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Bacterial diversity and functional interactions between bacterial strains from a phenanthrene-degrading consortium obtained from a chronically contaminated-soil

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ABSTRACT

A phenanthrene-degrading consortium (CON-Phe) was obtained from a chronically contaminated soil. The consortium degraded 58% of the phenanthrene supplied during the first 7 days of incubation with the concomitant accumulation of 1-hydroxy-2-naphthoic acid (HNA). The composition of CON-Phe and its dynamic during phenanthrene degradation were determined using culture-dependent and independent approaches (polymerase chain reaction-denaturing gradient gel electrophoresis and clone libraries). Among the detected bacteria by both methods, *Sphingomonadaceae* family frequently occurred, but some genera were only observed through culture-dependent methods (*Enterobacter* sp. *and Pseudomonas sp.*) and others only through culture-independent methods (*Ochrobacterium* sp., *Alcaligenes* sp.). Five different strains were isolated and identified; between them only the strain AM (*Sphingobium* sp.) showed phenanthrene degradation. And only in strains AM and B (*Enterobacter* sp) evidence of the presence of PAH-ring hydroxylating dioxygenases genes was found. In order to determine the role of the isolated strains in the CON-Phe and the interaction between them, the phenanthrene degradation by defined mixed cultures was studied. All the defined consortia, formed by strain AM together with another of the isolated strains, showed percentages of phenanthrene biodegradation significantly higher than the strain AM alone and the natural consortia. In addition, no accumulation of HNA was observed in these defined consortia. These results might suggest that in the soil consortia the competition between the species and the community dynamics could cause a negative effect in the phenanthrene degradation. On the other hand, a synergistic effect between the phenanthrene-degrading strain AM and the other isolated strains was observed.

1. Introduction

Environmental contamination due to xenobiotic compounds is mainly due to their large-scale manufacturing, processing and handling. Owing to long-term persistence and to acute toxic effects of these compounds, remediation of contaminated sites is necessary (Sahoo et al., 2012). Polycyclic aromatic hydrocarbons (PAHs) are thus amongst the most widespread organic contaminants in soils, water, and wastewater (Puglisi et al., 2007) and they are produced in many processes, including the burning of fossil fuels; gas, coal tar and wood processing; exhaust emissions from vehicles and the incineration of waste (Cai et al., 2007; Das et al., 2008). The presence of some PAHs in the environment causes acute health hazard due to their high recalcitrance ability and high toxicity to living organisms for their mutagenic or carcinogenic properties (Sinha et al., 2012).

It has been found that many bacteria degrade PAHs via either metabolism or co-metabolism and it is well known that a better understanding of the microorganism-mediated mechanisms of catalysis of PAHs will facilitate the development of new methods to enhance the bioremediation of PAHs-contaminated soil (Peng et al., 2008). Bioremediation of soils involves several technologies, including bioaugmentation of microorganisms with indigenous, exogenous, or genetically engineered microorganisms (GEMs) and also biostimulation to increase the removal of PAHs. Both bioaugmentation and biostimulation are strongly linked to the soil microorganism capacity to dissipate PAHs (Fernández-Luqueño et al., 2011).

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In some cases, bioaugmentation (inoculation of allochthonous microorganisms) is the best option to clean the polluted area when the autochthonous microbial populations do not have the appropriate metabolic ability to do so (Edgehill, 1999). The applications of bioaugmentation with bacterial consortia is of special concern since syntrophic interactions are an extremely interesting example of "support aptitude", especially in the biodegradation of aromatic compounds where the biochemical steps are shared among community members in order to completely mineralize recalcitrant and/or toxic substrates (Shim et al., 2005; Fazzini et al., 2010). Among the microbial consortia there are, defined and undefined; defined ones are those that are constituted by a combination of isolates with known degradative capabilities complementary to each other, which are well characterized but have the disadvantage that it is commonly necessary to have a great number of strains to achieve an extensive degradation (Leahy and Colwell, 1990). An undefined consortium is constituted as the result of a direct process of enrichment from environmental samples with a history of contamination (Venkateswaran et al., 1995; Sugiura et al., 1997; Budzinski et al., 1998), the microbial population was selected naturally for their cooperation in the metabolic degradation of the contaminant, which potentially offers greater efficiency than a defined consortium in the degradation (Grifoll et al., 1995). It is important to establish previous laboratory evaluations as the characterization of the microbial consortium to determine its capacity to remove the pollutant, increasing the bioremediation efficiency (Sabaté et al., 2004; Molina et al., 2009).

In this report, a PAH-degrading consortium was obtained and analyzed; their cultivable constituents were isolated and some physiological properties, related with the phenanthrene degradation, were studied with the aim of revealing bacterial interactions that can take place in the consortium during contaminant degradation. In addition, the isolated strains were combined in order to study their possible cooperation in phenanthrene degradation.

2. Materials and methods

2.1. Isolation of a phenanthrene-degrading bacterial consortium

A phenanthrene-degrading bacterial consortium was obtained from a chronically contaminated soil. The soil selected for this study was from Mosconi neighborhood (coordinates S 34° 52′31′ W 57° 55′10′), 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: the texture is a clay loam, a pH of 6.5, 4.73% (w/w) organic carbon, 8.16% (w/w) soil organic matter, 0.32% (w/w) total nitrogen, 14.8 C/N ratio, 6 mg kg^{-1} available phosphorus, and 643 mg kg^{-1} hydrocarbons. The number of heterotrophic cultivable bacteria of this soil was 1.8×10^7 cfu in R2A (Reasoner and Geldreich, 1985) per g of dry soil.

To obtain the consortium, 2 g of soil were suspended into an erlenmeyer with 20 ml of sterilized liquid mineral medium (LMM) (Coppotelli et al., 2010) and incubated at 150 rpm for 30 min. Once the soil of the suspension decanted after the incubation, 5 ml of the supernatant were transferred into an erlenmeyer with 250 ml of LMM, supplemented with 2000 mg l^{-1} of phenanthrene as a sole carbon and energy source, and incubated for 7 days at 28 °C, 150 rpm. After four sequential transfers in LMM with 2000 mg l^{-1} of phenanthrene, each one incubated for 7 days in the same previous conditions, the consortia was obtained. It was finally conserved in 15% of glycerol at −80 °C. When the consortium was required for an experiment it was reactivated in LMM supplemented with 2000 mg l^{-1} of phenanthrene for 7 days at 28 °C and 150 rpm.

