosms on day 14 of treatment (when all microcosms showed an active degradation), were profiled using pyrosequencing of PCR-amplified bacterial 16S rRNA gene fragments.

Pyrosequencing generated an average of 2032 (range 905–2917) quality-filtered sequence reads per sample; they were grouped into a total of 1023 OTUs using 97% minimum similarity. Table 1 shows the diversity estimations. The Hill-numbers, ^{0}D (species richness), ^{1}D and ^{2}D, differed among Control (uncontaminated soil) and Phe (contaminated) microcosms, indicating a reduction in microbial richness and diversity caused by the contamination.

About the contaminated and inoculated samples, the microcosm Phe + AM showed the higher ^{0}D suggesting that the inoculation with AM caused an increase in species richness in comparison to Phe microcosm; however it should be noted that ^{0}D is very sensitive to the sample size and in our study we have not replicates for pyrosequencing determinations. ^{1}D showed no notable differences among the three contaminated microcosms, implying the presence of a similar number of scarce and moderately common species, whereas the ^{2}D showed that Phe + AM microcosms have the more uneven assemblages, with a few species very dominants.

Table 1
Diversity parameters for the different communities obtained by analysis of pyrosequencing data of day 14 of incubation using EstimateS program.

	Number of sequences	Number of OTUs[a]	Hill Numbers		
			^{0}D	^{1}D	^{2}D
Control	2655	631	649.2	269.22	177.72
Phe	1652	349	416.87	103.57	27.68
Phe+CON	905	257	365.88	116.76	31.7
Phe+AM	2917	543	550.87	97.98	15.89

[a] Values based on 905 random sequences per sample.

Rarefaction analysis of the data showed that the number of sequences retrieved from each sample was enough to cover most of the diversity (Fig. 6). Rarefaction curves estimating OTU richness confirmed the difference between Control and contaminated samples (Table 1), where Control was predicted to have higher microbial species richness than contaminated samples.

The shared communities in different microcosms at OTUs level were further determined via the Venn diagram (Fig. 7). From a total of 1023 OTUs detected, 73 (7.13%) were shared between the four different microcosms on day 14 of treatment. In the contaminated microcosms, 799 OTUs were identified, and 85 of them (10.6%) are shared by the three microcosms. Between the inoculated microcosms, 132 OTUs are shared, accounting for 19.7% of the 668 OTUs analyzed in these microcosms.

All representative sequences of each OTU in the four microcosms were classified into the domain Bacteria (15 phyla). On average for the complete data set, the major microbial phyla were Proteobacteria (24.5%–49.7% of the reads), Acidobacteria (20.7%–34.2%), Actinobacteria (7.1%–15.5%), Verrucomicrobia (3.7%–8.9%), Chloroflexi (2.4%–9.6%), Firmicutes (1.7%–4.5%), and Gemmatimonadetes (1.3%–4.6%) (Table S1 in Supplemental material).

The contamination with phenanthrene triggered a predominance of the Proteobacteria phylum (more than 42% of all sequences) mainly at the expense of a marked increase in Alphaproteobacteria (Table S2 in Supplemental material). In addition relative abundance of Actinobacteria and Acidobacteria suffered significant variations according to the treatment (Table S1 in Supplemental material).

3.3.3. Impact of the different treatments on the relative abundance of specific soil bacterial populations

Many individual soil bacterial populations significantly increased or decreased in soil treated with phenanthrene or treated with a combination of phenanthrene contamination and inoculation. The differences in relative abundance in the treated microcosms in comparison to the Control were analyzed.

At order level, populations remarkably stimulated by phenanthrene contamination were Actinomycetales, Burkholderiales and Sphingomonadales. In inoculated microcosms the increase in Burkholderiales and Sphingomonadales was also marked (Table S3 in Supplemental material). However, increase in Sphingomonadales in the inoculated microcosms is not surprising, since AM and 87.9% of CON sequences were affiliated to this order (Fig. S1 in Supplemental material).

In order to facilitate the observations of the real impact of bioaugmentation strategies on the bacterial community at order level, the OTUs corresponding to the orders of the inoculants were removed. The analysis were performed after removing the OTU corresponding to AM in the Phe+AM and after removing the OTUs corresponding to the CON in Phe + CON, results are shown in Fig. 8.

Some soil bacterial populations, at order level, highly impacted by contamination were Anaerolineales, Acidobacteriales, Holophagales and Solirubrobacteriales, which were significantly repressed (2.1–6.5%) in the Phe microcosm with respect to the Control. Others were also considerably repressed but to a lesser extent (<2.0%) (Fig. 8 and Table S3 in Supplemental material).

