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Study of a bacterial consortium adapted to low bioavailability to phenanthrene, as potential inoculant to chronically PAH contaminated soils.

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Introduction

Polycyclic aromatic hydrocarbons (PAHs) are common environmental pollutants produced by industrial operations using fossil fuels as well as by natural events such as forest fires (Berthe-Corti et al. 2007). Microorganisms are the major agents mineralizing PAH in terrestrial environments, either using them directly as carbon and energy source or via co-metabolism - when other substrate constitute the primary carbon sourse (Ortega et al., 1998).
Microbial transformations of PAHs do not only have a special impact in nature but they also play key roles in various technological applications of microorganisms, such as wastewater treatment, biodegradation, bioremediation, and biocatalysis.
PAHs have a strong tendency to adsorb onto solid surfaces especially hydrophobic sites. A reduction in water concentration due to their adsorption can modify the accessibility and bioavailability of PAHs and thus, decrease or enhance their biodegradation (Semple et al., 2007).
The establishment of microniches of PAH degrading bacteria in a PAH contaminated soil represents a process strongly influenced by both, the manner in which the PAH are exposure in the soil and bacterial capability to develop physiological strategies to adapt to the different PAH bioavailability (Johnsen et al., 2005).
In order to reduce phenanthrene bioavailability to different degrees, Grosser and coworkers (Grosser et al., 2000) proposed the establishment of enrichment cultures in which solid organic phases were used. They also reported that different phenanthrene-utilizing bacteria inhabiting the same soils may be adapted to different phenanthrene bioavailabilities. In addition, the authors showed that the range of phenanthrene bioavailabilities obtained using solid phases with different sorptive properties provides a useful approach for determining the effects of contaminant sorption on microbial selection and for cultivating and characterizing microorganisms that may have more relevance in natural environments.
Due to the reduced bioavailability that characterizes chronically hydrocarbons contaminated soils, the bioaugmentation with cultures obtained by enrichment in solid phase systems, could be a promissing strategy to apply in aged contaminated soils.
In this context, the objective of this work was to study four bacterial component obtained from a degrader phenanthrene consortium by using solid phases with a resin XAD2 preloaded with phenanthrene, and to rebuild a defined degrading consortium from them.
The isolated strains were identified by 16S rRNA and phenotypically characterized using the conventional assays. Furthermore, their main phenotypic features and biochemical characteristics where analyzed by Fourier-transform infrared spectroscopy (FT-IR). The phenanthrene degradation was studied from both in the strain pure cultures and from the defined consortium. Our results showed that the application of XAD2 preloaded with phenanthrene culture systems represents a selective environment towards substrate colonizing bacterial species and allow us study the different strategies to enhance the PAH uptake and allow the bacterial survival in oligotrophic conditions.

Materials and Methods

Phenanthrene degrader inocula. An enrichment culture of phenanthrene degrading bacteria was previously obtained in liquid mineral medium (LMM) (Coppotelli et al. 2010) by using a petrochemical contaminated soil (10% w/v) and 0.1g of phenanthrene cristals l^{-1}.

Bacterial consortium obtained by enrichments in the presence of model organic phases.
Amberlite® XAD®-2 preloaded with phenanthrene (10.87mg of phenanthrene g^{-1} XAD2) was used as solid model in batch system with LMM. Amberlite® XAD®-2 is a porous resin with large surface area available for phenanthrene sorption. The extent of phenanthrene desorption was less than 7% after 30 days, under abiotic incubation condition.
The batch systems LMM-XAD2-PHE were prepared by inoculation of 1ml of phenanthrene enrichment culture in 20ml of LMM containing 2g of XAD2 preloaded with phenanthrene (1000ppm). The systems were incubated during 30 days at 28±2°C. A 10% of colonized solid phase was removed, washed and transferred to fresh systems. As a whole, four transfers were done.