2.2. Biodegradation studies

The phenanthrene degradation by the consortium was determined in LMM with 200, 500 and 2000 mg l^{-1} of phenanthrene as a sole carbon and energy source. The phenanthrene degradation by the defined consortia was determined in LMM with 200 mg l^{-1} of phenanthrene as a sole carbon and energy source. The systems were incubated at 28 °C, 150 rpm and monitored at 0, 4, 7 and 15 days in the case of the consortium and at 2 and 7 days in the case of the defined consortia. The total culture volume was extracted three times with ethyl acetate (1:5 ethyl acetate/culture ratio). The same extractions were carried out for the study of the degradation of phenanthrene (200 mg l^{-1}) and 1-hydroxy-2-naphthoic acid (HNA) (100 mg l^{-1}) by the isolated strains, incubated at the same conditions for 7 days. An abiotic control sample was analyzed and in all the cases the experiments were performed at least in triplicate.

A sequential extraction was made in the defined consortium cultures to determine the presence of salicylic acid, another phenanthrene metabolite. After the third extraction with ethyl acetate the remnant aqueous phase was acidified to pH 2.5 with concentrated HCl and re-extracted with ethyl acetate in the same way (Coppotelli et al., 2010).

The ethyl acetate extracts were analyzed by reversed-phase high-pressure liquid chromatography (HPLC) using a Waters® chromatograph with a Symmetry Waters C18 column (15 cm × 4.6 mm i.d., bead size 5 μm, pore size 100 Å) and a diode-array detector. A linear gradient of 15 mM phosphoric acid in nanopure water solution and methanol (20:80 to 5:95, vol vol^{-1}) over 15 min and a flow rate of 1 ml/min was used (Coppotelli et al., 2010).

The statistical analysis of the phenanthrene degradation data were performed by parametric one-way ANOVA test, using the SigmaPlot/SigmaStat software program (SPSS Inc., Chicago, Illinois, USA).

2.3. Microbiological characterization

Total heterotrophic cultivable bacteria and PAH-degraders were studied in the consortium. The former were quantified in duplicates by successive 1/10 dilutions. These suspensions were spread on R2A medium plates and colonies were counted after 7 days of incubation at 28 °C. R2A medium contains 0.5 g of yeast extract, 0.5 g of proteose peptone, 0.5 g of casamino acids, 0.5 g of glucose, 0.5 g of soluble starch, 0.3 g of K_2HPO_4, 0.05 g of $MgSO_4$ $7H_20$, 0.3 g of sodium pyruvate and 15 g of agar per liter of laboratory quality water, final pH 7.2.

PAH-degraders were quantified in sterile polypropylene microplates according to Wrenn and Venosa (1996). The wells contained LMM and a PAH-mix in pentane as a substrate. PAH mix contains 1 g/l phenanthrene, 0.5 g/l anthracene, 0.5 g/l fluorene and 0.5 g/L dibenzothiophene. The wells were inoculated with an aliquot of serial dilutions of the sample and incubated at 28 °C for 21 days. Most Probable Number (MNP) method was used to enumerate PAH degraders.

The isolation of the predominant culturable bacteria from R2A plates was performed. For the five different colonies isolated from the consortia, the biochemical characterization includes: Gram staining, motility, oxydase, nitrate reduction, gelatinase and catalase activity, growth in F and P agar, citrate, O/F (Hugh-Leifson oxidation/fermentation), TSI (triple sugar iron) and cetrimide (Gerhard et al., 1981).

2.4. Carbon source utilization

Growth with 200 mg l^{-1} of phenanthrene, 100 mg l^{-1} of HNA and 100 mg l^{-1} sodium salicylate as a sole carbon and energy source was tested in sterile LMM in duplicates. Cultures of the isolated strains were incubated at 28 °C, 150 rpm for 7 days.

Growth was monitored by counting colony-forming units (cfu) after spreading 0.1 ml of an appropriate dilution on R2A medium and incubating the plates in the dark for 10 days at 30 °C (Coppotelli et al., 2010).

2.5. Dioxygenase activity and presence of dioxygenase gene

Ring-hydroxylating dioxygenases related to polycyclic aromatic hydrocarbon catalyze the first step of the PAH degradation pathway (Jurelevicius et al., 2012) via the incorporation of molecular oxygen into the aromatic ring.

To determine the initial dioxygenase activity in the biodegradation of PAHs, the method used was the one described by Zocca et al. (2004). This method is based on the conversion of indole to indigo catalyzed by a dioxygenase. Once the bacterial colony was grown on an R2A agar plate, the indole crystals were positioned on the Petri dish lid and incubated at 28 °C until a blue color appeared due to the indigo formation.

The presence of the dioxygenase gene in the consortium and the isolated strains was determined with a PCR with the set of primer designed by Cébron et al. (2008), PAH-RHDα GN-F 610 (GAGATGCATACCACGTKGGTTGGA) and PAH–RHDα GN-R 916 (AGCTGTTGT TCGGGAAGAYWGTGCMGTT) in a reaction volume of 30 μl containing 2 μl of DNA, 0.2 mM of dNTPs, 0.2 μM of each primer, 1.25 U of GoTaq DNA Polymerase (Promega, Madison, WI) and the manufacturers' recommended buffer as supplied with the polymerase enzyme. Amplification was performed on an Eppendorf® Mastercycler® thermocycler (Eppendorf, Hamburg, Germany). The program consisted of an initial denaturation step for 5 min at 94 °C, followed at first by 5 cycles of 94 °C for 1; 46 °C for 2; and 1 min at 72 °C and then by 30 cycles of 1 min at 95 °C, 1 min at 55 °C; and 72 °C for 1 min. The final extension was carried out at 72 °C for 10 min. The PCR products were analyzed by agarose gel electrophoresis.