Few populations were significantly stimulated by phenanthrene contamination, the most stimulated was Sphingomonadales

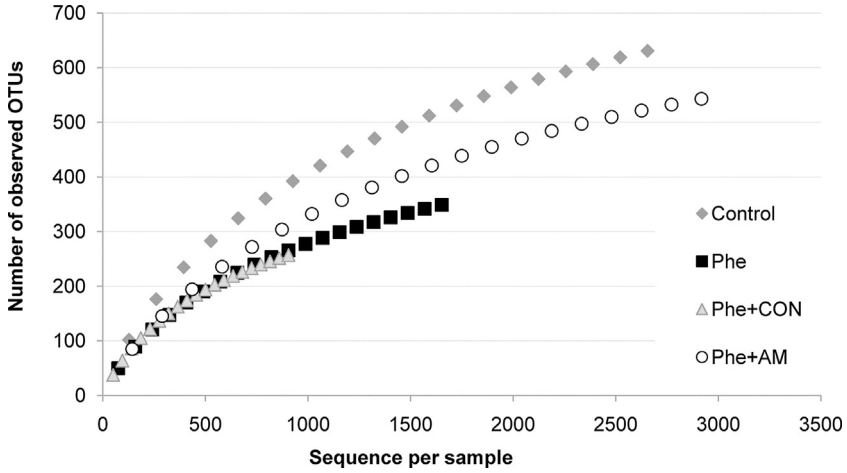

Fig. 6. Rarefaction analysis constructed using EstimateS software for pyrosequencing data. Rarefaction curves indicate the observed number of operational taxonomic units (OTUs) (*y*-axis) for Control and Phe, Phe + AM and Phe + CON microcosms as a function of number of reads (*x*-axis)

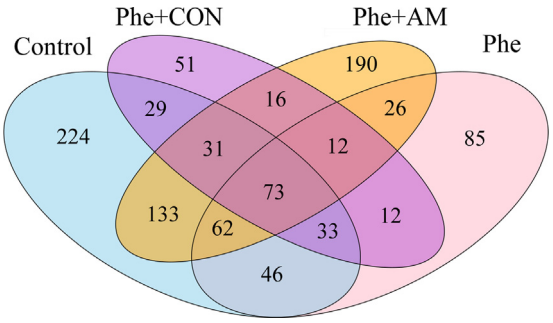

Fig. 7. Venn diagram showing the unique and shared OTUs (3% distance level) for Control and Phe, Phe + AM and Phe + CON microcosms.

(approximately 18.4%), followed by Actinomycetales (approximately 9.0%), and Burkholderiales (approximately 2.5%).

Inoculation with AM produced an additional increase of Burkholderiales by approximately 5.7% and Rhizobiales 5.0%, and a relative decline of Actinomycetales compared to the Phe microcosms (3.7%). In Phe + AM several populations significantly diminished (>2.0%) with respect to the Control, they are Verrucomicrobiales, Holophagales, Acidobacteriales, Gemmatimonadales, Bacillales and Pseudomonadales (Fig. 8).

With the inoculation of CON, the relative decline of Actinomycetales was also observed; and other populations were stimulated, Holophagales increased by approximately 4.4% and Acidobacteriales by approximately 3.6%.

We further explored the specific microbial groups within the orders Actinomycetales, Burkholderiales and Sphingomonadales, identified to be associated with contamination. Although these orders were found in almost all samples, the combined relative abundances in contaminated microcosm were more than 32% (compared to 9.9% in Control).

Relative phylotype frequency at the genus level is shown in Fig. 9. Order Actinomycetales were mainly represented by the genus *Arthrobacter*; order Burkholderiales by *Achromobacter* and *Cupriavidus*, Rhizobiales by *Rhodoplanes* and order Sphingomonadales by *Sphingomonas*,*Sphingobium* and *Novosphingobium* (Fig. 9 and Table S4 in Supplemental material).

The predominant genus varied from one microcosm to the other. While *Acidobacterium* (belonging to Acidobacteriales order) was the predominant genus in the Control (19.2%), with the addition of phenanthrene to the soil the *Novosphingobium* population became dominant (17.3%) and the genus *Arthrobacter* increased significantly (10.3%).

In Phe + AM microcosm, the genus of the inoculated strain AM (*Sphingobium*) became dominant (25.3%) and the *Novosphingobium*

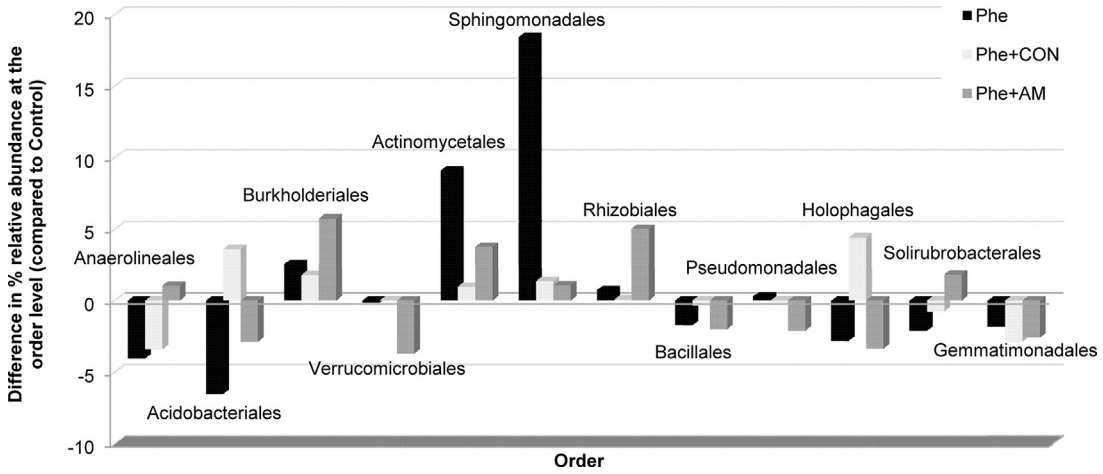

Fig. 8. Differences in relative phylotype abundance at the order level in soil contaminated microcosms compared to Control microcosm, the OTUs corresponding to the orders of the inoculants were removed. Only reads that changed more than 2% with respect to the Control microcosm are shown.