Isolation and identification of predominant degrader bacteria from the LMM-XAD2-PHE system.
The predominant strains were isolated by plating in R2 agar (Reasoner and Geldrich, 1985) and solid mineral medium (SMM) supplemented with phenanthrene as sole carbon source (Bogart, and Hemmingsen, 1992) from the colonized XAD2-PHE beads, from the fourth batch system.
The identity of the isolated bacteria was based on 16S rRNA gene sequence analysis (Macrogen, Korea). Nucleotide sequences were compared to those in the National Center for Biotechnology Information GenBank database by using BLAST program.

Phenotypic characterization of the isolates
Morphological features of the colonies were studied on R3 agar plates after 2 days of incubation at 28±2°C. Biochemical characterization included: Gram staining, motility, OF test, citochrome oxidase, hemolityc activity (Gerhard et al. 1981). Siderophore production and phosphorus solubilization were also determined (Aguado-Santacruz et al., 2012; Fernandez et al., 2005). For testing of growth substrates, the strains were inoculated in Biolog EcoPlate™ system (Biolog, Inc. USA). The microplates were incubated at 28±2°C and the absorbance of each well at 590 nm was measured daily with an automatic plate reader, for 5 days. The average well color development (AWCD) and the physiological profile of each strain were calculated. (van Heerden et al., 2002).
The isolates' ability to grow in LMM supplemented with crystal of phenanthrene (1000 mg phenanthrene l^{-1}) or with XAD2-PHE beads (10%, w/v) were studied. The capacity of growth in other PAHs: naphthalene, anthracene, fluoranthene, pyrene, dibenzothiophene, and a mix of the PAHs, as sole carbon and energy source, was analyzed in microplates with LMM supplemented with 0.1% of PAH. We also tested the indigo production, with indole crystals in the surface of SMM-PHE.
The kinetics were determined by measuring the increase of OD_{600nm} from the culture in R3 broth (Reasoner and Geldrich, 1985) and by plate count in R2 agar from LMM supplemented with phenanthrene.

FT-IR spectroscopy
FT-IR spectra analysis of the four individual strains was performed for bacteria growing on R3 agar for 4 days at 28±2°C. The bacterial sample was prepared as previously reported by Nauman et al., 2000. Three independent cultures for each strain were carried out.
Absorbance spectra of all samples were acquired from 4,000 to 600 cm^{-1} with a FT-IR spectrometer (Spectrum One, PerkinElmer, USA) with 6 cm^{-1} spectral resolution and 64 scan co-additions (Helm *et al.*, 1991). To avoid interference from biomass variations among the different samples, spectra were vector normalized in the whole spectral range of 4000 to 650 cm^{-1} (Naumann, 2000). OPUS software version 4.2 (Bruker Optics GmbH, Ettlingen, Germany) was used for data pre-processing and spectral calculations.
For data analysis the five typical windows which are usually used to carry out spectral correlations for the most important biological macromolecules in microorganisms were used: W_1, 3100–2800 cm^{-1} dominated by –C-H stretching vibrations of the functional groups usually present in the fatty acid components ($>CH_2$ and $-CH_3$); W_2, 1800–1500 cm^{-1} which includes the ester band, and the amide I and II bands from proteins and peptides (>C=O in esters, and C=O, N-H, C-N in Amide bands); W_3, 1500–1200 cm^{-1} known as the "mix region" which is assigned to proteins, phosphate compounds, and fatty acids absorption bands (C-O, P=O stretching bands); W_4: 1200–900 cm^{-1} recognized as the polysaccharide region (C-O, C-O-C); W_5, 900–700 cm^{-1} which contains bands which cannot be easily assigned to specific functional groups and is normally known as the "true fingerprint region" (Helm *et al.*, 1991; Naumann, 2000).

