FEMS Microbiology Ecology, 92, 2016, fiw125

doi: 10.1093/femsec/fiw125
Advance Access Publication Date: 7 June 2016
Research Article

RESEARCH ARTICLE

Monitoring the impact of bioaugmentation with a PAH-degrading strain on different soil microbiomes using pyrosequencing

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One sentence summary: The effect of a bioaugmentation technique on different soils microbiomes showed that inoculation managed to increase the richness and diversity producing a beneficial effect on soil ecology regardless of favouring the process of bioremediation.

Editor: Petr Baldrian

ABSTRACT

The effect of bioaugmentation with *Sphingobium* sp. AM strain on different soils microbiomes, pristine soil (PS), chronically contaminated soil (IPK) and recently contaminated soil (Phe) and their implications in bioremediation efficiency was studied by focusing on the ecology that drives bacterial communities in response to inoculation. AM strain draft genome codifies genes for metabolism of aromatic and aliphatic hydrocarbons. In Phe, the inoculation improved the elimination of phenanthrene during the whole treatment, whereas in IPK no improvement of degradation of any PAH was observed. Through the pyrosequencing analysis, we observed that inoculation managed to increase the richness and diversity in both contaminated microbiomes, therefore, independently of PAH degradation improvement, we observed clues of inoculant establishment, suggesting it may use other resources to survive. On the other hand, the inoculation did not influence the bacterial community of PS. On both contaminated microbiomes, incubation conditions produced a sharp increase on *Actinomycetales* and *Sphingomonadales* orders, while inoculation caused a relative decline of *Actinomycetales*. Inoculation of most diverse microbiomes, PS and Phe, produced a coupled increase of *Sphingomonadales*, *Burkholderiales* and *Rhizobiales* orders, although it may exist a synergy between those genera; our results suggest that this would not be directly related to PAH degradation.

Keywords: bioaugmentation; *Sphingobium* sp. AM genome; soil microbiome; inoculant establishment; community diversity; pyrosequencing

Received: 18 February 2016; **Accepted:** 2 June 2016

INTRODUCTION

Understanding the potential of microorganisms into a soil microbiome to improve polycyclic aromatic hydrocarbons (PAH) biodegradation efficiency and the influence of this inoculant on native microbial communities concerns bioremediation and ecological evaluation.

Bioremediation process efficiency of contaminated areas depends on several factors such as the presence of microorganisms with appropriate catabolic capacity on the contaminated site, environmental and nutritional conditions, contaminant composition and concentration and pollutant bioavailability to microbial attack (Cerqueira *et al.* 2014). Improved bioremediation may be obtained by bioaugmentation with catabolically relevant microorganisms. It has been previously postulated that the growth and spread of an inoculant is associated with the degree to which it can gain access to (previously unavailable) local resources (Mallon, Elsas and Salles 2015).

Successful effect on the introduction of foreign microorganisms into an environment is considered when the introduced species have the potential to alter the interactions within the community resulting in strong shifts in community structure, having neutral, beneficial or detrimental impacts in the new habitat (Mallon, Elsas and Salles 2015).

The introduced strain behaviour in a complex system like the native microbiome will depend on its ability to survive or grow in the new environment subjected to factors such as water availability, pH or temperature and accessibility of the target pollutant or other metabolisable substrates (Moreno-Forero and van der Meer 2015).

At a regional scale, many abiotic factors, such as soil type and texture, pH and carbon availability, play significant roles in shaping microbial communities (Hu *et al.* 2014). For this reason, it is feasible to expect that soils with different abiotic factors and different history of contamination differentially respond to bioremediation strategies (Coppotelli *et al.* 2008; Cerqueira *et al.* 2014; Pessacq *et al.* 2015; Ren *et al.* 2015).

In this study, soils with different history of contamination were used in preparation of microcosms, with the aim of evaluating the bioremediation efficiency and the effect of a bioaugmentation strategy on the soil microbiome using pyrosequencing through time.

Recent advancements in sequencing technologies and molecular techniques have opened new frontiers in microbial community analysis by providing unprecedented levels of coverage and resolution of the soil microbiome (Fodelianakis *et al.* 2015; Ren *et al.* 2015). Pyrosequencing is a powerful molecular tool for gaining insight into microbial diversity in contaminated soils and its dynamics throughout bioremediation processes (Llado *et al.* 2015).

In recently contaminated soil, the resource pulse modulates the species richness and could suppress competition from native microbiome, allowing an inoculant to successfully invade and integrate into a new locality (Mallon *et al.* 2015). On the other hand, soils exposed to long-term contamination with hydrocarbons may present extreme diverse structural and functional microbial communities (Pessacq *et al.* 2015); in these soils, higher number of available ecological niches may offer a good chance for one inoculant to establish, despite the low bioavailability of pollutants.

We hypothesised that inoculation success depends on the local microbiome, which in turn depends on its physicochemical properties and contamination history. Either in recently or in chronically contaminated soils, inoculation could be successful, since the inoculant has access to resources.

The obtained data will improve our understanding of the ecology that drives bacterial communities in response to inoculation and will help in designing appropriate management and cleanup of PAH-contaminated soil ecosystems.

MATERIALS AND METHODS

Bacterial strain and DNA extraction for genome sequencing

Sphingobium sp. AM was isolated from a chronically hydrocarbon contaminated soil near La Plata city, Argentina (Festa, Coppotelli and Morelli 2013). It was characterised in terms of degradation capacity in liquid mineral medium (Vecchioli, Del Panno and Painceira 1990) with different substrates (200 mg l^{-1}) (phenanthrene, anthracene and fluorene).

For the bioaugmentation strategies and prior to DNA extraction, the AM inoculum was cultured and treated as described in Festa, Coppotelli and Morelli (2016). Total DNA of AM strain for whole genome sequencing was extracted as described by Entcheva, Liebl and Johann (2001).

Whole genome sequencing assembly and annotation

The genome of AM strain was sequenced using HiSeq 1500 Illumina (2 × 100 bp paired-end reads) sequencing technology. Raw Illumina sequence data were quality trimmed and filtered using Nextera® XT Illumina protocol. *De novo* assembly of these reads was carried out with Illumina's A5-miseq Assembly Pipeline (v. 2.0) platform (Tritt *et al.* 2012). Two strategies were used to analyse the genome. First, the Rapid Annotation using Subsystem Technology (Aziz *et al.* 2008) server version 2.0 was used to predict and annotate the genes on the draft genome. The SEED viewer (Overbeek *et al.* 2005) was used to categorise predicted genes into functional subsystems. Later on, functional assignment of genes was performed by searching KEGG database followed by manual curation using the genome viewer Artemis (Rutherford *et al.* 2000) (http://www.sanger.ac.uk/Software/Artemis). To determine each probable coding sequence (CDS), a BLASTX alignment by the NCBI database was performed (http://blast.ncbi.nlm.nih.gov). The draft genome of AM strain has been deposited in GenBank WGS under the accession number LRUK00000000. Automated annotation, propagation from GenBank and curation by NCBI staff, was performed.