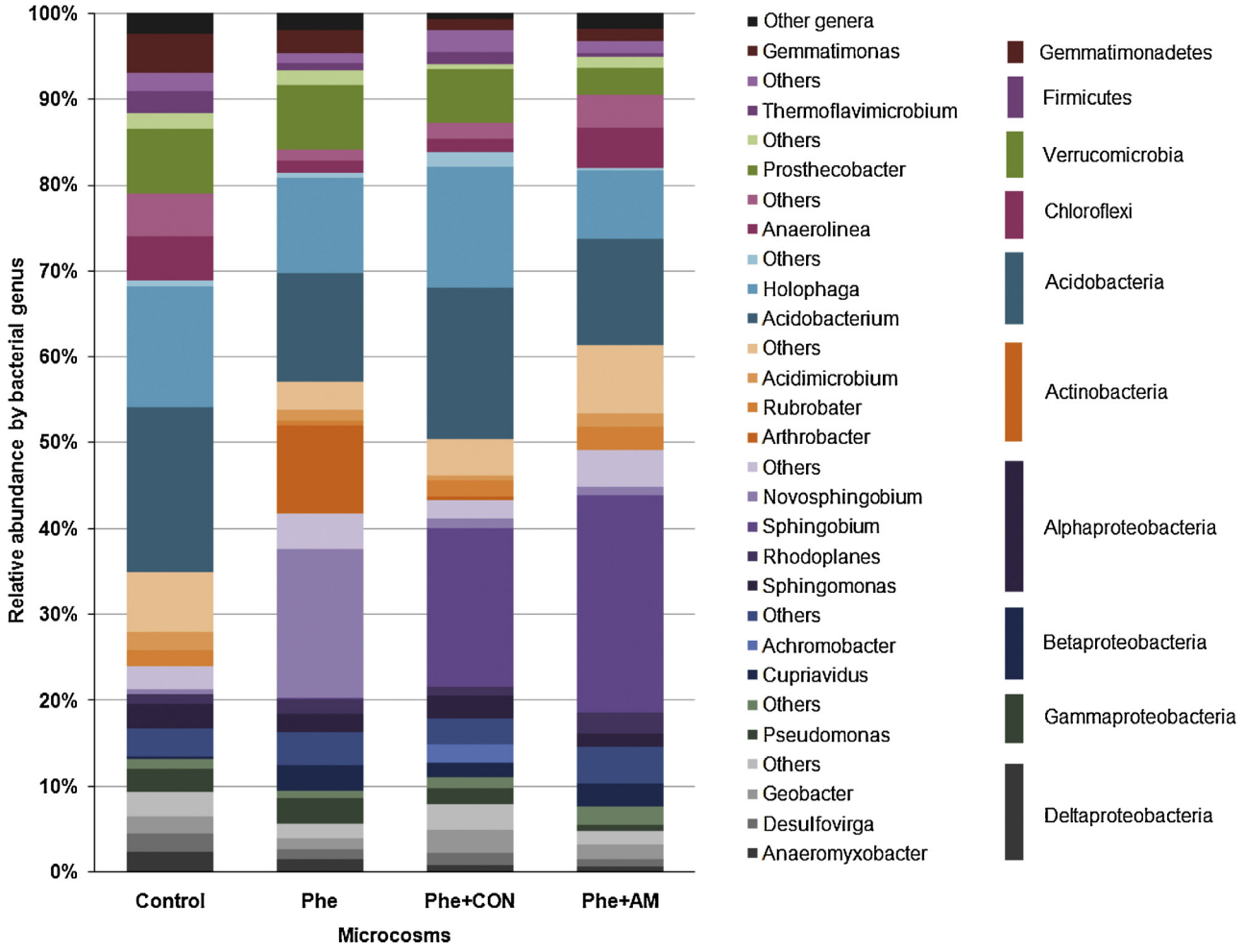

Fig. 9. Relative phylotype frequency at the genus level as revealed by pyrosecuencing in the four microcosms at day 14 of incubation. Affiliation determined with 97% likeness level using BLASTn against a curated GreenGenes database. Genera with <2% reads belonging to each phylum were grouped in "Others". Genera belonging to other phyla with <2% reads were grouped in "Other genera".

population decreased to 0.9%. In Phe+CON microcosm, the dominant populations was also *Sphingobium* (18.3%), in addition, a significant increase was observed in other population belonging to the consortium, *Achromobacter* (2.1%).

4. Discussion

The selection of microorganisms for inoculation should not be at random, since it was often observed that the performance of inocula exhibiting excellent properties during studies carried out under laboratory conditions was hindered during field studies under actual environmental conditions (Szulc et al., 2014).