Defined bacterial consortium

The defined consortium was built up from predominant bacterial isolates of the fourth LMM-XAD-PHE system. The isolated colonies from SMM-PHE plates were resuspended in LMM to an optical density at 600nm of 0.01. Based on direct cell counts, this was equivalent to 1.10^8 to 2.10^8 cells ml-1. Each isolate was added to 125-ml Erlenmeyer flasks containing 40 ml LMM with 4g of XAD2-PHE (1000ppm), yielding an initial population density of 1.10^4 to 2.10^4 cells $ml^{-1.}$ The consortium' ability to grow in LMM supplemented with crystal of phenanthrene (1000 mg phenanthrene l^{-1}) or with XAD2-PHE beads (10%, w/v) were studied. Aditionally, each strain and the defined consortia were inoculated in LMM with XAD2 with the aim to evaluate the inespecific adsortion to the resin beads.

Phenanthrene degradation in the presence of model solid phases.

With the intention to evaluate the consortium degrading capacity, the residual phenanthrene and two metabolic intermediates, 1-hydroxy-2-naphthoic acid and salicylic acid were determined by HPLC from LMM-XAD2-PHE. We also determine the degrading capacity from de each strain in LMM-XAD2-PHE cultures at 28±2ºC in a rotary shaker at low rpm, after 30 days of incubation.

The remaining phenantrene was extracted with ethyl acetate using exhaustive extraction method (Coppotelli et al. 2010). The PAH concentration in the ethyl acetate extracts was analyzed by reversed-phase high-pressure liquid chromatography (HPLC) using a Waters® chromatograph with a Symmetry Waters® C18 column and a diode array detector (Coppotelli et al., 2010).

Results and discussion

Identification and study of the predominant sessil cells isolated.

The predominant sessil cells were isolated from the fourth LMM-XAD2-PHE system. In all, four different colony morphotypes in R3 agar were obtained. They corresponding to two Gramnegative rods, and two Grampositive pleomorphic cells (Table 1).

Table 1: Physiological properties of the strains.

Strains	C1	C2	C3	C4
Cell Morphology	Regular rods	Regular rods	Rods	Cocci-rod cycle
Colony morphotype	Small, yellow	Small, extended, transparent	Pink, opaque	White, extended, mucous
Gramreaction	Gram-negative	Gram-negative	Gram-positive	Gram-positive
Motility	+	-	-	-
OF - glucose	O [a]	inert	O [a]	O [a]
Hemolytic activity	-	+	-	-
Cytochrome oxidase	+	-	-	-
Indigo production	+	-	-	-
Siderophores	-	+	+	-
Phosphorus solubilization	+	-	-	+
R3 broth (μ, hs^{-1})	0.38 ± 0,07	0,23 ± 0,01	0,20 ± 0,05	0,50 ± 0,05
LMM-D-glucose (μ, hs^{-1})	0.14±0.03	No growth	0.21±0.01	0.22±0.04
LMM-PHE (μ, hs^{-1})	0.12±0.05	No growth	No growth	0.05±0.01
SMM-PHE	halo	halo [b]	Growth without halo	halo

[a] Mild acidification

[b] The initial ability to produce a solubilization halo was lost through successive passages on SMM-PHE.

Submission of the segment of 1,400bp of the 16SrRNA gene to GenBank revealed that the strains C1, C2, C3 and C4, were most closely related to *Sphingobium, Acidovorax, Rhodococcus* and *Arthrobacter genus*, respectively (Table 2).
The C1 strain was related with maxima identity with *Sphingobium fuliginis (*strain TKP^{T}), that was described as efficiently phenanthrene degrading on solid medium as well as in liquid culture (Prakash and Lal, 2006).
The C2 strain was closely related to *Acidovorax avenae* strain, a strain isolated from rhizosphere from the Ni hyperaccumulator *Alyssum mural*.
Rhodococcus sp. ML-0004, a novel strain for producing epoxide hydrolase, isolated from soil was the most closely related to C3 strain. It is well known that Rhodococcus genus has a remarkable metabolic diversity (Alvarez, 1996). Direct adherence to hydrocarbons is a common uptake mechanism for Rhodococcus and could be attributed to cell-bound glycolipids (Lang and Philp, 1998). Alonso-Gutierrez et al., 2009, observed the dominance of the Class Actinobacteria at oiled rock, and explained the major presence of *Rhodococcus* genus, due to its hydrophobic surface development, that allowed the growth of the other microorganisms attached to the oil surface increasing their degrading capacity.