2.6. Molecular characterization

Genomic DNA of the consortia was extracted with Qiagen Genomic-tips Kit (20G) (Qiagen Inc., Chatsworth, CA, USA) following manufacturer's instructions. As for the isolated strains, genomic DNA was extracted with boiling method (Sambrook et al., 1989), 1 ml of each overnight culture grown in broth R3 werecentrifuged and resuspended in 1 ml of distilled H_2O. The suspensions were boiled for 10 min and the supernatants werecollected after spinning for 2 min in a microcentrifuge. Soluble DNA was recovered from supernatants. R3 broth contains 1 g of yeast extract, 1 g of proteose peptone, 1 g of casamino acids, 1 g of glucose, 1 g of soluble starch, 0.6 g of K_2HPO_4, 0.1 g of $MgSO_4 \cdot 7H_20$ and 0.6 g of sodium pyruvate per liter of laboratory quality water, final pH 7.2.

In order to obtain the complete amplification of the 16S ribosomal ribonucleic acid (rRNA) gene of the isolated strains, a PCR using 27 forward primer (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492 reverse primer (5′TACGGCTACCTTACGACTT-3′) was carried out. The PCR program consisted of initial denaturation step at 95 °C for 2 min, followed by 30 cycles of 95 °C for 1 min, 60 °C for 30 s, 72 °C for 35 s, and the final extension step was carried out at 72 °C for 5 min, on a Eppendorf® Mastercycler® thermocycler (Eppendorf, Hamburg, Germany).

The positive amplicons were purified using PCR purification kit QIAquick (Qiagen Inc., Chatsworth, CA, USA) and were 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.

DNA extracted from the consortia and from the isolated strains was amplified in order to obtain the partial amplification of the 16S rDNA sequence to be analyzed by DGGE (Denaturing Gradient Gel Electrophoresis).

The set of primers for eubacteria used were GC-341F (5′-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCCTCCTACG-GGAGGCAGCAG-3′) and 907R (5′-CCGTCAATTCCTTTGAGTTT-3′) (Muyzer et al., 1998). The PCR reaction contained 1 μl of DNA, 1 U of GoTaq, manufacturers' recommended buffer as supplied with the polymerase enzyme, 200 mM of BSA, 0.2 mM of dNTPs and 5 μM of each primer in a total reaction volume of 30 μl. Amplification was performed on an Eppendorf® Mastercycler® thermocycler (Eppendorf, Hamburg, Germany). The 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. The PCR products were analyzed by agarose gel electrophoresis and then the positive amplicons were purified using a purification kit QIAquick (Qiagen Inc., Chatsworth, CA, USA).

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.

To obtain sufficient amount of DNA for cloning, the band of interest was re-amplified by PCR. It was cut from de gel and washed with 100 μl of sterile deionized water, vortexed and frozen at −80 °C for 30 min, later it was vortexed and held 24 h at 4 °C to release the DNA into the water.

2.7. Clone library

DNA clone library was constructed from the consortium after 4 and 15 days of incubation. 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. 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.

To study the phylogenetic relationship between the 16S rDNA sequences from the isolated strains, the clones, the DGGE bands 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). The robustness of the phylogeny was tested by bootstrap analysis with 500 iterations.

3. Results

3.1. Obtaining of a phenanthrene-degrading consortium

A phenanthrene degrading bacterial consortium (CON-Phe) was obtained from hydrocarbons-contaminated soil of Mosconi neighborhood, Ensenada, Argentina, by four sequential enrichments in

LMM with 2000 mg l^{-1} of phenanthrene as sole carbon and energy source. The phenanthrene utilization of the consortium was evidenced by the apparition of brownish color caused by the production of water-soluble intermediates (Tao et al., 2007).

When the capacity of the consortium to degrade phenanthrene was tested with three different phenanthrene concentrations (2000; 500 and 200 mg l^{-1}), the phenanthrene eliminated was 16, 25 and 59% respectively after 15 days of incubation. The studies continued using the phenanthrene concentration which showed the highest percentage of phenanthrene degradation (200 mg l^{-1}).

3.2. Biodegradation of phenanthrene by CON-Phe bacterial consortium

The kinetic of phenanthrene degradation was determined in LMM cultures supplemented with 200 mg l^{-1} of phenanthrene. Fig. 1a shows the remaining phenanthrene concentration in the CON-Phe cultures and abiotic control cultures (Control) for 15 days of incubation. The phenanthrene degradation took place during the first 7 days of incubation, after that, the phenanthrene concentration remained constant. Concomitantly, the apparition of the HNA, an intermediate phenanthrene metabolite, was detected (Fig. 1b). The accumulation of the HNA showed a behavior in agreement with the kinetic of phenanthrene degradation, increasing its concentration during the first 7 days of incubation, and then remained constant.

The degradation of phenanthrene by consortium CON-Phe was accompanied with an increase in the number of the heterotrophic viable bacteria, only during the first 5 days of incubation (Fig. 1c). On the other hand, the number of PAH-degrading bacteria of the consortium showed an increase until day 7 of incubation, after that the PAH-degrading count declined sharply, this decrease was coincident in time with the interruption in phenanthrene degradation (Fig. 1c).

3.3. Diversity and dynamics of CON-Phe growing in LMM with phenanthrene

The first appreciation about consortium diversity was based on the morphological analysis of colonies on R2A plates. Three different morphotypes were clearly distinguishable, according to the color of colonies: white colonies (WC), yellow colonies (YC) and transparent colonies (TC).

A dynamic in the bacterial composition was observed at cultivable level in the R2A plates. Fig. 2 shows a differential count considering the three predominant morphologies. At the beginning of the experiment, the R2A plates showed a predominance of WC (96%), nevertheless they presented similar values of count during the whole experiment. After four days of incubation an important increase of YC (more than 2 orders of magnitude) was observed, constituting then 76% of the heterotrophic viable bacteria; then declined rapidly. At the fifteenth day (when the phenanthrene degradation was stopped) the YC only reached 1% of the cultivable populations. The TC colonies showed a progressive increase during the phenanthrene degradation experiment, reaching their highest count and becoming the cultivable predominant population (68%) at the end of the incubation time.