In previous works we obtained a bacterial consortium by the enrichment culture technique from a chronically hydrocarbon-contaminated soil, with the aim of obtaining a functional, natural degrading consortium (Festa et al., 2013). Despite the fact that both, culture dependent and independent techniques (DGGE and clone libraries) provided information about the consortium composition diversity, the lack of correspondence between the results of these two strategies did not allow us to obtain information about the composition of the consortium in terms of relative abundance (Festa et al., 2013). Here, using a high-throughput technique was possible to find that some of the sequences obtained by culture-independent approaches were consistent with the sequences of the isolated strains from the consortium. This approach showed that the genera present in the consortium were *Enterobacter* sp., *Achromobacter* sp., *Inquilinus* sp., *Bordetella* sp., *Luteibacter* sp., *Sphingomonas* sp., *Pseudomonas* sp., *Bradyrhizobium* sp. and *Sphingobium* sp., the latter being the most abundant (87.4%) (Fig. S1 in Supplemental material).

Other consortia with similar bacterial associations were reported in literature, such as the naphthalene degrading bacterial consortium DV-AL (Patel et al., 2012); a bacterial consortium efficient in degrading petroleum sludge and polluted sandy soil (Gojgic-Cvijovic et al., 2012) and a PAHs degrading consortium (González et al., 2011). It is clear that a habitual association occurs between strains of *Achromobacter* sp., *Pseudomonas* sp., *Enterobacter* sp. and *Sphingobium* sp. whose nature is still unknown.

In order to determine if the associations present in the consortium confer advantages as inoculum in bioaugmentation processes of PAH-contaminated soil, we compared the efficiency of the consortium with one *Sphingobium* sp. strain (AM), belonging to the consortium and selected by its PAH-degrading capacity. Biodegradation and changes in the abundance of specific bacterial taxa in response to shifting treatments on the soil using two of the 16S rRNA gene-based approaches (DGGE and pyrosequencing) were evaluated.

Both inoculation strategies (using CON or AM as inoculants) managed to stimulate the degradation of phenanthrene (Fig. 1), with a significant increase in the number of cultivable heterotrophic bacteria (Fig. 2a) and dehydrogenase activity (Fig. 3). When we compared the two inoculated microcosms, a significant lower

degradation rate was observed in Phe + CON microcosm between day 14 and 63 of treatment, causing Phe + AM to reach the lowest values of residual concentration of phenanthrene at the end of treatment. Although these microcosms had similar cultivable heterotrophic bacteria counts during the whole experiment, the number of PAH-degrading bacteria (Fig. 2 b) was significantly lower in Phe + CON microcosm during the first 21 days of incubation.

With this partial result AM seems to be more effective in soil (as it could be hypothesized from its degradation potential in liquid medium) causing higher elimination rate of phenanthrene, higher PAH-degrading bacterial counts and higher initial dehydrogenase activity. However, at the end of experiment (63 days), Phe + AM was the treatment with the lowest dehydrogenase activity.

Performance of AM strain in relation to phenanthrene degradation in liquid medium (Festa et al., 2013) was not different from that of strain *S. paucimobilis* 20006FA (Coppotelli et al., 2008). The difference in degradation efficiency in soil could be related to the different inoculation protocol used, while here we used a unique inoculation event (1×10^8), Coppotelli and collaborators used three successive inoculations (1×10^8 each inoculation). They obtained a high abundance of the inoculant in the soil, what was hypothesized to have produced the observed pause in the decrease in the phenanthrene concentration because of the accumulation of toxic phenanthrene metabolites caused by the metabolic activity of a bacterial community with a lower genetic and functional diversity.

In the fingerprint analysis used in this study, it was revealed that dominant members of the soil bacterial community were influenced positively or negatively by the addition of contaminant and inocula (Fig. 4). A reduction in the number of some bands and an increment in the intensity of others were observed when phenanthrene was incorporated to the soil (Phe microcosm). Inoculation of soil with AM or CON resulted in, a more dramatic reduction in the number of bands (highly marked in Phe + AM microcosm) and a few populations stimulated. A remarkable dominance of the bands corresponding to the inoculants was observed in both inoculated microcosms, this is in agreement with the results previously found by Coppotelli (Coppotelli et al., 2008) where the predominance of the band belonging to the inoculant was observed until the end of treatment. MDS analysis (Fig. 5) evidenced the highest impact on the community produced by contamination and inoculation.

It has been discussed that the high abundance of the inoculant cells in the soil may cause disturbances on DGGE profiles (Gomes et al., 2005), although that hypothesis was recently discussed by Madueño (Madueño et al., 2015), who showed that the inoculation of a strain under no competitive conditions did not cause a significant reduction of the number of bands in the DGGE profiles of soil bacterial communities.

Our results regarding changes in abundance of different microbial populations and dominance of some microbial members caused by the phenanthrene contamination and bioaugmentation strategies correlates with the analysis of pyrosequencing data (Figs. 7–9), which were performed with a focus on bacterial taxa and their perceived ecological functions.

The Hill numbers (Table 1) showed a significant reduction in microbial richness and diversity in the contaminated microcosm (Phe) with respect to the Control; these results were confirmed with the rarefaction analysis (Fig. 6). Whereas the inoculation with CON caused a reduction in the species richness (^{0}D) compared to Phe microcosm, the inoculation with AM caused the reverse effect (probably due to the higher number of sequences retrieved) with a decrease in diversity index ^{2}D (Table 1). In spite of not having replicates of the pyrosequencing data, the results obtained with this technique can be correlated with the diversity observed on the DGGE profile of Phe + AM microcosm which showed a dramatic reduction in number of bands (Fig. 4). It has been described that a decline in microbial diversity may reduce the soil community resilience toward other natural or manmade perturbations (Degens et al., 2001).