Table 2: Results of 16s rRNA gene sequence and phylogenetic affiliations of the strains isolated by solid phase model with resin XAD 2 preloaded with phenanthrene.

Strain	Most closely related bacterial 16S rRNA gene sequence	Cover[a]	Max identity	Accesion number [b]
C1	*Sphingobium fuliginis strain TKP*	1324/1485	99	DQ092757.1
C2	*Acidovorax avenae*	1285/1285	99	AY512827.1
C3	*Rhodococcus opacus strain ML0004*	1351/1485	99	DQ474758.1
C4	*Arthrobacter globiformis strain A2S3*	1354/1485	99	EU221407.1

[a] Results are expresed as no.of similar nucleotides/no. tested
[b] GenBank sequence accesion number of most closely related bacterial sequence.

The C4 strain was most closely related to *Arthrobacter globiformis strain A2S3* isolated from the microbial biota associated with wheat rhizosphere. This strain was described as Nitrogen fixer that exhibited antifungal activity. As C4 strain, A. *globiformis A2S3* was not a siderophore producer. We observed a mucoid morphotype from C4 strain colony, indicating the polymeric extracellular matrix (EPS) production. This property has been shown to profoundly influence the rate and degree of attachment of microbial cells to hydrophobic surfaces and could be also involved in the storage of PAH in the polymeric matrix (Johnsen et al., 2005). *Arthrobacter phenanthrenivorans* type strain Sphe3T, described as phenanthrene degrader by Kallimanis et al., 2009, was related with a 97% of maxima identity with our C4 strain.
The physiologycal profile of the strains was tested to assess the potential metabolic diversity of each strain. The average well color development (AWCD) was also calculated.

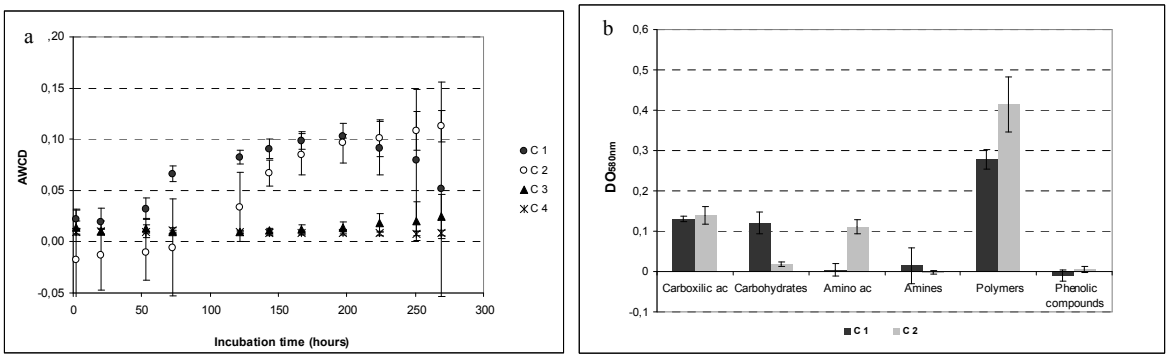

Figure 1: Average well color development (AWCD) of substrate utilization profiles in Biolog EcoPlate^{TM} microplates from the isolated strains (a). Total utilization of carbon substrates from different substrate groups by C1 and C2 strain, at 200hs of incubation (b).

