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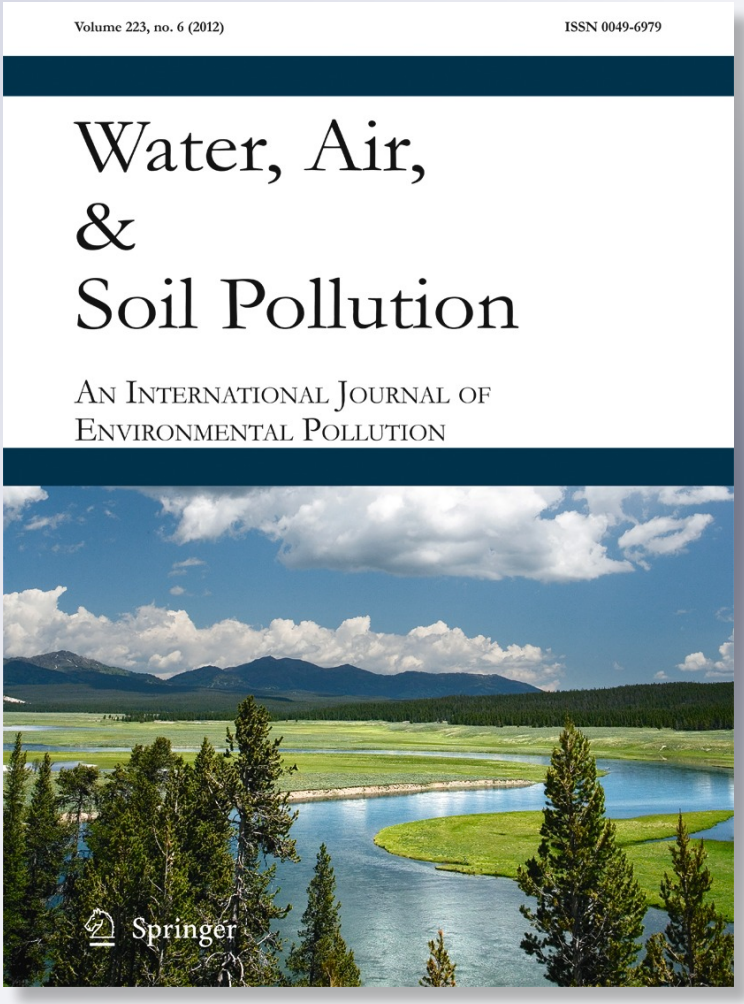

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Evaluation of the Influence of Multiple Environmental Factors on the Biodegradation of Dibenzofuran, Phenanthrene, and Pyrene by a Bacterial Consortium Using an Orthogonal Experimental Design

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Abstract For a bioremediation process to be effective, we suggest to perform preliminary studies in laboratory to describe and characterize physicochemical and biological parameters (type and concentration of nutrients, type and number of microorganisms, temperature) of the environment concerned. We consider that these studies should be done by taking into account the simultaneous interaction between different factors. By knowing the response capacity to pollutants, it is possible to select and modify the right treatment conditions to enhance bioremediation.

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Keywords PAH degradation · Orthogonal · Microbial consortium · Optimization

1 Introduction

Polycyclic aromatic hydrocarbons (PAH) are a group of organic compounds composed of two or more aromatics rings. High molecular weight PAH (HMW-PAH) with four (i.e., pyrene) or more aromatics rings and other heterocyclic aromatic compounds as dibenzofuran, both with high molecular mass, are often more difficult to biodegrade that other low molecular weight PAH (LMW-PAH), due to their lower solubility and biodegradability. Many of them have toxic, mutagenic, and carcinogenic properties, and the effects of PAH as naphthalene or phenanthrene in animals and humans, their toxicity and carcinogenic activity, has been reported and well documented (Sudipt et al. 2002). In addition, PAH are bioaccumulated in the environment and trophic chains, properties that increase with the numbers of rings. There is a natural degradation carried out by microorganism able to use PAH as carbon source, which represents a considerable portion of the bacterial communities present in polluted soils (Heitkamp and Cerniglia 1988). However, this natural biodegradation may be affected by environmental factors which optimization allows us to achieve a more efficient process. Temperature is a key factor in the physicochemical properties of PAH as well

as in the metabolism of the microorganisms. Although it has been shown that biodegradation of PAH is possible even at temperatures lower than 5°C (Eriksson et al. 2001), it is usually more efficient at mild temperatures (15–25°C; Mohn and Stewart 2000). The carbon, nitrogen, and phosphorus (C/N/P) molar ratio is another important factor in biodegradation process because it affects the dynamics of the bacterial metabolism changing growth and PAH depletion rates of PAH-degrading species (Leys et al. 2004). The form in which these essential nutrients are supplied affects the bioavailability for the microorganism, being more soluble and efficient in the oxidized forms (such as nitrates) than reduced forms (such as ammonium; Schlessinger 1991).