Five different strains (AM, T, Bc, B and B1) were isolated and identified according to biochemical characteristics (Table 1) and 16S rDNA sequence analysis (Table 2). The strain AM was classified

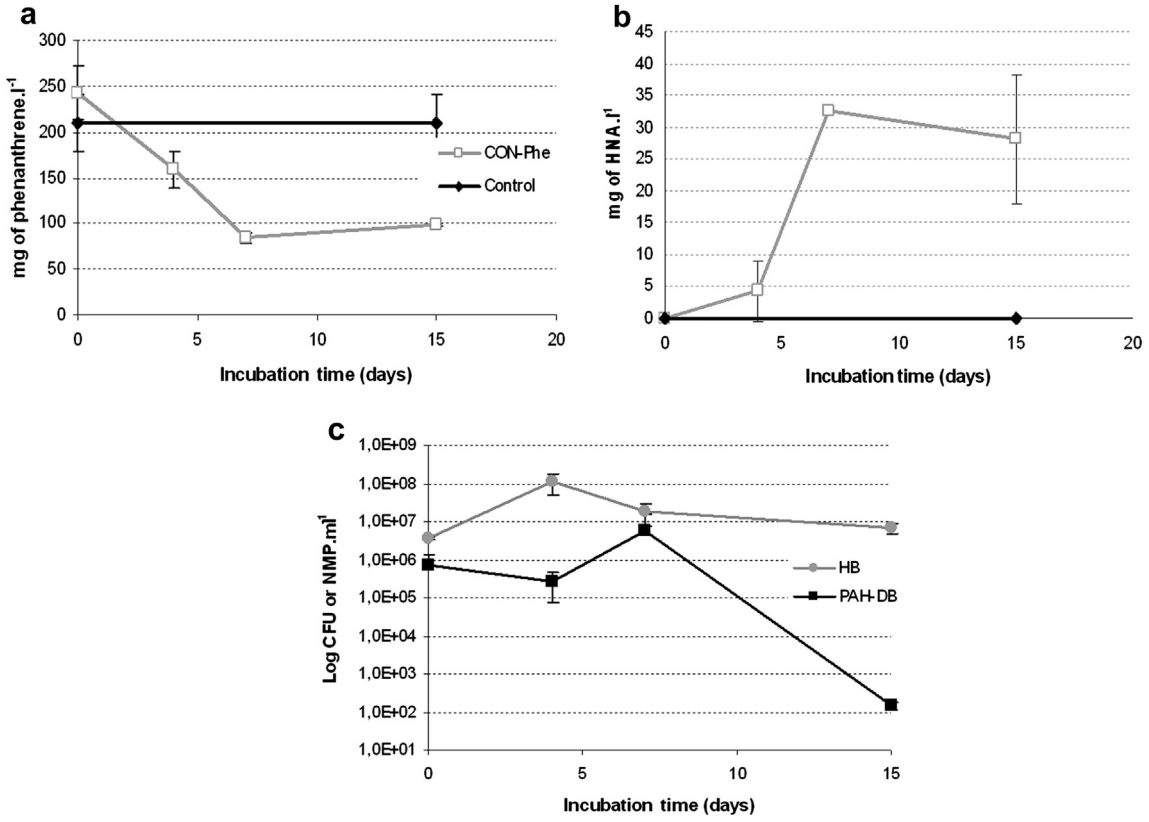

Fig. 1. Concentration of phenanthrene (a) and 1-hydroxy-2-naphthoic acid (HNA) (b) and counts of heterotrophic viable bacteria (HB) and PAH-degrading bacteria (PAH-DB) (c); in the CON-Phe culture growing in LMM with phenanthrene as a sole carbon and energy source during 15 days incubation. Results are means of triplicate independent experiments. Bars represent standard deviations.

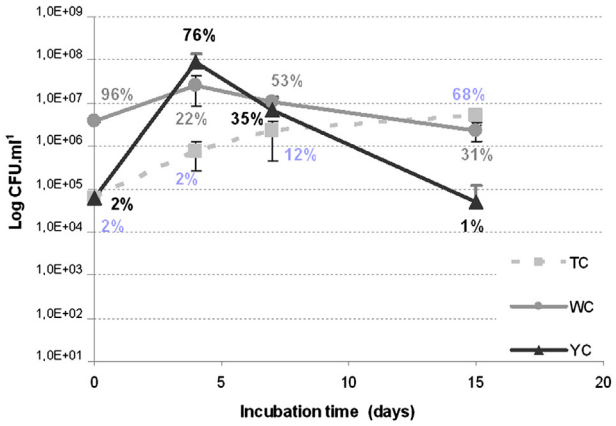

Fig. 2. Differential count of heterotrophic viable bacteria in the consortium during phenanthrene degradation, white colonies (WC), yellow colonies (YC) and transparent colonies (TC).

as *Sphingobium* sp., the strain T and Bc were assigned to *Pseudomonas* sp. and the strain B and B1 to *Enterobacter* sp.

To investigate the genetic bacterial diversity of the consortium and the dynamic changes in the course of phenanthrene degradation assay, DGGE was performed for CON-Phe growing in LMM with phenanthrene at 0, 4, 7 and 15 days of incubation, also two 16S rDNA libraries were derived from the consortium after 4 and 15 days of incubation.

Fig. 3 shows the phylogenetic analyses of 16S rDNA sequences of clones from libraries, clones from a predominant DGGE band and the isolated strains. The correspondence between a DGGE migration band derived from the consortium and a particular clone and strain was established using PCR-DGGE co-migration analysis (Fig. 4).

The DGGE patterns indicate the changes in bacterial diversity of the consortium over time (Fig. 4), successions in consortium composition are shown by bands that appeared or disappeared during the experiment. However a band with a very high intensity (B8) can be observed in all DGGE profiles. This band was excised, re-amplified and cloned; the partial 16S rRNA gene sequence of three randomized clones was determined (clones 18, 19 and 20). Phylogenetic analysis (Fig. 3) revealed that the B8 clones showed a phylogenetic relationship with the *Sphingomonadaceae* family. The 16S rDNA sequence of the three B8 clones showed a 96% similarity with the 16S rDNA sequence of the strain AM (identified as *Sphingobium* sp), although they showed a different position in the DGGE gel (Fig. 4) and grouped in separate sub-clusters within the Sphingomonadaceae cluster (Fig. 3). Furthermore, the band corresponding to the isolate strain AM, as well as the other isolated strains, did not appear in the studied DGGE profiles of the CON-Phe, suggesting that it is not a predominant member in terms of genetic diversity.