The barcoded pyrosequencing technique used here yielded results that were qualitatively similar to those of the fingerprint-based analysis of soil bacterial communities, but the technique provides a far more robust description of the changes in bacterial community enabling the determination of stimulation or reduction of relative abundances of specific populations compared to their levels in the control microcosm.

The microbial communities of the four microcosms were dominated by seven major bacteria phyla (Proteobacteria, Acidobacteria, Actinobacteria, Verrucomicrobia, Chloroflexi, Firmicutes and Gemmatimonadetes) accounting for more than 95% of the sequences in each of the soils examined (Table S1 in Supplemental material). Overall, bacterial community composition and diversity were strongly influenced by the presence of the contaminant and inoculants (Figs. 7–9) even at a very low level of taxonomic resolution.

The analysis of the sequences obtained showed that the relative abundances of the predominant bacterial phyla (e.g., Proteobacteria, Actinobacteria, and Acidobacteria) changed values significantly according to treatment (Table S1 in Supplemental material). The observed increase of Proteobacteria in contaminated soils (Table S2 in Supplemental material) is not surprising since their ability to utilize aliphatic and aromatic compounds has previously been established, and shifts in their abundance are often noted upon contamination with petrol or during bioremediation (Sutton et al., 2013).

At order level, the higher number of populations repressed was observed as consequence of the inoculation with AM strain to the soil (Fig. 8 and Table S3 in Supplemental material), this corresponds to the previous observation in the DGGE profile (Fig. 4).

Populations remarkably stimulated by phenanthrene contamination were Sphingomonadales, Actinomycetales and Burkholderiales (Fig. 8). Sphingomonadales were mainly represented by the genus *Novosphingobium* and Actinomycetales were mainly represented by the genus *Arthrobacter* (Fig. 9 and Table S4 in Supplemental material). *Arthrobacter* and relatives are environmentally widespread and they have been routinely associated with hydrocarbon-contaminated environments (Hennessee and Li, 2010) maybe because of its physiologic characteristics as biosurfactant production (Kosaric, 2001) and cell membrane fluidity alteration (Kallimanis et al., 2007), what indicates that they can be highly useful in bioremediation.

In the three contaminated microcosms, Sphingomonadales were mainly represented by the genera *Sphingomonas*, *Sphingobium* and *Novosphingobium* (Fig. 9 and Table S4 in Supplemental material). The cause of their increase is expected in inoculated microcosms, since this is the order of the major percentage of strains contained in the inocula (*Sphingobium*). Although when only phenanthrene was incorporated to the soil (Phe microcosm) the highest increase observed was in *Novosphingobium*, another member of Sphingomonadaceae family which is a well known player in the degradation of this kind of contaminants (Seo et al., 2009; Ibarrolaza et al., 2011; Madueño et al., 2011). Despite not having duplicates for pyrosequencing analysis, the shift between the predominance of *Novosphingobium* to *Sphingobium* was also evidenced by DGGE and sequence similarity of cloned bands 2 and 4 (Fig. 4 and Fig. S2 in Supplemental material).

In a degrading community it is useful to have different physiological responses to hydrocarbons in order to increase degradation potential (Bundy et al., 2002). The noticeable increase

of Actinomycetales, slow growing, and Sphingomonadales, fast growing and efficient PHA degraders, in all studied microcosms (Fig. 8) can be evidencing the different strategies developed by the soil community studied here to degrade the contaminant. It is clear that inoculation produced a relative inhibition of Actinomycetales (Figs. 8 and 9), what could be related to the incorporation of a significant mass of cells capable of growing at higher velocity.

Burkholderiales were stimulated in the three contaminated microcosm studied, the differences observed in their relative abundance in inoculated microcosms may be due to the composition of the inocula (in the case of CON consisted of *Achromobacter* in a 2.9%) but also to the cooperation needed for degradation of phenanthrene by the inoculated strain (in Phe + AM microcosm). *Achromobacter* and *Cupriavidus* have been reported as PAH degraders (Goyal and Zylstra, 1996; Perez-Pantoja et al., 2008; Wang et al., 2008).

With the inoculation with AM, additionally to the increase in Sphingomonadales, Burkholderiales and Actinomycetales, an increase in populations of Rhizobiales was observed (Fig. 8). Sequences of Rhizobiales were also found in CON. There have been a few reports dealing with the life of rhizobacteria under hydrocarbon contamination and with the interaction of these bacteria with pollutants. Recently Muratova et al. (2014) (Muratova et al., 2014) demonstrated that *Ensifer meliloti* was able to degrade phenanthrene in the presence of succinic acid as a co-substrate.

Two orders, Holophagales and Acidobacteriales, belonging to Acidobacteria phylum were solely stimulated on Phe + CON microcosm. Although Acidobacteria are widespread and abundant in soils, the vast majority for which 16S rRNA gene sequences have been obtained, still remain uncultured, and consequently, their role in the environment is poorly understood (Xie et al., 2011).

The ecology and behavior of microbial community in soil are more complex than prospected from their physiological characteristics or abundance in PAH contaminated soils. Some members of the microbial community might be able to secrete important degradative enzymes or growth factors, whereas others may produce biosurfactants leading to the enhanced solubilization of hydrophobic hydrocarbons for their better utilization by microbes (Mukherjee and Bordoloi, 2011).