Soils characteristics

Two different soils were selected, an uncontaminated soil (pristine soil: PS) and a soil chronically polluted with petrochemical sludge (IPK), both from an area near La Plata city, Argentina. Both soils were analysed in the Laboratory of Soil Science at the University of La Plata and their physicochemical properties are shown in Table 1.

Preparation of soils microcosms and bioaugmentation assay

Soil microcosms were performed with of 0.5 kg of sieved soil (2-mm mesh) in a glass container with 1 kg capacity. For recently contaminated treatment, PS was artificially contaminated with 2000 mg of phenanthrene (Carlo Erba, Milan, Italy, >99.5% purity) per kilogram of dry soil. Phenanthrene was delivered in acetone solution (150 mg ml^{-1}) and mixed into the soil manually.

Table 1. Physicochemical properties of the two soils used in this study.

	PS	IPK
Coordinates	34°51′24.6″S 58°06′54.2″W	34°53′19″S 57°55′38″W
Soil texture	Clay loam	Loam
pH	5.8–5.9	7.71
Carbon (%)	3.60	2.2
Organic matter (%)	6.21	3.78
Nitrogen (%)	0.30	0.2
Phosphorus (mg kg^{-1})	4.20	8.33
C/N	12.16	11
EC (mS cm^{-1})	1.1	2.72
Aliphatic hydrocarbons(mg kg^{-1})	Not determined	199.70
PAHs (mg kg^{-1})	Not determined	885.96
Total hydrocarbons (mg kg^{-1})	50	Not determined

With PS, three treatments were carried out in triplicates:(1) PS + AM: a non-contaminated and inoculated microcosm with 1×10^8 cells of AM strain per gram of dry soil; (2) Phe: a contaminated and non-inoculated microcosm; (3) Phe + AM: a contaminated and inoculated microcosm with 1×10^8 cells of AM strain per gram of dry soil 1 day after phenanthrene was added to the soil. In regard to the chronically contaminated soil, one treatment was performed in triplicates: (1) IPK + AM: microcosm inoculated with 1×10^8 cells of AM strain per gram of dry soil.

A PS microcosm and a non-inoculated microcosm (IPK) were used as controls of incubation conditions (made in triplicate). Control microbiomes were the original soils on day 1 of incubation, control PS and control IPK.

In both inoculated treatments (Phe + AM and IPK + AM), a specific volume of the obtained cells suspension was added to achieve the desired inoculum density. The same volume of bidistilled water was added to Phe and control microcosms to standardise 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 ± 2% by adding distilled water.

Chemical extraction of hydrocarbons and analysis

The phenanthrene concentration of the artificially contaminated soil microcosms was determined by HPLC-UV using an exhaustive extraction methods described in a previous work (Festa, Coppotelli and Morelli 2016). Total hydrocarbon (PAH and aliphatic hydrocarbons) concentration in soil samples of the chronically contaminated microcosms was extracted with dichloromethane: acetone according to Mora *et al.* (2014) and analysed in a Perkin–Elmer autosystem gas chromatograph according to Del Panno *et al.* (2005).

Microbial enumeration and dehydrogenase activity

The determination of total heterotrophic cultivable bacteria was performed on R2A medium plates according to Reasoner and Geldreich (1985), and determination of PAH degraders was performed in sterile polypropylene microplates with a mixture of PAH as substrate according to Wrenn and Venosa (1996). The dehydrogenase activity, an indicator of the broad range of oxidative processes in soil, was determined according to Andreoni *et al.* (2004). For detailed protocols, see Festa, Coppotelli and Morelli (2016). All determinations were performed in triplicate.

The statistical analysis of dehydrogenase activity and counts data were performed by parametric one-way ANOVA test, followed by Tukey's honestly significant difference post-hoc test, using XLStat-Pro statistical package v7.5.2 (Addinsoft SARL, France).

Soil DNA extraction, barcoded PCR and pyrosequencing of the 16S rRNA gene

From 1 g of soil sample of each microcosm, total DNA was extracted at different days in the incubation period (1, 14 and 63) with E.Z.N.A.® Soil DNA Kit (Omega Bio-Tek, Inc., Norcross, GA, USA) following manufacturer's instructions.

All samples were used for PCR amplification using the 16S rRNA universal bacterial primers, 341Fbac (CCTACGGGAGGCAGCAG) (Muyzer, De Waal and Uitterlinden 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 as described in a previous work (Festa, Coppotelli and Morelli 2016). 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 utilising 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 on established and validated protocols (http://www.mrdnalab.com/). These sequence data are available at the NCBI Short-Read Archive under accession number SRP071329. Because we have only duplicates, results were interpreted with caution.

Processing and analysis of the pyrosequencing dataset

The sequence data derived from the high-throughput sequencing process were analysed 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 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 utilised 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.* 2008). Taxonomy was defined based on the following percentages: >97%, species; between 97% and 95%, unclassified genus; between 90% and 85%, unclassified order; between 85% and 80%, unclassified class; between 80% and 77%, unclassified phylum; <77%, unclassified.

These sequences data are available at the NCBI Short-Read Archive under accession number SRP071329.

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 as in previous work (Festa, Coppotelli and Morelli 2016).

Rarefaction curves, diversity and richness measurements were calculated using EstimateS (Version 9) (Colwell 2013). Because these measurements are influenced by sequencing depth, normalisation was performed through resampling and estimated diversity was computed from 906 sequences (corresponding to the number of reads in the shallowest sampled community) that were randomly drawn from each sample. Correspondence analysis (CA) was performed on the pyrosequencing data using the frequency values of each OTU to identify samples with similar bacterial composition for pristine and contaminated soils. The bacterial communities were also explored constructing a Heat map. Clustering was based on the Bray–Curtis dissimilarity index calculated by OTUs at a distance of 3%. To identify the number of OTUs shared between different samples, we generated Venn diagrams of pristine and contaminated soils. The CA, Heat map and Venn diagrams were implemented in R Statistical Software version 3.1.2 (R Core Team 2014) using libraries 'vegan' (Oksanen *et al.* 2015), 'VennDiagram' (Chen 2014) and 'fields' libraries (Nychka, Furrer and Sain 2014).

RESULTS

AM strain

AM strain was isolated from a chronically contaminated soil, identified by 16S rRNA gene sequence and biochemical characteristics as *Sphingobium* sp. (Festa, Coppotelli and Morelli 2013). In pure culture, AM strain showed the ability to degrade phenanthrene, anthracene and fluorene.

The assembled draft genome of AM strain (*Sphingobium* sp.) has a total length of 5318 831 bp, consisting of 112 scaffolds (N50 = 105 075 bp; mean scaffold size = 47 490 bp; median scaffold size = 23 840 bp) with a GC content of 61%. Annotation identified 435 subsystems (network of metabolites and enzymes that comprise, for example, a metabolic or a signal transduction pathway), 5001 CDS and 52 RNAs.

Genes for a complete set of enzymes involved in the degradation of aromatic (49 CDS) and aliphatic (35 CDS) hydrocarbons (Table S1, Supporting Information) are encoded in the genome of AM. Proteins for the alpha and beta subunits of aromatic-ring-hydroxylating dioxygenase, the initial enzyme in PAH degradation pathway and all the enzymes needed to mineralise PAH compounds were predicted from the annotated genome.