Only the Gramnegative strains, C1 and C2, developed in the Eco microplates (Fig 1a). The Figure 1a shows de evolution of AWCD of C1 and C2 strains during the incubation time. The

AWCD values increased slowly for both strains, reaching peak colour development in 200hs, aproximately. The Figure 1b shows the physiological profiles obtained for both strains when the AWCD maximun was reached. The mainly assimilated substrates were the polymers, tween 40 and tween 80 for C2, and α cyclodextrine for C1.
When the strains were inoculated in LMM with D-glucose as sole carbon source the C2 strain was unable to grow, a ubiquitous trait in *Acidovorax* strains (Willems et al., 1990). All the isolated, except of C2, produced mild acidification in arobic condition from glucose in OF test (Table 2).
With reference to PAH degrader capacity, C1, C2 and C4 evidenced the ability to produce a solubilization halo on SMM-PHE. After the early successive passages in SMM-PHE the C2 degrading capacity was lost. The C1 and C4 strains grew en LMM with cristals of phenanthrene (Table 2). Only C1 strain developed in the microplates with LMM and PAHs as sole carbon and energy source. Color development was detected in the wells supplemented with anthracene, dibenzothiophene and the mix of PAH, due to metabolic byproducts. The addition of indole to SMM-PHE agar plates resulted in purple colonies from the production of indigo only for C1 strain and was indicative of an active dioxygenase. The hemolytic activity was tested by the blood agar method, as a preliminary screening of microorganisms for the ability to produce biosurfactants on hydrophilic media (Walter et al., 2010). Only the C2 strain evidenced hemolytic activity in the assay conditions. The siderophore production and phosphorus solubilization was evidenced among the isolates (Table 2). The presence of these properties in the defined consortium could be interesting at the moment to propose apply it in a nutrient deficient soils, as the chronically hydrocarbon contaminated soils are.

FT-IR spectroscopic characterization of the individual strains
FT-IR spectroscopy of intact cells produced highly specific bacterial patterns which have successfully been applied to characterize the structural and biochemical composition of a wide range of microorganisms, including Grampositive and Gramnegative bacteria, in a non-destructive manner (Kacurakova et al 2000). We applied this technology to study the more distinct biochemical characteristics of each of the genus encountered in the consortium.
Figure 2 shows the overlaid FT-IR absorbance spectra of one of the three replicate spectra obtained for each strain. The mains distinct aspect that spectra of the strain C1 assigned to *Sphingobium fuliginis* species showed, was the ability of these bacteria to synthesize and accumulate a significant amount of polyhydroxyalkanoates (PHAs) when they are grown under the above mentioned conditions. Spectra of these bacteria showed a strong ester carbonyl band at 1,738 cm^{-1}, accompanied by a number of additional bands at 1,383, 1,304, 1,187, 1,135, 1,101, 1,059, and 976 cm^{-1}, which were assigned to poly(β-hydroxybutyrate) (PHB) (Naumann 2000, Bosch et al. 2008), one of the most common PHAs usually accumulated by bacteria as carbon and energy reserve.
Spectra acquired from the strain C2, identified as *Acidovorax avenae,* showed the typical spectral features of Gramnegative bacteria. Most the absorbance peaks displayed by these bacteria cultured on R3 agar are similar to those recently reported for *A. citturli* (Wang et al. 2012), showing no spectral bands associated to the presence of ester groups.
As Grampositive organisms both C3 and C4 strains displayed the general spectral features of this type of bacteria showing the typical peaks at 1,240, 1,154, 1,080 and 1,026 due to the peptidoglycan moieties and the phosphorus-containing carbohydrates such as teichoic acids usually present in the bacterial cell walls (Naumann 2000). These two bacterial strains, however, seemed to express significant different phenotypes when they are cultured on R3. Inspection of spectra aquired for the strain C3, identified as *Rhodococcus opacus,* revealed the presence of a high intense bands in the regions assinged to antisymmetric and symmetric stretching modes of C-H in $>CH_2$ (2,927 and 2,854 cm^{-1}, respectively), together with a significant peak at 1,746 (assigned to C=O in esters). We could thus infer that the *Rodoccocus* strain member of the consortium, as occurred with other Grampositive bacteria belonging to *Mycobacterium* and *Streptomyces* genera, has the *ability* of synthesize and intracellularly accumulate neutral lipids such as triacylglycerols (TAG) (Alvarez et al. 1996). The accumulation of these lipids was attributed to be part of a strategy of these bacteria to survive under environmental fluctuating conditions (Bequer Urbano, 2012).
Finally, the spectra recovered for the C4 strain identified as *Arthrobacter globiformis,* showed in addition the typical bands associated to Grampositive bacteria, a significant increase in the intensity of the absorption band in the W4 region, (assigned to C-O-C, C-O dominated by ring vibrations in various polysaccharides together with a low amount of biomass (represented by a weak Amide II band). These results could be indicating that bacterial colonies grown on R3 agar