5. Conclusion

In spite of the knowledge of the advantages of using microbial consortia for bioaugmentation, in this study, since both inoculations produced a positive effect in phenanthrene degradation, inoculation with single strain AM reached the lowest phenanthrene concentration at the end of treatment; the use of this inoculant, became the best strategy of those two analyzed here to remediate phenanthrene contaminated soil mainly on the basis of the degradation efficiency.

However, the results obtained from the fingerprint and the high throughput techniques, revealed that inoculation with AM caused more drastic changes in microbial community than inoculation with CON, what can be compromising the ulterior functionality of the soil. A first evidence of this impact was observed in dehydrogenase activity. Observing the minor impact produced on the bacterial community, inoculation with CON is a strategy that should be considered.

This study was able to describe the main behavior of community composition and diversity during phenanthrene degradation in the bioaugmented microcosms, and DGGE has proven to be a simple and valid method to monitor these systems, since a good correlation with pyrosequencing data was obtained; although a large amount of the variability in bacterial community structure remains unexplained. It would be of interest to perform further studies to monitor whether the original bacterial community reestablishes itself in terms of composition and ecological function after contaminations and inoculations such as those described here.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.apsoil.2015.08.025.

References

Alexander, M., 1999. Biodegradation and Bioremediation, 2nd ed. Academic Press, Inc., San Diego, Calif.

Amann, R.I., Ludwig, W., Schleifer, K.H., 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. 59, 143–169.

Andreoni, V., Cavalca, L., Rao, M.A., Nocerino, G., Bernasconi, S., Dell'Amico, E., Colombo, M., Gianfreda, L., 2004. Bacterial communities and enzyme activities of PAHs polluted soils. Chemosphere 57, 401–412.

ATSDR (Agency for Toxic Substances and Disease Registry), 2005. CERCLA Priority list of hazardous substances US Department of Health and Human Service. Atlanta, G., USA [WWW.document]. URL http://www.asdr.cdc.gov/cercla/05list.html.

Bundy, J.G., Paton, G.I., Campbell, C.D., 2002. Microbial communities in different soil types do not converge after diesel contamination. J. Appl. Microbiol. 92, 276–288.

Cole, J.R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R.J., Kulam-Syed-Mohideen, A.S., McGarrell, D.M., Marsh, T., Garrity, G.M., Tiedje, J.M., 2009. The Ribosomal database project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res. 37, D141–D145.

Colwell, R.K., 2013. EstimateS: Statistical estimation of species richness and shared species from samples., Version 9. User's Guide and application published at: http://viceroy.eeb.uconn.edu/estimates.

Coppotelli, B.M., Ibarrolaza, A., Del Panno, M.T., Morelli, I.S., 2008. Effects of the inoculant strain *Sphingomonas paucimobilis* 20006FA on soil bacterial community and biodegradation in phenanthrene-contaminated soil. Microb. Ecol. 55, 173–183.

Coppotelli, B.M., Ibarrolaza, A., Dias, R.L., Del Panno, M.T., Berthe-Corti, L., Morelli, I. S., 2010. Study of the degradation activity and the strategies to promote the bioavailability of phenanthrene by *Sphingomonas paucimobilis* strain 20006FA. Microb. Ecol. 59, 266–276.

Chao, A., 2005. Species estimation and applications, 2nd ed. Encyclopedia of Statistical Sciences, vol. 12. Wiley, New York, pp. 7907–7916.

Chao, A., Chiu, C.-H., Hsieh, T.C., 2012. Proposing a esolution to debates on diversity partitioning. Ecology 93, 2037–2051.

Chen, H., Boutros, P.C., 2011. VennDiagram: a package for the generation of highly-customizable Venn and Euler diagrams in R. BMC Bioinf. 12 (1), 35.

de Araujo, J.C., Schneider, R.P., 2008. DGGE with genomic DNA: suitable for detection of numerically important organisms but not for identification of the most abundant organisms. Water Res. 42, 5002–5010.

Degens, B.P., Schipper, L.A., Sparling, G.P., Duncan, L.C., 2001. Is the microbial community in a soil with reduced catabolic diversity less resistant to stress or disturbance? Soil Biol. Biochem. 33, 1143–1153.

Dejonghe, W., Boon, N., Seghers, D., Top, E.M., Verstraete, W., 2001. Bioaugmentation of soils by increasing microbial richness: missing links. Environ. Microbiol. 3, 649–657.

Dowd, S.E., Callaway, T.R., Wolcott, R.D., Sun, Y., McKeehan, T., Hagevoort, R.G., EdringtonT, S., 2008a. Evaluation of the bacterial diversity in the feces of cattle using 16S rDNA bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP). BMC Microbiol. 125.

Dowd, S.E., Sun, Y., Wolcott, R.D., Domingo, A., Carroll, J.A., 2008b. Bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) for microbiome studies: bacterial diversity in the ileum of newly weaned Salmonella-infected pigs. Foodborne Pathog. Dis. 5, 459–472.