Original soil microbiomes

The taxonomic composition and the diversity of the bacterial microbiomes of the two soils used for the inoculation experiments, uncontaminated soil (PS) and soil chronically polluted with petrochemical sludge (IPK), were profiled using pyrosequencing of PCR-amplified bacterial 16S rRNA gene fragments. Pyrosequencing analysis was performed in duplicate.

The number of bacterial sequences obtained by pyrosequencing ranged from 2293 to 7226, and they were clustered into 1371 OTUs at a 97% similarity threshold (see in Table 3).

The rarefaction curve of pristine soil (control PS) did not reach a plateau even though more than 7000 reads were sequenced (Fig. S2, Supporting Information), indicating that its microbial community was very complex. On the other hand, in control IPK soil microcosm, rarefaction analysis showed that even with less than 2300 sequences retrieved, it was enough to cover most of the diversity.

Good's coverage values for control PS and control IPK soil were of 0.94 and 0.99 (Table 3), respectively. As the values approached 1.0, that is, although the rarefaction curve did not reach a plateau for control PS, the achieved coverage was enough to cover the diversity.

To assess the complexity of the individual bacterial community, the diversity and richness estimators described by Hill (1973) were obtained (see in Fig. 2). The PS shows a high richness (^{0}D), a very high diversity (^{1}D and ^{2}D) being a community slightly uneven. On the contrary, the chronically contaminated soil (IPK) possesses very low diversity and richness estimators in comparison to PS microcosms, with a highly uneven community.

IPK soil was dominated by the orders *Pseudomonadales* (88.3%) and *Bacillales* (11.0%), where the main representing genera were *Acinetobacter* (88.3%) and *Bacillus* (8.5%), respectively (Tables S3 and S4, Supporting Information).

In PS, the predominant orders were *Acidobacteriales* (21.8%), *Holophagales* (14.2%), *Verrucomicrobiales* (13.8%) and their main representing genera were *Acidobacterium* (20.3%), *Holophaga* (13.9%) and *Prosthecobacter* (12.6%) (Tables S3 and S4).

Bioremediation efficiency

The effects of bioaugmentation strategy on PAH bioremediation efficiency in both recently contaminated soil (Phe) and soil chronically polluted with petrochemical sludge (IPK) are shown in Table 2.

Chronically polluted soil contained 10 priority PAH in a total concentration of 885.9 mg kg^{-1} of dry soil and aliphatic hydrocarbons in a total concentration of 199.7 mg kg^{-1} of dry soil (IPK).

The PS artificially contaminated contained 2000 mg of phenanthrene kg^{-1} of dry soil (Phe).

During incubation time (63 days), bioaugmentation with AM strain significantly enhanced degradation of phenanthrene ($P < 0.05$) in recently contaminated soil (Phe + AM) reaching a degradation of 99.67 ± 0.29% of the phenanthrene supplied in comparison to Phe (95.86 ± 0.18%). On the contrary, chronically contaminated soil inoculation with AM (IPK + AM) did not significantly enhance the specific degradation of phenanthrene (67.89 ± 6.74%), since no difference was observed in comparison to the control (IPK) (50.30 ± 13.16%) (Table 2A and B).

The PAH degradation either in IPK or in IPK + AM occurred mainly during the first 14 days of incubation (Table 2B), considering no differences were observed in comparison to degradation on day 63.

The addition of the inoculant to chronically contaminated soil (IPK + AM) did not significantly increase ($P < 0.05$) the degradation of the other nine PAH (Table 2B) or aliphatic hydrocarbon (data not shown) after 63 days of incubation.

Microbial enumeration and dehydrogenase activity

Total counts of heterotrophic and PAH-degrading microorganisms were determined over 63 days of treatment. Heterotrophic bacterial counts revealed an initial concentration of cells between 7.8 ± 0.11 and 9.1 ± 0.16 $\log_{10}$ CFU g^{-1} in the six microcosms analysed. After 63 days, no significant differences ($P < 0.05$) with initial values and throughout the different treatments were observed (data not shown).

Throughout the experimental trial, MPN of PAH-degrading bacteria (Fig. 1) ranged from 3.1 to 8.9 $\log_{10}$ MPN g^{-1} for the different treatments. Inoculation produced a significant increase ($P < 0.05$) in number of PAH-degrading bacteria in the three inoculated microcosms (PS + AM, Phe + AM and IPK + AM), in comparison to the non-inoculated ones. The number of PAH-degrading bacteria gradually decreased ($P < 0.05$) in each

Table 2. Degradation percentage of PAH in **(A)** Phe, Phe + AM and **(B)** IPK and IPK + AM microcosms after 14 and 63 days of incubation. Results are means of triplicate independent microcosms. STD: standard deviations.

A	PAH	Phe d14		Phe d63		Phe + AM d14		Phe + AM d63	
		%	STD	%	STD	%	STD	%	STD
	Phenanthrene	53.74	6.68	95.86	0.18	91.41	0.58	99.67	0.29

B	PAH	IPK d14		IPK d63		IPK + AM d14		IPK + AM d63	
		%	STD	%	STD	%	STD	%	STD
	Acenaphthylene	71.12	1.14	61.39	9.48	73.83	2.13	61.80	9.45
	Fluorene	71.46	1.69	71.42	4.84	82.72	3.79	68.26	10.12
	Phenanthrene	70,772	1,94	50.30	13.16	71.29	5.90	67.89	6.74
	Anthracene	67.99	3.32	59.42	8.41	71.29	4.22	59.45	8.48
	Fluoranthene	74.23	2.38	59.32	3.89	78.37	6.50	68.29	4.22
	Pyrene	65.40	8.47	50.36	3.31	68.90	7.37	44.22	10.02
	Benzo [a] anthracene	68.08	1.84	49.47	3.78	68.65	4.87	56.21	3.15
	Crisene	57.84	13.03	72.89	2.18	80.31	10.87	76.39	5.71
	Benzo [b] fluoranthene + Benzo [k] fluoranthene	74.23	2.07	57.79	12.59	74.91	9.18	59.83	4.55
	Benzo [a]pyrene	70.20	2.36	55.61	13.61	77.30	10.65	55.27	13.20