Table 1
Morphologic and biochemical characteristics of strains AM, T, Bc, B and B1 isolated of CON-Phe consortium.

Characteristic	AM	T	Bc	B	B1
Colony color	Y	T	W	W	W
Microscopy morphology	R	R	R	R	R
Gram stain	−	−	−	−	−
Nitrate reducing	−	+	+	+	+
Nitrite reducing	−	+	+	+	+
Production of N_2	−	+	−	+	+
Oxidase (Kovacs)	+	+	+	−	−
OF glucose	N	O	O	F	F
Citrate (Simmons)	−	+	+	+	+
Gelatin hydrolysis	−	−	−	−	−
Growth in Agar Cetrimide	−	+	+	−	−

Y: yellow; T: transparent; W: white; R: rods; N: neuter; O: oxidative, F: fermentative; "+" indicated positive reaction; "-" indicated negative reaction.

Table 2
Results of partial sequence and tentative phylogenetic affiliations of strains isolated from the consortium AM, T, B, B1 and Bc.

Strain	Accession number[a]	Most closely related bacterial 16Sr RNA 2gene sequence	Similarity	Accession number[b]
AM	JQ886663	*Sphingobium* sp.	98%	EU159274.1
T	JQ886664	*Pseudomonas* sp.	100%	AY520572.1
Bc	JQ886665	*Pseudomonas* sp.	98%	DQ127532.1
B	JQ886666	*Enterobacter aerogenes*	99%	AB244467.1
B1	JQ886667	*Enterobacter aerogenes*	99%	AB244467.1

[a] GenBank accession number of the sequence from isolated strain.
[b] GenBank sequence accession number of most closely related bacterial sequence.

Based on sequence analyses of the 16S rDNA library, four different phylotypes were found at day 4 of incubation, three of which (represented by 6 clones) were affiliated to Alphaproteobacteria; another phylotype (represented by 1 clone) showed 94% of identity with *Alcaligenes faecalis* grouped within Betaproteobacteria. Three of the clones affiliated to Alphaproteobacteria (7, 9 and 8) clustered within the Sphingomonadaceae family and matched closely with sequences of clones from the predominant band in the DGGE profiles. The other clones were affiliated to another order of Alphaproteobacteria, two (10 and 11) were affiliated to *Inquilinus ginsengisoli* and another one (21) to *Ochrobactrum* sp.

At the end of the incubation time (15 days), the DGGE profile showed that whereas the B8 band, affiliated to *Sphingomonadaceae*, persisted as predominant band, a higher genetic diversity could be observed. The 16S rRNA gene library also showed a preponderance of Sphingomonadaceae family (five clones). Another phylotype (represented by 1 clone) was affiliated to Betaproteobacteria, *Burkholderiales* order, which includes the genus *Achromobacter*, *Bordetella* and *Alcaligenes*.

3.4. Study of degrading capacities of the isolated strains AM, T, Bc, B and B1

The capacity of the strains to grow using phenanthrene, HNA and sodium salicylate as the sole carbon source was determined in LMM after 7 days of incubation (Table 3).

The strains AM, T and Bc grew with phenanthrene as carbon source, however only the strain AM showed phenanthrene degradation in these conditions (approximately 87% in 7 days) (Table 3). Also, the strain AM was the only one that produced the formation of blue-indigo in presence of indol, indicating its aromatic ring cleavage activity. The strain AM did not grow in HNA but grew in salicylate.

The *Pseudomonas* sp strains (T and Bc) showed growth with the three carbon sources assayed, nevertheless in no case it was possible to demonstrate the substrate degradation as it was similar to control values. On the other hand, the *Enterobacter* sp. strains (B and B1) showed no growth with any of the carbon sources studied.

The presence of PAH-ring hydroxylating dioxygenases genes was screened in the isolated strains and in the CON-Phe at different incubation times, using the primer set designed by Cébron et al. (2008). Visualization of PCR products on agarose gel showed a single band of the expected size (306 bp) for the phenanthrene-degrading strain AM and *Enterobacter* sp strain B (Fig. 5). The