Dowd, S.E., Zaragoza, J., Rodriguez, J.R., Oliver, M.J., Payton, P.R., 2005. Windows .NET network distributed basic local alignment search toolkit (W.ND-BLAST). BMC Bioinform. 6, 93.
Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26, 2460–2461.
Epelde, L., Mijangos, I., Becerril, J.M., Garbisu, C., 2009. Soil microbial community as bioindicator of the recovery of soil functioning derived from metal phytoextraction with sorghum. Soil Biol. Biochem. 41, 1788–1794.
Festa, S., Coppotelli, B.M., Morelli, I.S., 2013. Bacterial diversity and functional interactions between bacterial strains from a phenanthrene-degrading consortium obtained from a chronically contaminated-soil. Int. Biodeterior. Biodegrad. 85, 42–51.
Fromin, N., Hamelin, J., Tarnawski, S., Roesti, D., Jourdain-Miserez, K., Forestier, N., Teyssier-Cuvelle, S., Gillet, F., Aragno, M., Rossi, P., 2002. Statistical analysis of denaturing gel electrophoresis (DGE) fingerprinting patterns. Environ. Microbiol. 4, 634–643.
Ghazali, F.M., Rahman, R.N.Z.A., Salleh, A.B., Basri, M., 2004. Biodegradation of hydrocarbons in soil by microbial consortium. Int. Biodeterior. Biodegrad. 54, 61–67.
Gojgic-Cvijovic, G.D., Fau, M.J., Solevic, T.M., Beskoski, V.P., Ilic, M.V., Djokic, L.S., Narancic, T.M., Vrvic, M.M., 2012. Biodegradation of petroleum sludge and petroleum polluted soil by a bacterial consortium: a laboratory study. Biodegradation 23, 1–14.
Gomes, N., Kosheleva, I.A., Abraham, W.R., Smalla, K., 2005. Effects of the inoculant strain *Pseudomonas putida* KT2442 (pNF142) and of naphthalene contamination on the soil bacterial community. FEMS Microbiol. Ecol. 54, 21–33.
González, N., Simarro, R., Molina, M., Bautista, L., Delgado, L., Villa, J., 2011. Effect of surfactants on PAH biodegradation by a bacterial consortium and on the dynamics of the bacterial community during the process. Bioresour. Technol. 102, 9438–9446.
Goyal, A.K., Zylstra, G.J., 1996. Molecular cloning of novel genes for polycyclic aromatic hydrocarbon degradation from *Comamonas testosteroni* GZ39. Appl. Environ. Microbiol. 62, 230–236.
Hennessee, C., Li, Q., 2010. Micrococcineae: arthrobacter and relatives. Handbook of Hydrocarbon and Lipid Microbiology. Springer, pp. 1853–1864.
Hervé, V., Le Roux, X., Uroz, S., Gelhaye, E., Frey-Klett, P., 2014. Diversity and structure of bacterial communities associated with *Phanerochaete chrysosporium* during wood decay. Environ. Microbiol. 16, 2238–2252.
Hill, M.O., 1973. Diversity and evenness: a unifying notation and its consequences. Ecology 54 (2), 427–432.
Ibarrolaza, A., Coppotelli, B., Del Panno, M., Donati, E., Morelli, I., 2011. Application of the knowledge based approach to strain selection for a bioaugmentation process of phenanthrene and Cr (VI) contaminated soil. J. Appl. Microbiol. 111, 26–35.
Jacques, R.J.S., Okeke, B.C., Bento, F.M., Teixeira, A.S., Peralba, M.C.R., Camargo, F.A.O., 2008. Microbial consortium bioaugmentation of a polycyclic aromatic hydrocarbons contaminated soil. Bioresour. Technol. 99, 2637–2643.
Jost, L., 2006. Entropy and diversity. Oikos 113, 363–375.
Kallimanis, A., SF, Drainas, C., Koukkou, A.I., 2007. Taxonomic identification, phenanthrene uptake activity, and membrane lipid alterations of the PAH degrading *Arthrobacter* sp. strain Sphe3. Appl. Microbiol. Biotechnol. 76, 709–717.
Kosaric, N., 2001. Biosurfactants and their application for soil bioremediation. Food Technol. Biotechnol. 39, 295–304.
Labud, V., Garcia, C., Hernandez, T., 2007. Effect of hydrocarbon pollution on the microbial properties of a sandy and a clay soil. Chemosphere 66, 1863–1871.
Madueño, L., Alvarez, H., Morelli, I., 2015. Autochthonous bioaugmentation to enhance phenanthrene degradation in soil microcosms under arid conditions. Int. J. Environ. Sci. Technol. 12, 2317–2326.
Madueño, L., Coppotelli, B., Alvarez, H., Morelli, I., 2011. Isolation and characterization of indigenous soil bacteria for bioaugmentation of PAH contaminated soil of semiarid Patagonia, Argentina. Int. Biodeterior. Biodegrad. 65, 345–351.
Mrozik, A., Piotrowska-Seget, Z., 2010. Bioaugmentation as a strategy for cleaning up of soils contaminated with aromatic compounds. Microbiol. Res. 165, 363–375.
Muckian, L.M., Grant, R.J., Clipson, N.J., Doyle, E.M., 2009. Bacterial community dynamics during bioremediation of phenanthrene-and fluoranthene-amended soil. Int. Biodeterior. Biodegrad. 63, 52–56.