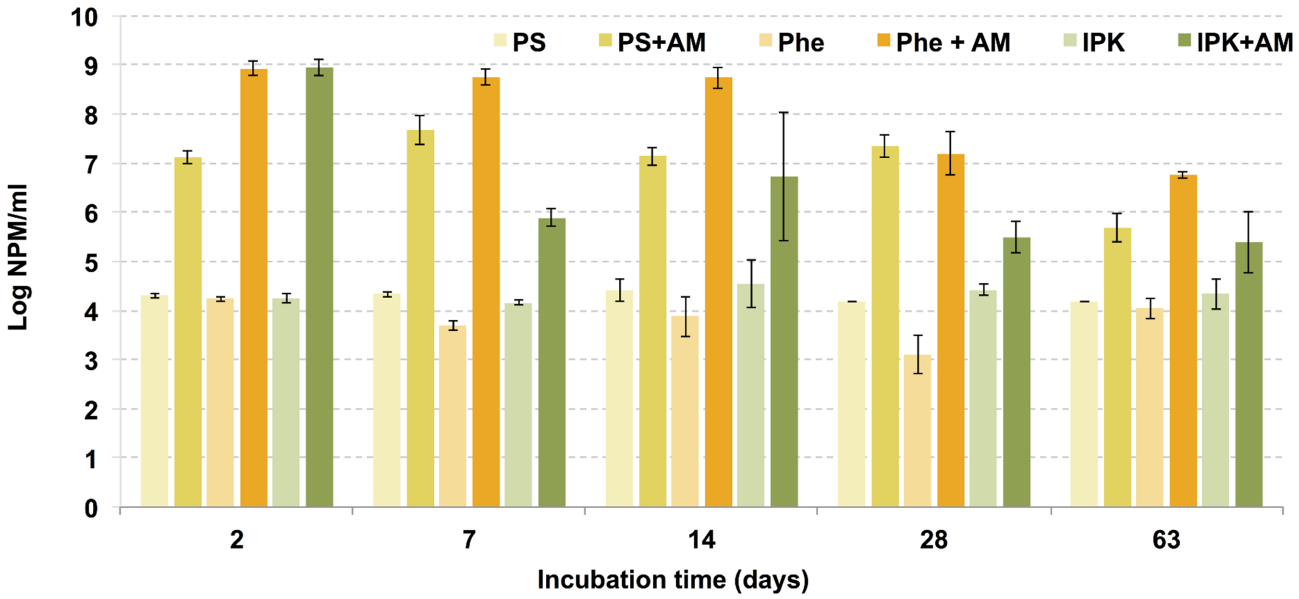


Figure 1. Dynamic of PAH-degrading bacteria populations (log_{10} MPN g^{-1} of dry soil) in PS, PS + AM, Phe, Phe + AM, IPK and IPK + AM microcosms during bioremediation process. Results are means of triplicate independent microcosms. Bars represent standard deviations.

inoculated microcosm until the end of the experiment, with the more marked decrease observed in IPK + AM microcosm (Fig. 1). Despite this decrease, the values were still significantly higher in the inoculated microcosms during the whole experiment (63 days).

Regarding dehydrogenase activity, the incorporation of phenanthrene (Phe) produced a significant increase ($P < 0.05$) in comparison to the control microcosm (PS). Additionally, while inoculation in Phe + AM microcosm significantly increased ($P < 0.05$) dehydrogenase activity after 7 days of incubation in comparison to Phe, IPK + AM and PS + AM microcosms, it did not show any significant difference ($P < 0.05$) with the non-inoculated microcosms (IPK and PS) throughout the whole experiment (Fig. S1, Supporting Information).

Bacterial diversity, structure and composition of the studied microcosms

The taxonomic composition and the diversity of the bacterial microbiomes present in the six soil microcosms on day 14 and 63 of treatment were profiled using pyrosequencing. The number of bacterial sequences obtained by pyrosequencing ranged from 906 to 10 598, and they were clustered into 3490 OTUs at a 97% similarity threshold (Table 3).

Table 3. Number of sequences retrieved by the pyrosequencing analysis, number of OTUs clustered at 97% similarity level and Good's index, for the different communities studied.

	Number of sequences	Number of OTUs[a]	Good's Index
Control PS	7226	1346	0.94
PS d14	5624	1164	0.93
PS d63	3790	950	0.89
PS + AM d14	4740	847	0.91
PS + AM d63	6026	973	0.95
Phe d14	3245	881	0.93
Phe d63	8934	1440	0.95
Phe + AM d14	6691	847	0.94
Phe + AM d63	10598	1252	0.96
Control IPK	2293	24	0.99
IPK d14	4084	508	0.95
IPK d63	2988	404	0.99
IPK + AM d14	2463	48	0.94
IPK + AM d63	906	267	0.85

[a]Values based on 906 random sequences per sample.

The rarefaction curves for most soil microcosms did not appear to reach a plateau, even though more than 2000 reads had been sequenced (Fig. S2, Supporting Information), this indicate that the microbial communities of those microcosms are very complex after incubation, contamination or inoculation. Only for IPK d63 microcosm, rarefaction analysis showed that most of the diversity was covered, even with low number of sequences retrieved (906).

Good's coverage values of all samples, in a range of 0.85–0.99 (Table 3), approached 1.0, indicating that the achieved coverage was enough to cover the diversity.

Diversity

To assess the complexity of the individual bacterial community, Hill numbers were obtained (Fig. 2). In recently contaminated and inoculated soils (Phe and Phe + AM), the Hill numbers, ^{0}D (species richness), ^{1}D and ^{2}D, diminished in comparison to PS microcosms indicating a reduction in microbial richness and diversity caused by contamination. After 63 days of incubation, only the inoculated microcosm (Phe + AM) partially recovered ^{0}D values, but the diversity was still low in comparison to control PS, showing a strongly uneven community. However, it should be noted that ^{0}D is very sensitive to the sample size and the number of sequences retrieved from this microcosm was very high.

Regarding the chronically contaminated soil microcosms, all of them possessed lower diversity and richness estimators than PS microcosms (Fig. 2). The IPK d14 and IPK + AM (d14 and 63) microcosms showed significantly higher ^{0}D values and inoculation with AM caused an increase in species richness in comparison to control IPK microcosm; this occurred despite the fact that a low number of sequence were retrieved in these microcosms.

Concerning the pristine soil microcosms (PS and PS + AM), no significant differences in richness or diversity, caused by inoculation or incubation, were observed over the whole experiment with respect to control PS.

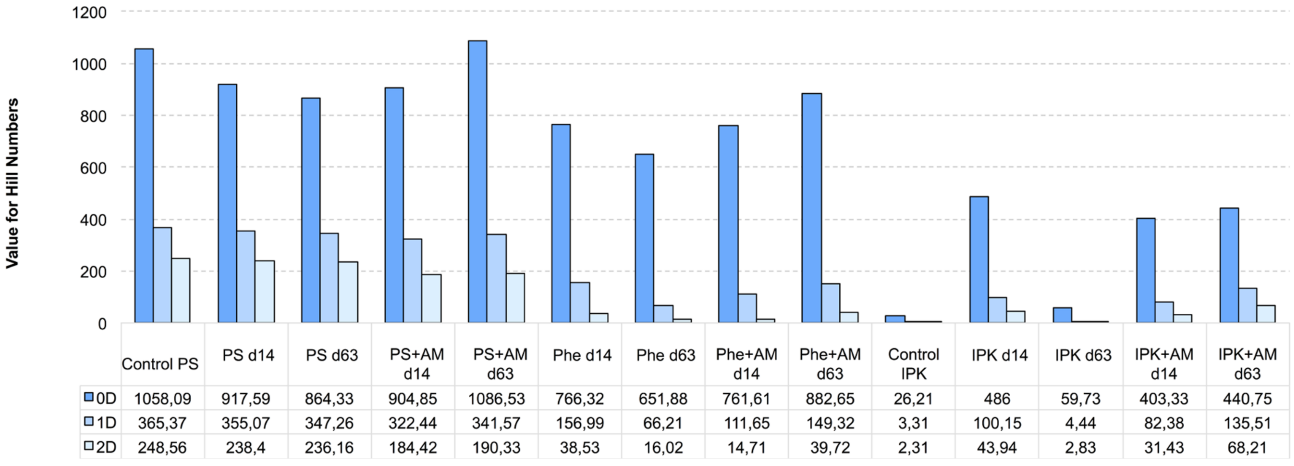
Structure

The OTUs shared among the microcosm's communities and controls were determined via a Venn diagram in order to compare the relationships between the communities in detail (Fig. S3 and description, Supporting Information).