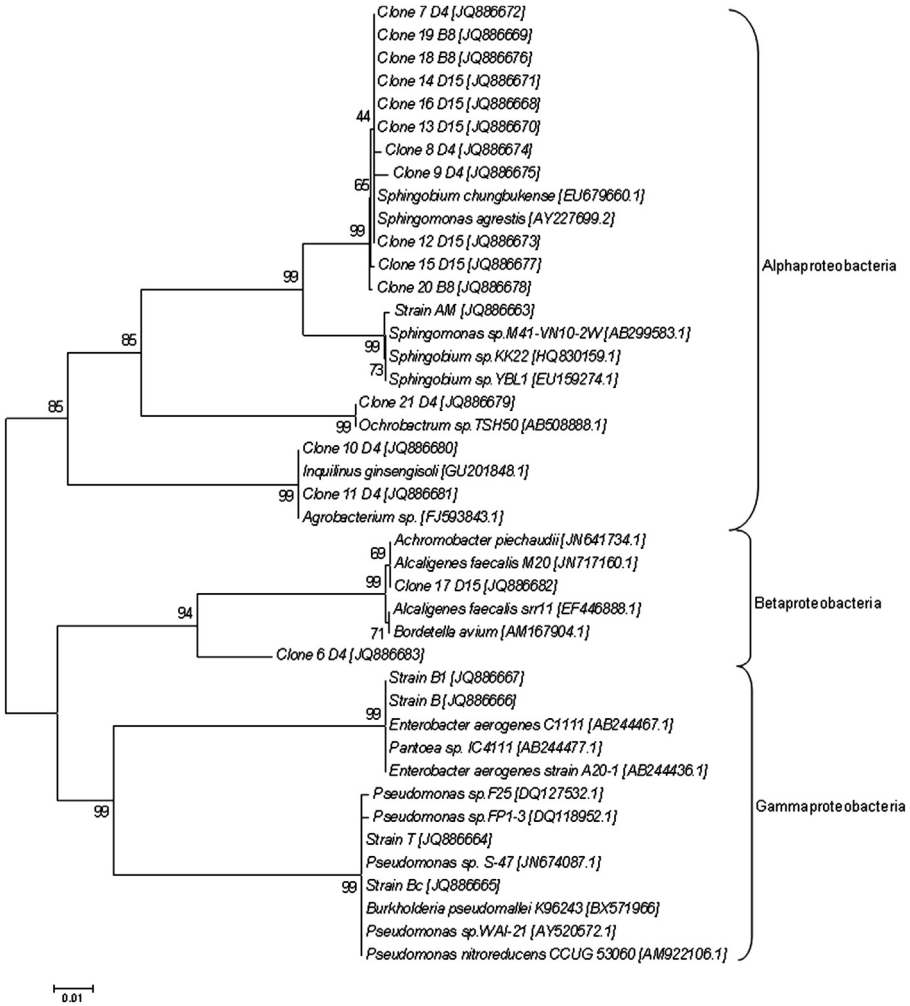

Fig. 3. Neighbor-joining tree based on a distance matrix analysis of 16S rDNA partial sequences of DGGE bands, isolated strains, clones and selected sequences available in GenBank (http://www.ncbi.nlm.nih.gov/Genbank) was constructed using the MEGA software version 4 (Tamura et al., 2007) .The numbers at each node correspond to the bootstrap per cent values. The space bar indicates 0.01 sequence variation. The numbers of clones with identical sequence are indicated in parentheses. The GenBank accession numbers of the isolated strains, the clones and selected sequences from GenBank are indicated.

strain T showed a PCR product with a slightly larger size than the expected one.

At the beginning of the incubation time, the PCR product from the CON-Phe showed a multiplicity of bands, one weak band of the expected size and other four bands with a larger size, between which two intense bands located among 1000–1500 bp were prominent. After 4 days of incubation only the two intense bands of larger size were visualized and no bands were observed in the PCR products from the total DNA of CON-Phe after 7 and 15 days of incubation.

3.5. Phenanthrene biodegradation in liquid medium by defined consortia

The phenanthrene degradation abilities of different defined consortia, formed by the combination of the phenanthrene-degrading strain AM with one of the other isolated strains (AM + T; AM + Bc and AM + B), were compared with the phenanthrene degradation performed by the AM strain. Table 4 shows that after 2 days of incubation the residual phenanthrene concentration in the defined consortia cultures (AM + T; AM + Bc and AM + B) showed no significant differences ($P < 0.05$) from the AM culture; reaching percentages of degradation between 56 at 66% of the phenanthrene supplied. Also, the accumulation of studied phenanthrene metabolites, HNA and salicylic acid in the consortia cultures were not significantly different from the AM culture.

After 7 days of incubation the defined consortia showed percentages of phenanthrene biodegradation higher to 97% and significantly higher ($P < 0.05$) than that reached by the strain AM (87.5%). But, at the same time and the same condition, the AM culture and defined consortia presented residual phenanthrene concentration significantly lower ($P < 0.05$) than the CON-Phe consortium (Fig. 1a). Also, the accumulation of HNA observed in the CON-Phe culture after 7 days of incubation was not detected either in the defined consortia or in the AM culture.

4. Discussion

In the present study a phenanthrene-degrading consortium (CON-Phe) was obtained from a chronically contaminated soil by sequential enrichment in liquid culture. Under studied conditions, the consortium showed phenanthrene degradation even when high initial phenanthrene concentrations were supplied (500 and 2000 mg l^{-1}). To lower initial phenanthrene concentration (200 mg l^{-1}) the consortium managed to degrade 58% of the phenanthrene supplied during the first 7 days of incubation

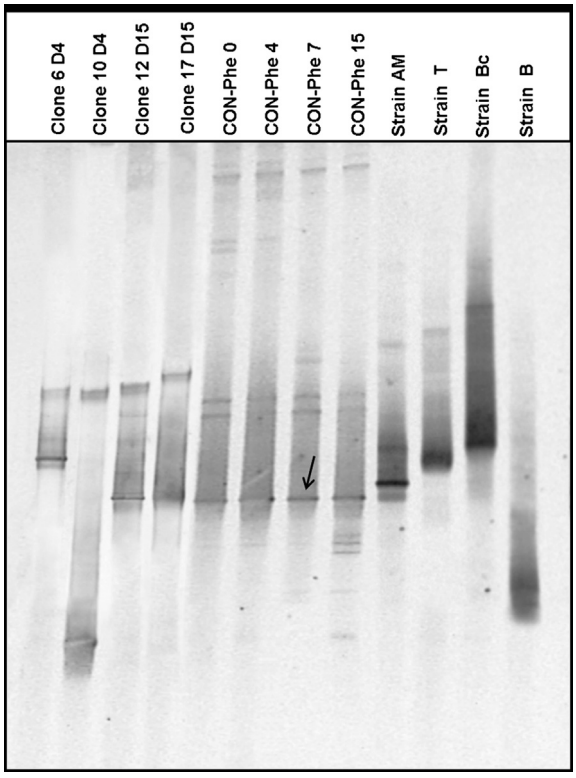

Fig. 4. PCR-DGGE analysis of isolated strains clones from libraries and bacterial populations of CON-Phe over the course of 15 days of incubation. The sequenced band (named as band B8 in the text) is indicated by the arrow.

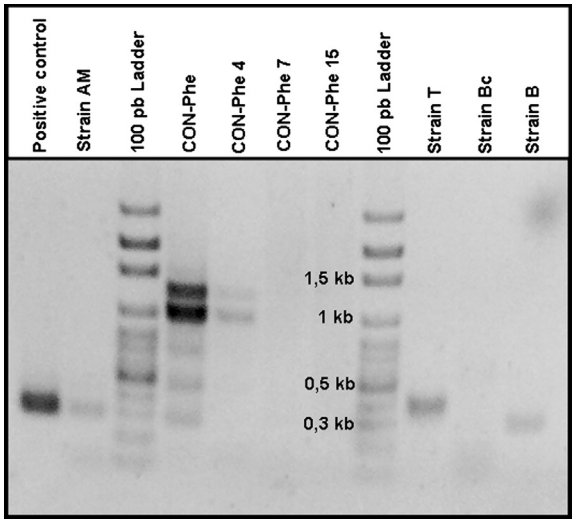

Fig. 5. Agarose gel electrophoresis of PCR products of PAH-ring hydroxylating dioxygenases genes.