are embedded externally by a mucous as determine in the morphotype analysis (Table 1) mainly constituted by carbohydrates.

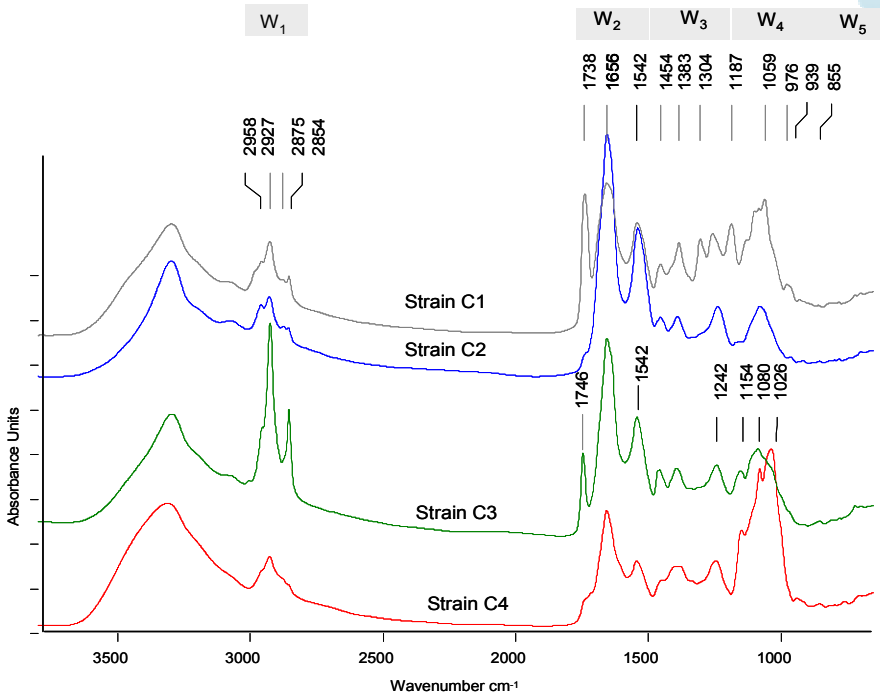

Figure 2: FTIR absorbance spectra of the 4 individual strains recovered from the consortium. The 5 typical windows which contain information of the different cell components usually used to study biological samples (see method section) are indicated.

Growth of the isolated strains and the defined consortium in LMM-XAD2-PHE system.
The strains were inoculated in LMM XAD-PHE systems and after 10 and 30 days samples were taken and analized separately by plate count, from the sessil and the planktonic cells.
The Table 3 shows the results obtained from the sessil cells of the strains growing in XAD2-PHE and LMM-XAD2. The last condition allowed us estimate the inespecific adsortion to the resin.
It is possible to appreciate that in general, the strains were adsorbed more significantly to XAD beads than XAD-PHE when they grew in independent batch systems.

Table 3: Bacterial growth on the resine beads preload with phenantrene XAD2-PHE and on XAD, in LMM batch systems.