In order to visualise the differences in community structure, at OTUs level, among different microcosms, a CA ordination plot of unconstrained data was performed (Fig. 3). The CA revealed that profiles from soil samples containing phenathrene were differentiated from those containing only inoculant and PS. It was observed that profiles from all microcosms containing pristine soil (control PS, PS and PS + AM) kept close throughout the complete experiment on the right of cero in CA1 (X-axis). The contaminated microcosms, Phe d14 and 63, were located on the left of cero in CA1 (X-axis) showing the higher differences with microcosms containing PS. The inoculated and contaminated microcosms (Phe + AM) were separated from pristine microcosms on CA2 (Y-axis), which represents minor differences.

Although the inoculation with AM caused effects on community structure (Phe + AM), the samples containing the contaminant (Phe) were the most spatially distant from microcosms containing PS, being the most marked differences attributed to the contamination.

In regard to microcosm containing chronically contaminated soil, the CA revealed high differences between control IPK and IPK d63 with the other microcosms, indicating that incubation and inoculation caused a marked effect on the community profiles at OTUs level, since the longer distances were observed on



	Control PS	PS d14	PS d63	PS+AM d14	PS+AM d63	Phe d14	Phe d63	Phe+AM d14	Phe+AM d63	Control IPK	IPK d14	IPK d63	IPK+AM d14	IPK+AM d63
0D	1058,09	917,59	864,33	904,85	1086,53	766,32	651,88	761,61	882,65	26,21	486	59,73	403,33	440,75
1D	365,37	355,07	347,26	322,44	341,57	156,99	66,21	111,65	149,32	3,31	100,15	4,44	82,38	135,51
2D	248,56	238,4	236,16	184,42	190,33	38,53	16,02	14,71	39,72	2,31	43,94	2,83	31,43	68,21

Figure 2. Diversity parameters for the different communities obtained by analysis of pyrosequencing using EstimateS program. ^{q}D values were obtained based on 906 random sequences per sample.

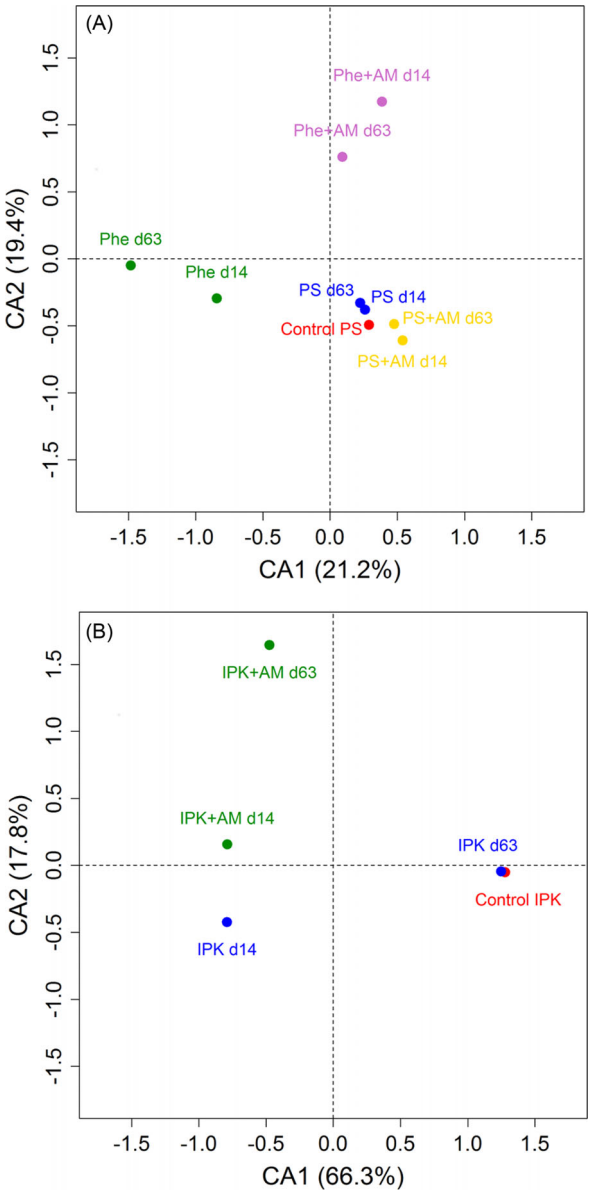


Figure 3. CA based on frequency values of OTUs of the bacterial diversity in (**A**) the four microcosms containing pristine soil (PS, PS + AM, Phe and Phe + AM) compared to control PS; and (**B**) in the two microcosms containing chronically contaminated soil (IPK and IPK + AM) compared to control IPK.

X-axis which offered the greatest contribution to the analysis (66.3%).

Composition

The pyrosequencing results showed that all the OTUs belonging to the six studied microcosms and controls were classified into the domain Bacteria (20 phyla) (Table S2, Supporting Information).

The predominant phyla in the bacterial community of PS microcosms (contaminated or inoculated) were the members of *Proteobacteria* (21.7%–53.2%), *Acidobacteria* (6.15%–36%), *Actinobacteria* (10.1%–34.0%), *Firmicutes* (1.1%–6.8%), *Verrucomicrobia* (1.4%–16.0%) and *Chloroflexi* (3.7%–10.7%).

In the bacterial community of IPK soil (inoculated or not), the predominant phyla were *Proteobacteria* (40.2%–88.3%), *Actinobacteria* (1.0%–29.0%), *Verrucomicrobia* (0.1%–11.3%), *Bacteroidetes* (0.6%–11.9%) and *Firmicutes* (0.6%–9.5%) (Table S2, Supporting Information).

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

The differences in relative abundance of bacterial populations at order level in the treated microcosms in comparison to control microcosms (control PS or control IPK) were analysed and are shown in Fig. 4.

In Fig. 4A, it is clearly observed that phenanthrene addition and inoculation led to remarkable shifts in the bacterial population of the microcosms containing PS, while minor variations were produced by incubation of PS.

The data obtained for contaminated and inoculated soils after 14 days of incubation showed that populations belonging to the order *Sphingomonadales* became dominant in PS + AM, Phe and Phe + AM (relative abundance with respect to control were 9.5%, 16.7% and 29.5%, respectively) (Fig. 4A and Table S3, Supporting Information). After 63 days, *Sphingomonadales* remained dominant in the three mentioned microcosms and a significant increase in *Actinomycetales* was also observed in Phe microcosms (relative abundance of 21.3%).

The dominance of *Sphingomonadales* in these three microcosms was accompanied by a decrease in populations of *Acidobacteriales*, *Holophagales* and *Verrucomicrobiales* on day 14, and at the end of the incubation time the decrease was even higher in Phe and Phe + AM.