(Fig. 1a) with the concomitant accumulation of at least one phenanthrene intermediate metabolite (Fig. 1b). One of the possible advantages of the use of microbial consortium to improve PAH degradation, opposite to pure bacterial cultures is that due to the commensal or syntrophic contribution between the diverse members of the consortium, they could perform a complete degradation pathway of PAHs (Bouchez et al., 1999). This was not found with the CON-Phe growing in LMM with phenanthrene as sole carbon source, where the accumulation of HNA was observed (Fig. 1b). Kanzuga and Aitken (2000) proposed that the accumulation of highly reactive metabolites either reduces the viability of PAH-degrading bacteria or causes inhibition of the degradation of PAH. These both effects were observed in the CON-Phe after 7 days of incubation (Fig. 1a and c).

The composition of CON-Phe and its dynamic during phenanthrene degradation was determined using culture-dependent and independent approaches. One important observation is that the 16S rDNA sequences of strains isolated from R2A plates were not closely related with the sequences obtained from the 16S rDNA mini-library of the CON-Phe (Fig. 3), and neither showed a co-migration with any DGGE bands from CON-Phe profiles (Fig. 4). However, the sequence of the dominant band (B8) of the DGGE profiles was also the most frequently obtained by 16S rDNA library. In addition, some clones from 16S rDNA library showed co-migration with the bands observed in DGGE profile of CON-Phe (Fig. 4). These results proved a good correlation between the culture-independent approaches.

The fact that the sequences obtained by culture-independent approaches were not correlated with any isolated strains clearly might be caused by the different culturability of the bacteria present in the CON-Phe in the conditions used in the culture-dependent approach (culture medium, temperature, etc.). In other words, the culture-independent approaches are affected by the biases of 16S rDNA PCR amplification, so that the 16S rDNA from some species are preferentially amplified (Hansen et al., 1998). These biases have been attributed to the differences in the genome size and 16S rDNA copy number of the species present in the sample (Farrelly et al., 1995), the interference from DNA flanking the template region (Hansen et al., 1998) or mismatches between primer and its binding site on the genome (Sipos et al., 2004). In consequence, the culture-independent approaches based on PCR amplification of 16S rDNA were suitable for the identification of some numerically important members of the community, but not for the determination of the dominant organisms (De Araujo and

Table 3
Utilization of carbon substrate by the strains AM, T, Bc, B and B1. A control system was added as abiotic elimination measured.

Carbon source	(Growth[a])/% of carbon source degradation (mg l^{-1})					
	AM	T	Bc	B	B1	Control
Phenanthrene	(+)/87.4 ± 4.0*	(+)/9.7 ± 4.0	(+)/12.1 ± 5.9	(−)/9.1 ± 1.4	(−)/1.0 ± 1.9	−/10.9 ± 2.3
1-hydroxy-2-naphthoic acid	(−)/15.6 ± 3.2	(+)/14.2 ± 7.0	(+)/10.8 ± 0.8	(−)/13.9 ± 10.7	(−)/5.7 ± 4.1	−/10.7 ± 9.5
Salicylic acid[b]	(+)/nd	(+)/92.3 ± 0.3	(+)/nd	(−)/nd	(−)/nd	−/92.9 ± 0.8

(+) Corresponds to an increase ≥2 orders of magnitude.
Data corresponding to the carbon source degradation are mean of three independent experiments ± standard deviation.
*Indicates difference significant with the abiotic control ($P < 0.05$).
nd: not detected.
−: not determined.
[a] Growth was followed by measuring the increase of cfu in R2A during 7 days of incubation.
[b] The results of salicylic acid degradation corresponding to 48 h of incubation.

Table 4
Concentration of Phenanthrene, HNA and Salicylic Acid during Phenanthrene biodegradation in liquid medium by the strain AM and the defined consortia (AM + T; AM + Bc and AM + B). A control system was added for abiotic elimination measurement.

Concentration (mg l^{-1})	2 days				7 days				
	AM	AM + T	AM + Bc	AM + B	AM	AM + T	AM + Bc	AM + B	Control
Phenanthrene	$87.3^{a} \pm 7.4$	$67.9^{a} \pm 30.6$	$76.1^{a} \pm 29.5$	$77.2^{a} \pm 35.2$	$25.1^{b} \pm 8.0$	$2.9^{c} \pm 1.8$	$5.8^{c} \pm 4.3$	$2.8^{c} \pm 0.5$	$166.8^{d} \pm 7.3$
1-hydroxy-2-naphthoic acid	$64.6^{a} \pm 13.9$	$50.5^{a} \pm 12.5$	$56.5^{a} \pm 36.6$	$51.3^{a} \pm 9.5$	nd	$0.7^{b} \pm 0.2$	$0.6^{b} \pm 0.1$	$0.4^{b} \pm 0.1$	–
Salicylic acid	$0.5^{a} \pm 0.4$	$12.0^{a} \pm 21.6$	$21.1^{a} \pm 27.9$	$12.3^{a} \pm 13.3$	nd	nd	nd	nd	–

Values are the means of six tuplicate independent experiments (standard deviation). Values in a row followed by the same letter are not significantly different ($P < 0.05$).
nd: not detected.
–: not determined.

Schneider, 2008). Hence, the use of the polyphasic assessment is necessary for a better understanding of the diversity of the consortium, as it was previously proposed by Viñas et al. (2005), but he has not given us information about the composition of the community, in terms of relative abundance.