System		XAD2 (Ufc of sessil cells g-1)		XAD-PHE (Ufc of sessil cells g-1)	
Incubation time		10 days	30 days	10 days	30 days
Strains	C1	$^{a}5.13.10^{7}$	$^{bc}1.97.10^{6}$	$^{c}9.52.10^{5}$	$^{b}4.21.10^{6}$
	C2	$^{b}4.21.10^{6}$	$^{a}7.73.10^{5}$	$^{a}1.22.10^{6}$	$^{a}2.81.10^{5}$
	C3	$^{b}4.21.10^{5}$	$^{a,b}1.36.10^{6}$	$^{b}9.63.10^{5}$	$^{a}7.65.10^{6}$
	C4	$^{a}1.37.10^{7}$	$^{b,c}4.90.10^{5}$	$^{a,b}6.61.10^{6}$	$^{c}1.32.10^{5}$

Values, gives as means of triplicate independent experiments with the same letter are not significantly different (P< 0.01).

An opposite behavior was observed when the strains were inoculated toghether in LMM-XAD2-PHE system, as defined consortium, Figure 3. In this culture condition the strains grew more significantly adsorbed to XAD-PHE beads during all incubation time.

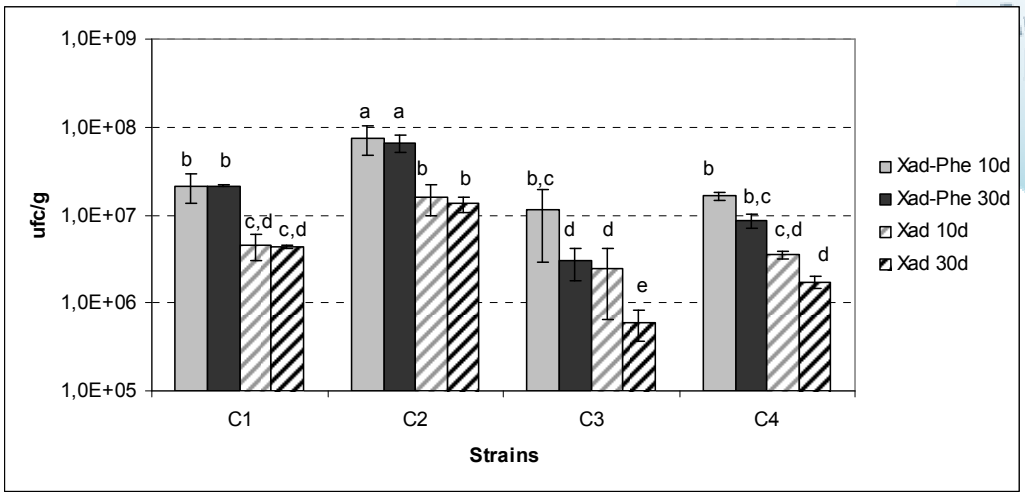

Figure 3: Growth of the sessil cells after 10 and 30 days in the model organic phases, with XAD2 and XAD2 preloaded with phenanthrene. The different cell morphotypes coud be distinguished and plate counted during the incubation of the defined consortium in LMM-XAD and LMM-XAD-PHE. Values, gives as means of triplicate independent experiments with the same letter are not significantly different (P< 0.01).

The C2 strain, identified as *Acidovorax* genus, developed very small and transparent colonies in SMM-PHE, without phenanthrene solubilization halo. Despite not growing in pure culture with cristal or adsorbed phenanthrene, the C2 strain showed the highest development during the 30 days, adsorbed to XAD-PHE in the defined consortia (Fig.3).
Similarly, the C3 strain, identified as *Rhodococcus* genus grew neither in LMM-PHE nor LMM-XAD2-PHE, in pure culture. After 10 days in the defined consortium, the C3 cells were significantly adsorbed to XAD-PHE. After that, the adsorbed cells decreased in presence or not of phenanthrene.
The C1 and C4 strains, identified *as Sphingobium* and *Arthrobacter* genus respectively, developed colonies with solubilization halo on SMM-PHE and grew in LMM with phenanthrene. These strains remained significantly adsorbed to XAD2-PHE in the consortium during the 30 days of incubation. This result suggests C1 and C4 strains kept the phenanthrene dissolution maximal by effective removal of aqueous phenanthrene, without extra bioavailability promoting properties when are growing with adsorbed phenanthrene.
Instead, C2 and C3 strains, wich did not grow in LMM with cristal phenanthrene, demonstrated to have the ability to remain adhered to XAD2-PHE. Due to highest sessil count cells, C2 strain could express a specific bioavailability promoting property when is growing in the defined consortium with adsorbed phenanthrene.
The results of the residual phenanthrene from the strains growing in pure culture and all toghether in the defined consortium in LMM-XAD2-PHE are shown in the Table 4.