Inoculation in Phe + AM and PS + AM microcosms produced an increase in *Burkholderiales* and *Rhizobiales* orders, and a decrease in *Actinomycetales* regarding non-inoculated microcosms (PS and Phe) (Fig. 4A).

The data obtained for IPK soil microcosms, inoculated or not, are shown in Fig. 4B. After 14 days of incubation, a substantial variation with respect to the control was observed, since incubation produced particular dramatic decline in *Pseudomonadales* (around 80%) and the addition of the inoculant produced a significant increase in *Sphingomonadales* (approximately 17.2%). In addition, both incubation and inoculation produced an increase in *Actinomycetales* (approximately 17%).

After 63 days as is clearly observed that while IPK soil returned to the initial soil condition, the addition of the inoculant maintained the differences observed on day 14 (Fig. 4B and Table S3, Supporting Information).

In IPK + AM microcosm, inoculation increased *Rhodospirilales* and *Cythophagales*, and decreased *Sphaerobacterales* and *Actinomycetales* (both *Actinobacteria*) in comparison to IPK microcosm (Fig. 4B).

Comparison of bacterial community structures at genus level

The hierarchically clustered heat-map analysis based on the bacterial community profiles at the genus level is shown in Fig. 5. It was obtained based on the sequence frequencies of the 34 more abundant genera that covered more than 70% of all sequences.

The hierarchical heat-map analysis clearly identified three groups, indicating similar degrees of bacterial community structures among the samples.

The chronically contaminated soil bacterial profile (IPK) was distant from the IPK + AM profile (Fig. 5). The composition of contaminated and inoculated soil microcosm (Phe and Phe + AM) shared higher similarity when compared with the non-contaminated soil (PS and PS + AM).

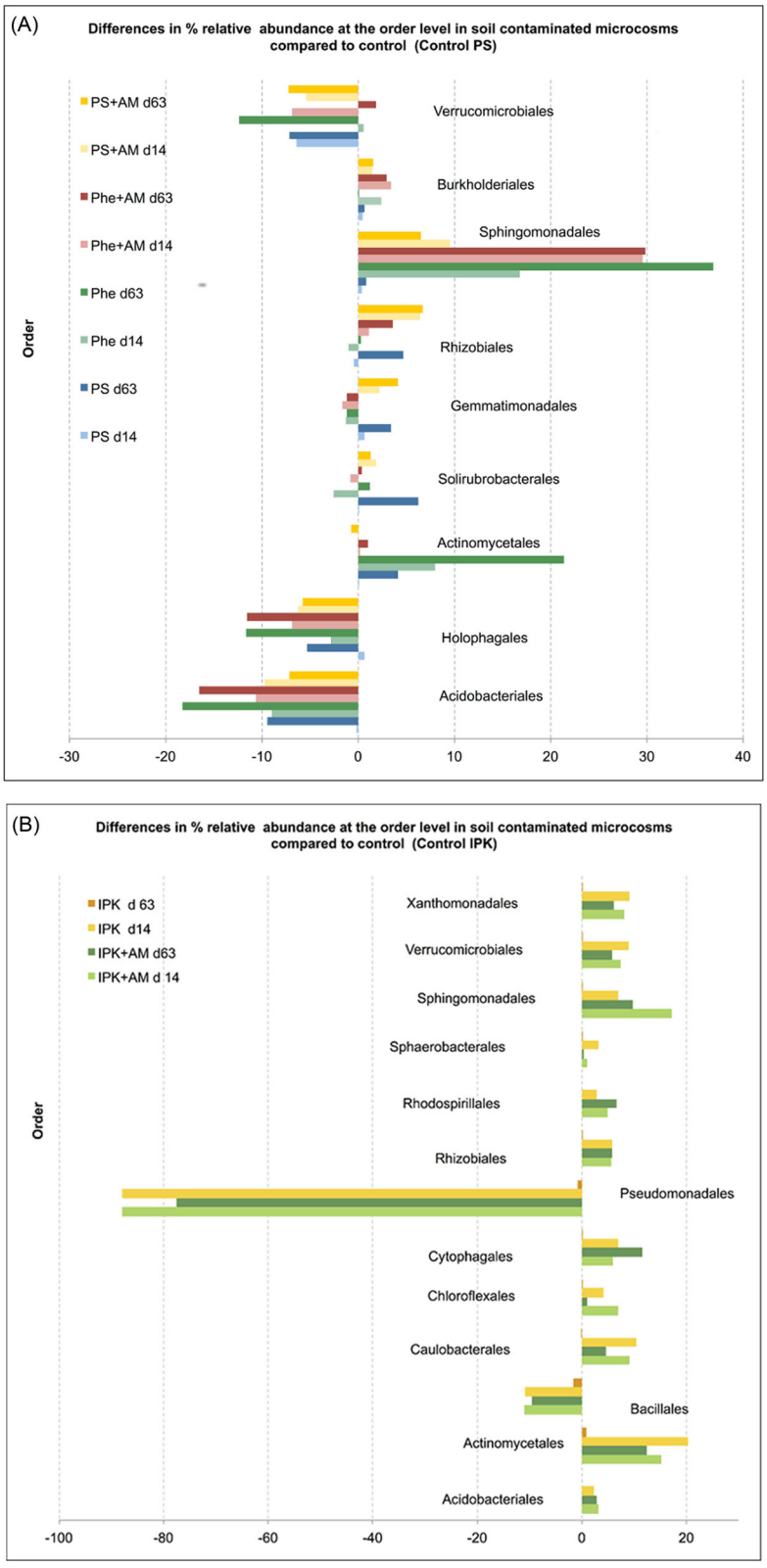


Figure 4. Differences in relative phylotype abundance at the order level in the microcosm containing PS compared to control microcosm (**A**) and in the microcosms containing chronically contaminated soil compared to control (**B**). Only reads that changed more than 3% with respect to the control microcosm are shown.