Within the isolated strains, the *Sphingobium* sp (strain AM) showed phenanthrene degradation capacity (Table 3), together with clear evidence of the presence and activity of PAH-ring hydroxylating dioxygenases (indol test and Fig. 5). In addition, the differential counts of the yellow colonies (with similar morphologic characteristic of the strain AM) performed during phenanthrene degradation by CON-Phe showed that the growth curve of YC (Fig. 2) exhibits a similar behavior to PAH-degrading bacteria counts (Fig. 1c). Also the dynamics of DGGE profiles of the consortium during the phenanthrene degradation showed that at 4 and 7 days of incubation (Fig. 4) the number of detected bands was lower than in the 15 day profile and was dominated by the presence of the B8 band that was also phylogenetic related with the *Sphingomonadaceae* family (Fig. 3). These results suggest that the Sphingomonadaceae species present in CON-Phe would be functionally related to the first steps of the phenanthrene degradation pathway.

Two of the isolated strains (T and Bc) from CON-Phe belong to *Pseudomonas* sp. Bacteria of the genus *Pseudomonas* are well-known PAH-degraders (Janbandhu and Fulekar, 2011). Although we could not demonstrate the capacity of this strain to degrade any of the studied compounds (Table 3), they were able to grow in phenanthrene, HNA and salicylic acid. On the other hand, the dynamic of the characteristic colony of the strain T (Fig. 2) that showed a clear predominance toward the end of the incubation period, when phenanthrene elimination ceased (Fig. 1a), might be indicating that the strain T would play a functional role related with the degradation of the phenanthrene metabolites.

The other isolated strains (B and B1) were identified as *Enterobacter* sp. *Enterobacter* genus has been traditionally studied by its animal gut symbiotic function, but rarely recognized as a PAH degrading group (Toledo et al., 2006). However, the strain B and B1 did not grow with any of the carbon source studied (Table 3), evidence of the presence of PAH-ring hydroxylating dioxygenases gen was found in the strain B (Fig. 5). Though we could not establish the possible role of these strains, *Enterobacter* sp strain has been previously isolated of the PAH-degrading consortia (Molina et al., 2009), some strains have demonstrated PAH-degrading capacity (Toledo et al., 2006; Molina et al., 2009) and other authors demonstrated that the EPS excreted by *Enterobacter cloacae* strain TU exhibited bioemulsifying activity, increasing the aqueous solubility of hexadecane (Hua et al., 2010). On the other hand, it is important to notice that neither in this work nor in Molina et al. (2009) work it was possible to identify the isolated *Enterobacter* strains with any DGGE bands of its consortium. The fact that *Enterobacter* sp. strains were isolated from both consortia, but not detected by PCR-DGGE might be attributed to the previously enumerated PCR biases.

With respect to other members of the CON-Phe that were determined only by culture-independent approaches, several phylotypes could be affiliated to bacterial genera that include phenanthrene-degrading species. Phenanthrene degradation capacity were reported to soil isolated strains of *Ochrobacterium* sp. (Ghosal et al., 2010), *Agrobacterium* sp (Aitken et al., 1998), *Achromobacter insolitus* (Janbandhu and Fulekar, 2011), *Alcaligenes faecalis* (Xiao et al., 2010) and *Bordetella petrii* (Yuan et al., 2009). Two of the sequenced clones found in CON-Phe at 4 days of incubation were affiliated to *I. ginsengisoli*, this species has been recently isolated from soil and described by Jung et al. (2011). Although this species has not been reported as PAH degrader, the presence of catechol 1, 2-dioxygenase gene was determined in a strain isolated from soil and identified as *Inquilinus* sp. (Tuan et al., 2011), suggesting that this species might be involved in the degradation of metabolites generated in the consortium.

As a complementary approach to determine the role of the isolated strains in the CON-Phe and the interaction between them, the phenanthrene degradation by defined mixed cultures was studied. All the defined consortia, formed by the phenanthrene-degrading strain AM together with another of the isolated strains, showed percentages of phenanthrene biodegradation significantly higher than that reached by the strain AM alone (Table 4), but also higher than the natural consortia (Fig. 1a). In the same way, the accumulation of HNA observed in the CON-Phe (Fig. 1b) was not detected in the AM and defined consortium cultures (Table 4). These results together with the behavior of the phenanthrene-degrading populations of the CON-Phe (Fig. 1c) might suggest that in the soil consortia, the competition between different species and the community dynamics, observed at level of cultivable (Fig. 2) and genetic diversity (Fig. 4), could cause a negative effect in the phenanthrene degradation.

On the other hand, a synergistic effect between the phenanthrene-degrading strain AM and the other isolated strains was observed (Table 4) since the defined consortia showed phenanthrene residual concentrations significantly lower than the AM culture. The cooperative metabolic activities in bacterial consortia during degradation of organic pollutants generally involve two known mechanisms. The first one is metabolic deficiencies, where the degrading bacteria are fastidious and depend on secondary strains providing various growth factors or nutrients (Sorensen et al., 2002). The second one is associated metabolism, where cross-feeding with metabolites from the degradation pathway occurs within the consortium (Bouchez et al., 1999; Dejonghe et al., 2003).

Another important observation is that whereas any of the studied strains shows a significant elimination of the HNA, this phenanthrene by-product, which increased its concentration after 2 days of incubation in all phenanthrene cultures (Table 4), showed

an almost complete elimination after 7 days of treatment, even in the AM culture. This could be attributed to the fact that enzymes needed for its degradation are induced by phenanthrene. In a recent work Wang et al. (2008) observed that *Cycloclasticus* sp. P1 can use neither salycilate nor catechol as carbon sources; however, a catechol pathway was confirmed to exist in P1 by an enzyme assay performed with lysates prepared from P1 cells grown on pyrene, naphthalene or phenanthrene. It is postulated that this pathway could be induced by pyrene, naphtalene and phenanthrene instead of catechol itself.

5. Conclusion

Despite the fact that both, culture dependent and independent techniques provided information about the diversity of the phenanthrene-degrading consortium; 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.

The incursion in the study of physiological interactions (related with phenanthrene degradation) between the strains isolated from the consortium demonstrated the existence of synergistic effects between them, suggesting that the incomplete phenanthrene degradation by the CON-Phe might be due to the existence of negative interactions between the complex diversity of consortium. More studies are needed to elucidate the complex mechanisms that govern the phenanthrene degradation by the consortium.

Our results suggest that the defined consortia designed from the strains isolated of the CON-Phe could be more efficient than the consortium itself, in bioaugmentation processes of PAH-contaminated soil.

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