Table 4: Phenanthrene remaining and metabolic intermediates from the strain pure cultures and the defined consortium in LMM-XAD2-PHE, after 30 days of incubation.

	Phenanthrene elimination (%)	1-Hydroxy-2-naphthoic acid [1] (%)	Salicylic acid[1] (%)
C1	60.5 ± 1.6 a,b	85.5 ± 23.3 a	0.19 ± 0.13 a
C2	19.9 ± 11.1 c	13.8 ± 10.3 b	Nd
C3	24.4 ± 5.4 c	38.8 ± 1.0 a	Nd
C4	47.1 ± 7.8 b,c	17.0 ± 0.3 b	Nd
Consortium	69.7 ± 3.2 a	Nd	0.04 ± 0.02 a
Abiotic Control	7 ± 3.1 d	-	-

Nd: no detected
[1]The hydroxi naphtoico and salicilic acids values represent the percent of the metabolite of the total eliminated phenanthrene. Value, gives as means of triplicate independent experiments with the same letter are not significantly different (P< 0.01).

The C1 strain showed the highest phenanthrene removal efficiency, and there was no significant difference with the consortium efficiency. No significant differences in removal efficiency existed between C2 and C3 and C4 strains.
The bacterial degradation of phenanthrene starts with the incorporation of two oxigen atoms in the molecule during a reaction catalized by dioxygenase enzyme (Habe and Omori, 2003).This enzyme was detected by the production of indigo in C1 strain, in the indole test.
The high value of 1hydroxi 2naphtoic acid produced by C1 strain indicated that the early steps of the degradation occured but then the degradation route resulted blocked in that culture condition. Also C4 strain produced significant phenanthrene elimination, but with less 1hydroxi 2naphtoic acid accumulation. However, the dioxygenase activity in C4 by the indol test was nor detected.
The phenanthrene removal efficiency was almost 70% when all strains were inoculated together in the defined consortium. There was no 1hydroxi 2naphtoic acid accumulation and lower salicilic acid percentaje was detected. The results suggest the behavior of the strains in consortium does not only depend on their own physiological characteristics and the applied environmental conditions, but also on interactions among them.

Conclusion

The predominant bacterial strains from a degrader consortium adapted to low bioavailability to phenanthrene were studied applying conventional biochemical and physiologycal analysis and the FT-IR methology. The four isolated strains were identified as belonging to *Sphingobium*, *Acidovorax*, *Rhodococcus* and *Arthrobacter* genera, suggesting the strains could develope different strategies to integrate the phenanthrene degrader consortium. Interesting properties of each strain were detected, as phenanthrene degrading capacity in pure culture; ability to produce and accumulate a significant amount of reserve material like as PHB or TAG; abundant extracellular polysaccharide production; siderophore production and phosphorus solubilization and capacity to adherence to hydrophobic surfaces. The strains *Acidovorax avenae* C2 and *Rhodococcus opacus* C3 could not degrade phenanthrene in pure culture, regardless whether the PAH was provided as cristals or adsorbed to XAD2. When the strains were cultured toghether in the defined consortium with the phenanthrene adsorbed to XAD2, the degrader capacity was significantly higher than in the pure culture of its constituent members. This suggests the consortium growth in the biofilm increased the phenanthrene degrading capacity and promoted the individual strains growth. This synergic relationship between the strains, possible due to growth factors or metabolic intermediates, could explain the higher phenanthrene-removal efficiency of the consortium, with no significant intermediates accumulation.

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