Mukherjee, A.K., Bordoloi, N.K., 2011. Bioremediation and reclamation of soil contaminated with petroleum oil hydrocarbons by exogenously seeded bacterial consortium: a pilot-scale study. Environ. Sci. Pollut. Res. 18, 471–478.
Muratova, A., Pozdnyakova, N., Makarov, O., Baboshin, M., Baskunov, B., Myasoedova, N., Golovleva, L., Turkovskaya, O., 2014. Degradation of phenanthrene by the rhizobacterium *Ensifer meliloti*. Biodegradation 25, 787–795.
Muyzer, G., Brinkahoff, T., Nübel, U., Santegoedes, C., Schäfer, H., Wawer, C., 1998. Denaturing gradient electrophoresis (DGGE) in microbial ecology. In: Akkermanns, A., Van Elsas, J., deBrujin, F. (Eds.), Molecular Microbial Ecology Manual. Kluwer Academic Press, Dordretch, pp. 1–27.
Muyzer, G., de Waal, E.C., Uitterlinden, A.G., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59, 695–700.
Neuwirth, E. 2014. RColorBrewer: ColorBrewer palettes. R package version 1.1–2. http://CRAN.R-project.org/package=RColorBrewer.
Patel, V., Jain, S., Madamwar, D., 2012. Naphthalene degradation by bacterial consortium (DV-AL) developed from Alang–Sosiya ship breaking yard, Gujarat, India. Bioresour. Technol. 107, 122–130.
Pearson, E., 1926. Review of Statistical Methods for Research Workers (R.A. Fisher), First edition, Sci. Prog., 20, 733–734.
Perez-Pantoja, D., De la Iglesia, R., Pieper, D., Gonzalez, B., 2008. Metabolic reconstruction of aromatic compounds degradation from the genome of the amazing pollutant-degrading bacterium *Cupriavidus* necator JMP134. FEMS Microbiol. Rev. 32, 736–794.
Poder Ejecutivo Nacional, Ley Nacional 24051, 1992. Residuos Peligrosos, generación, manipulación, transporte y tratamiento., República Argentina. Decreto Reglamentario 831. Boletín Oficial 03/01/1993.
Puglisi, E., Cappa, F., Fragoulis, G., Trevisan, M., Del Re, A.A., 2007. Bioavailability and degradation of phenanthrene in compost amended soils. Chemosphere 67, 548–556.
Reasoner, D., Geldreich, E., 1985. A new medium for the enumeration and subculture of bacteria from potable water. Appl. Environ. Microbiol. 49, 1–7.
Samanta, S.K., Singh, O., Jain, R.K., 2002. Polycyclic aromatic hydrocarbons: environmental pollution and bioremediation. Trends Biotechnol. 20, 243–248.
Seo, J.-S., Keum, Y.-S., Li, Q.X., 2009. Bacterial degradation of aromatic compounds. Int. J. Res. Public Health 6, 278–309.
Simarro, R., Gonzalez, N., Bautista, L.F., Molina, M.C., 2013. Assessment of the efficiency of in situ bioremediation techniques in a creosote polluted soil: change in bacterial community. J. Hazard Mater. 262, 158–167.
Sun, M.Y., Dafforn, K.A., Johnston, E.L., Brown, M.V., 2013. Core sediment bacteria drive community response to anthropogenic contamination over multiple environmental gradients. Environ. Microbiol. 15, 2517–2531.
Sutton, N.B., Maphosa, F., Morillo, J.A., Al-Soud, W.A., Langenhoff, A.A., Grotenhuis, T., Rijnaarts, H.H., Smidt, H., 2013. Impact of long-term diesel contamination on soil microbial community structure. Appl. Environ. Microbiol. 79, 619–630.
Szulc, A., Ambrożewicz, D., Sydow, M., Ławniczak, Ł., Piotrowska-Cyplik, A., Marecik, R., Chrzanowski, Ł., 2014. The influence of bioaugmentation and biosurfactant addition on bioremediation efficiency of diesel-oil contaminated soil: feasibility during field studies. J. Environ. Manag. 132, 121–128.
Tamaki, H., Wright, C., Li, X., Lin, Q., Hwang, C., Wang, S., Thimmapuram, J., Kamagata, Y., Liu, W.-T., 2011. Analysis of 16S rRNA amplicon sequencing options on the roche/454 next-generation titanium sequencing platform. PLoS One 6.
Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596–1599.
Tyagi, M., da Fonseca, M.M.R., de Carvalho, C.C., 2011. Bioaugmentation and biostimulation strategies to improve the effectiveness of bioremediation processes. Biodegradation 22, 231–241.
Vecchioli, G., Del Panno, M., Painceira, M., 1990. Use of selected autochthonous soil bacteria to enhanced degradation of hydrocarbons in soil. Environ. Pollut. 67, 249–258.
Wang, B., Lai, Q., Cui, Z., Tan, T., Shao, Z., 2008. A pyrene degrading consortium from deep sea sediment of the West Pacific and its key member *Cycloclasticus* sp. P1. Environ. Microbiol. 10, 1948–1963.
Wrenn, B.A., Venosa, A.D., 1996. Selective enumeration of aromatic and aliphatic hydrocarbon degrading bacteria by a most-probable-number procedure. Can. J. Microbiol. 42, 252–258.
Xie, S., Sun, W., Luo, C., Cupples, A.M., 2011. Novel aerobic benzene degrading microorganisms identifiedin three soils by stable isotope probing. Biodegradation 22–71.