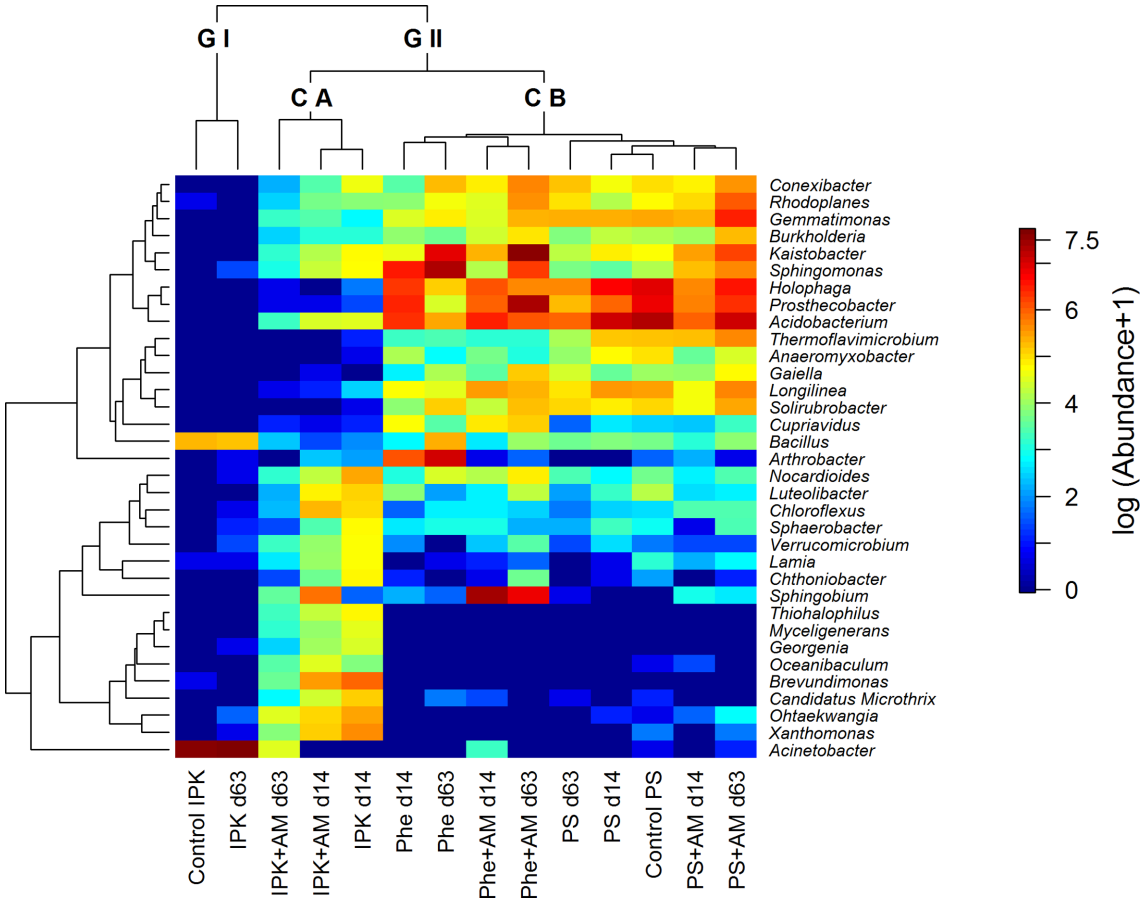


Figure 5. Bacterial distribution among the six microcosms showed in a double hierarchical dendrogram. The bacterial phylogenetic cluster and the relationship among samples were determined by the Bray–Curtis distance and the complete clustering method. The heat-map plot depicts the frequency of each bacterial genus (variables clustering on the Y-axis) within each sample (X-axis clustering). The values in the heat map represent the Log-transformed Abundance (Log X + 1) of each bacterial genus, which are depicted by colour intensity with the legend indicated at the upper right corner of the figure. G: Group; C: Cluster.

Group I was clustered by samples control IPK and IPK d63. Both shared a highly similar community, characterised by the abundance of *Acinetobacter* and *Bacillus* genus. The dominant populations were clearly dissimilar to all other populations in the microcosm samples (Table S4, Supporting Information).

Group II was composed by two clusters A and B. Cluster A formed by samples IPK d14 and IPK + AM d14 and d63 shared a highly similar community structure, demonstrating a repeatable succession of bacterial compositions mainly based on incubation and inoculation effect. However, while *Ohtaekwangia* genus was dominant in the cluster, some differences between the three bacterial communities were observed. Both IPK d14 and IPK + AM d14 were characterised by a prevalence of *Brevundimonas* and *Xanthomonas*, and only in IPK + AM d14 microcosm *Sphingobium* was prevalent, whereas IPK + AM d63 was distinguished by the dominance of *Acinetobacter*. Cluster B comprised all the microcosms prepared with PS, and was distantly branched from cluster A and Group I. (Fig. 5).

On the basis of cluster heat-map analysis, the populations related to the genus *Acidobacterium, Prosthecobacter, Holophaga, Sphingomonas, Kaistobacter and Gemmantimonas* were the main contributors to the differences observed in bacterial community structures.

Into cluster B, there were two distinctive subclusters, one comprised the microcosms contaminated and inoculated (Phe and Phe + AM) and the other comprised the pristine and inoculated microcosms (PS and PS + AM). Into the first subcluster, the branch that grouped the microcosms Phe d14 and d63 was characterised by the prevalence of *Arthrobacter* and *Sphingomonas*. While the branch formed by the microcosms Phe + AM d14 and 63 was characterised by the prevalence of *Sphingobium*. Control PS, PS and PS + AM formed the other subcluster, where all branches were characterised by the prevalence of *Acidobacterium, Holophaga* and *Prosthecobacter.*

DISCUSSION

The overall goal of this study was to determine the effect of a bioaugmentation strategy on different soil microbiomes in PS, chronically contaminated soil (IPK) and recently contaminated soil (Phe). We used pyrosequencing technique to examine the microbial diversity in soil microcosms throughout 63 days of treatment and resolve changes associated with inoculation and degradation of high concentrations of hydrocarbons.

In a previous work, we isolated the strain *Sphingobium* sp. AM from a chronically hydrocarbon-contaminated soil, which was characterised by Festa, Coppotelli and Morelli (2013). The strain was selected due to its phenanthrene-degrading capacity. Here, the characterisation of AM strain was forward, finding that it was able to degrade several PAH (phenanthrene, anthracene and fluorene), and its draft genome was obtained using a high-throughput technique. As in other *Sphingomonads*

genomes (Kertesz and Kawasaki 2010), a rich set of genes for metabolism of aromatic compounds and aliphatic hydrocarbons was present in the subsystems of AM strain (Table S1, Supporting Information), indicating that is a promissory candidate for bioaugmentation studies.

The application of AM strain in bioaugmentation experiments was performed taking into account that *Sphingomonads* to grow in many complex soils and at the expense of PAH (Coppotelli *et al*. 2008; Fida *et al*. 2013). Consequently, rapid adaptation and evolution of the inoculant was expected, since they are key factors that contribute to the establishment and spread success depending on ecological interactions between species (Whitney and Gabler 2008).

In recently contaminated soil (Phe + AM), the inoculation of AM strain significantly improved the elimination of phenanthrene during the 63 days of treatment, whereas in chronically contaminated soil (IPK + AM) no degradation improvement of any of the 10 PAH present in that soil was observed (Table 2). This is in agreement with the lack of stimulation in dehydrogenase activity produced by inoculation in IPK + AM microcosm (Fig. S1, Supporting Information). However, in both microcosms there are clues that indicate the inoculant establishment, like the number of PAH-degrading bacteria (Fig. 1) and the analysis of pyrosequencing data (Fig. 5, Supporting Information), where many shifts in community structure were observed.

In IPK soil, despite the inoculant established, its presence did not improve the degradation. There, biodegradation seems to have been performed mostly by the autochthonous population and occurred during the first 14 days of incubation. Fodelianakis *et al*. (2015) also observed that in oil-polluted sediments when environmental conditions were optimised, bioaugmentation did not enhance the remediation process while the indigenous microbiome at the polluted site seems to have outperformed the inoculation. Manipulation, such as mixing, homogenisation and sieving, is generally thought to increase availability of organic compounds because of disruption of the equilibrium with organic carbon in the pore/water particle system (DeWitt 2001). The absence of degradation observed between days 14 and 63 might be due to the fact that only the bioavailable fraction of the pollutant is being degraded.