Surfactants are compounds used to increase the PAH solubility, although both, positive (Boochan et al. 1998; Jin et al. 2007) and negative effects (Boochan et al. 1998; Laha and Luthy 1992) on the biodegradation process has been reported. The nature of the effect depends on several factors such as the type and concentration of surfactant, due to the toxic properties of some of them (Jin et al. 2007), and the increasing of toxicity of PAH produced by increasing their solubility (Thibault et al. 1996). Another factor considered is the inoculum size, related to the diversity and effectiveness of the biodegradation, because in a diluted inoculum, the minority microorganisms which likely have an important role in the biodegradation process, can be removed (Szabó et al. 2007). Moreover, it has been reported (Szabó et al. 2007) that the addition of a readily metabolized carbon source (i.e., glucose) improves the PAH degradation, possibly due to the increased biomass, although in others cases, (Wong et al. 2000) this better bacterial growth reduced significantly PAH degradation.

We consider that the study of the individual effect of abiotic factors on the biodegradation capacity of the microbial consortium is incomplete because the effect of one factor can be influenced by other factors. In this work, the combination between factors was optimized by an orthogonal experimental design fraction of the full factorial combination of the selected environmental factors.

Hence, our two mains goals are to determine the optimal conditions for the biodegradation of low (phenanthrene and dibenzofuran) and high (pyrene) molecular weight PAH by a bacterial-degrading consortium (C2PL05) and the study of the influence of the factors (temperature, C/N/P molar ratio, type of nitrogen and iron sources, iron source concentration, carbon source, surfactant concentration, and inoculum dilution) on the biodegradation. In order to achieve these objectives, we elaborated an orthogonal experimental design to take into account all combination between eight factors: temperature, C/N/P molar ratio, nitrogen and iron sources, iron concentration, addition of glucose, surfactant concentration and inoculum dilution at three and two levels.

2 Materials and Methods

2.1 Chemicals and Media

Dibenzofuran, phenanthrene, and pyrene (>99 % purity) were purchased from Sigma-Aldrich (Steinheim, Germany). Stock mix of the three PAH was prepared by dissolving the necessary amount in *n*-hexane (Fluka, Steinheim, Germany). In previous work (Bautista et al. 2009), we tested that the optimal surfactant for the consortium was the biodegradable and nontoxicTween-80 (Sigma-Aldrich, Steinheim, Germany). Bushnell–Haas broth medium (BHB) was purchased from Panreac (Barcelona, Spain) and its original composition (0.2 gL^{-1} $MgSO_4 \cdot 7H_2O$, 0.02 gL^{-1} $CaCl_2$ $2H_2O$, 1 gL^{-1} $KHPO_4$, 1 gL^{-1} K_2HPO_4, 1 gL^{-1} NH_4NO_3, 0.05 gL^{-1} $FeCl_3$) was modified according to the treatment (see Table 1).

2.2 Bacterial Consortium

PAH-degrading consortium C2PL05 was isolated from a soil in a petrochemical complex in Puertollano (Spain) and was identified and described in Molina et al. (2009). All strains of the consortium C2PL05 isolated by culture-dependent techniques were *γ-proteobacteria*, and the strains presents belong to the genera *Enterobacter*, *Pseudomonas*, and *Stenotrophomonas* (Molina et al. 2009). In addition, the diversity of the enriched microbial consortium was characterized by a non culture-dependent molecular technique such as denaturing gradient gel electrophoresis (DGGE) following the procedure described elsewhere (Molina et al. 2009) using the primers 341 F-GC and 907R (GC clamp: 5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCC CCG CCC-3′; Muyzer et al. 1995).