The ability of AM strain to get involved in the carbon and energy flux by providing its enzymatic battery, metabolising not only hydrocarbons but also their metabolites (Table S1, Supporting Information), might let us assume that the inoculant could have been participating in the carbon flux associated with intermediate degradation products between day 14 and 63 of incubation. This is in agreement with the community shifts caused by inoculation (Figs 3–5).

Through the study of both soils (PS and IPK), we have found that they possess different physicochemical properties what redounds in very different soil microbiomes as assessed by pyrosequencing. Differences were observed either in their diversity (Fig. 2) or in their bacterial composition (Fig. 5).

However, interpretation of 16S rRNA gene-derived results must be approached with caution when used to quantify the relative abundance of different taxa as 16S rRNA gene copy numbers per genome vary from 1 to 15 or more copies depending on bacterial species, which could produce an overestimation of abundance values (Větrovský and Baldrian 2013). It should be considered that in our case the bias was minimised due to a comparison and deltas calculation were performed in most phylogenetic analyses.

IPK soil owns a highly uneven microbiome with much lower diversity and richness estimators than PS microcosms (Fig. 2). This is consistent with the aged contaminated soil, since contaminants exert a selective pressure, resulting in more competent populations (often a few) occupying the available niches (Niepceron *et al*. 2014). Here, those populations were *Acinetobacter* and *Bacillus* accounting for 97% of the community (Fig. 5 and Table S4, Supporting Information).

Acinetobacter and Bacillus species were found to possess hydrocarbon-degrading capabilities (Das and Mukherjee 2007; Golby *et al*. 2012). Moreover, they were proved to display a substantial capacity to decrease surface tension and increase emulsification activity (Menezes Bento *et al*. 2005), useful strategies both for enhancing hydrocarbon degradation.

Inoculation in pristine soil (PS + AM) did not produce modifications in richness or diversity. But when PS was contaminated (Phe), a reduction in Hill numbers was observed; however, inoculation on this soil (Phe + AM) seems to have partially recovered diversity and richness after 63 days (Fig. 2 and S2, Supporting Information).

In a high diverse environment such as PS (Fig. 2), the amount of available resources is limited and reduces the possibilities of the inoculant to establish. We found evidence of the strain AM survival in PS + AM microcosms, because PAH-degrading bacteria (Fig. 1) and *Sphingobium* genus had higher values in PS + AM than in PS during the 63 days of treatment (Fig. 5 and Table S4, Supporting Information). Despite the evidence, the invasion of the inoculant was not observed, since no significant shifts occurred in community structure (Figs 2, 4 and 5).

When resources are added to experimental communities (as in the case of phenanthrene in Phe + AM), by changing its availability from low to high, invaders may take full advantage and grow, even when confronted with highly diverse communities (Liu *et al*. 2012; Mallon *et al*. 2015).

In the chronically contaminated soil microcosms, two effects were observed. While initially incubation conditions and aeration produced an increase in richness and diversity (IPK d14) and later on a reduction in those parameters (IPK d63), the inoculation with AM strain (IPK + AM) maintained high Hill numbers values over the whole experiment (Fig. 2). Thomson *et al*. (2010), using molecular fingerprinting (T-RFLP), found that sieving-homogenisation method significantly altered soil bacterial community structure. Our study showed that these changes were particularly important in chronically contaminated soil (IPK d14), where the inaccessible PAH entrapped within the soil aggregated could have been exposed to the soil microbiome by the sieving procedure.

Overall, inoculation with AM strain managed to increase the richness and diversity in both recently and chronically contaminated microbiomes (Fig. 2, Table S2, Supporting Information), therefore, independently of PAH degradation improvement, we observed clues of inoculant establishment.

In PS, bacterial community composition and diversity were mainly influenced by the contaminant, whereas in IPK soil bacterial community composition and diversity were strongly influenced by incubation conditions even at a very low level of taxonomic resolution (Figs 3–5).

In PS + AM, Phe and Phe + AM microcosms underwent the most significant shifts in comparison to control PS, the major population stimulated by contamination and inoculation (Phe and Phe + AM) was *Sphingomonadales* (Fig. 4A).

Additionally, inoculation in Phe + AM and PS + AM microcosms produced an increase in *Burkholderiales* and *Rhizobiales* orders. There have been many described PAH-degrading communities composed by genera belonging to those orders (Wang *et al*. 2008; González, Simarro and Molina 2011; Festa, Coppotelli

and Morelli 2016) suggesting that these members are cooperative. Although there may be a synergy between those genera, our results suggest that this may not have been directly related to PAH degradation since an increase in them was also observed in PS + AM microcosms, where no PAH was present.

In Phe microcosm, an especially marked increase in *Actinomycetales* was observed (Table S3 and S4, Supporting Information). *Actinomycetales* also experienced a sharp increase in soil with oil pollution in comparison to controls in the work of Bastida *et al.* (2015). However, in metaproteomic studies no key catabolic proteins related to hydrocarbon degradation were identified with *Actinobacteria,* suggesting that they can take advantage of the intermediate metabolites generated by other degraders, or occupy the niche of disappearing groups rather than participating directly in hydrocarbon degradation (Bastida *et al.* 2015).

In IPK soil microcosm, incubation produced a dramatic decline in *Pseudomonadales* but later on (day 63) the community returned to the initial soil condition observed in control. Communities of control IPK and IPK d63 showed high similarity, due to their high selected and low diverse profiles (Figs 3B and 5).

At order level, incubation and inoculation of IPK soil caused the increment of *Sphingomonadales* and *Actinomycetales* (Fig. 4B and Table S3, Supporting Information). Regarding *Sphingobium* population, it was observed a prevalence in IPK + AM d14 and a decrease towards the end of incubation time (day 63) (Fig. 5). Nevertheless, the community maintained the shifts caused by the introduction of the inoculant in relation to control IPK (Figs 3B, 4B and 5; Tables S2 and S3, Supporting Information), giving support to the theory of establishment.

In IPK + AM microcosm, a decrease in *Actinomycetales* order was observed in comparison to IPK microcosm (day 14) (Fig. 4B and Table S2, Supporting Information). Because of the role explained before for *Actinobacteria*, here the inoculant may have been exerting an ecological displacement of some degraders of intermediate metabolites.

CONCLUSIONS

Through the pyrosequencing analysis, it was observed that inoculation managed to increase the richness and diversity in both recently and chronically contaminated microbiomes.

This study evidences the establishment of an inoculant in soils with different available resources. Thus, while inoculation could be successful, it may not guarantee improved degradation, as the inoculant may have been using other resources to survive.

Inoculation of a PAH-degrading strain (*Sphingobium* sp. AM) managed to improve PAH degradation in recently contaminated soil but did not improve the degradation in chronically contaminated soil.

These results showed the importance of implementing bioremediation experiments combined with microbiome assessment to gain insight on the potential functions of complex microbial communities involved in bioremediation processes.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

FUNDING

This research was partially supported by the Agencia Nacional de Promoción Científica y Tecnológica (PICT 2013-0103). Festa S. and Macchi M. have doctoral fellowships supported by CONICET. Cortés F. is a research member of INIDEP. Morelli I.S. is a research member of CIC-PBA. Coppotelli B.M. is a research member of CONICET.

Conflict of interest. None declared.

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