2.3 Experimental Design

An orthogonal design form of L_{18} (3^7) (2^1) selected from the module of Statistica (Version 6.0), was used

Table 1 Experimental design

Treatment	T (°C)	C/N/P (molar)	N source	Fe source	Iron source concentration (mM)	Glucose/PAH (%)	Surfactant concentration	Inoculum dilution
1	30	100/5/0.5	$(NH_4)_2SO_3$	$Fe_2(SO_4)_3$	0.2	0/100	CMC	10^{-3}
2	20	100/21/16	$(NH_4)_2SO_3$	$FeNO_3$	0.05	0/100	+20 % CMC	10^{-2}
3	25	100/10/1	$NaNO_3$	$FeNO_3$	0.2	0/100	+20 % CMC	10^{-1}
4	20	100/5/0.5	$NaNO_3$	$Fe_2(SO_4)_3$	0.2	50/50	+20 % CMC	10^{-2}
5	25	100/5/0.5	NH_4NO_3	$FeNO_3$	0.1	50/50	CMC	10^{-2}
6	30	100/10/1	NH_4NO_3	$Fe_2(SO_4)_3$	0.05	80/20	+20 % CMC	10^{-2}
7	30	100/10/1	$NaNO_3$	$FeCl_3$	0.1	0/100	CMC	10^{-2}
8	20	100/5/0.5	$NaNO_3$	$FeCl_3$	0.05	80/20	CMC	10^{-1}
9	25	100/21/16	$(NH_4)_2SO_3$	$FeCl_3$	0.2	80/20	CMC	10^{-2}
10	20	100/21/16	NH_4NO_3	$Fe_2(SO_4)_3$	0.1	0/100	CMC	10^{-1}
11	20	100/10/1	NH_4NO_3	$FeNO_3$	0.2	80/20	CMC	10^{-3}
12	25	100/10/1	$(NH_4)_2SO_3$	$Fe_2(SO_4)_3$	0.05	50/50	CMC	10^{-1}
13	25	100/21/16	$NaNO_3$	$Fe_2(SO_4)_3$	0.1	80/20	+20 % CMC	10^{-3}
14	30	100/21/16	NH_4NO_3	$FeCl_3$	0.2	50/50	+20 % CMC	10^{-1}
15	25	100/5/0.5	NH_4NO_3	$FeCl_3$	0.05	0/100	+20 % CMC	10^{-3}
16	30	100/21/16	$NaNO_3$	$FeNO_3$	0.05	50/50	CMC	10^{-3}
17	30	100/5/0.5	$(NH_4)_2SO_3$	$FeNO_3$	0.1	80/20	+20 % CMC	10^{-1}
18	20	100/10/1	$(NH_4)_2SO_3$	$FeCl_3$	0.1	50/50	+20 % CMC	10^{-3}

to do the multifactor combination. A total of 18 experiments, each in triplicate, were carried out in 100 ml Erlenmeyer flasks with a total volume of 50 ml of BHB medium (Panreac, Barcelona, Spain) with an original composition modified according to the treatments requirements (see Table 1). The replicates were incubated in an orbital shaker (Innova 40, New Brunswick Scientific; Edison, NJ, USA) at 150 rpm under dark conditions, but prior to inoculating the consortium, the flasks were shaken overnight to equilibrate and solubilize most of the PAH. Table 1 shows a summary of environmental conditions and incubation of each treatment. Tween-80 concentration was 0.012 mM, the critical micellar concentration (CMC), 100 % of PAH was equivalent to 0.3 gL^{-1} (0.1 gL^{-1} of each PAH). The initial cell concentration of the inoculum consortium was determined by the most probable number method (Wrenn and Venosa 1983). The number of heterotrophic microorganisms (3.15×10^6 cell mL^{-1}) was measured with Luria base broth (Panreac, Barcelona, Spain) with glucose as carbon source and the PAH degrading microorganisms of the consortium (6.95×10^5 cell mL^{-1}) with BHB with PAH mix as carbon source.

2.4 Cell Density

Bacterial density during the PAH-degrading process was monitored at 0, 15, 24, 39, 48, 63, 72, 87, 95, and 159 h by the increase in absorbance of the culture media at 600 nm in a spectrophotometer (Spectronic Genesys™, England). Throughout the cell growth curve, we calculated the average of the cell densities increments (CDI) applying Eq. 1:

$$\Delta A_i = \exp\left(\frac{\ln(A_i) - \ln(A_{i-1})}{t_i - t_{i-1}}\right), \quad (1)$$

where, A is the absorbance at 600 nm, t is the time elapsed in hours, and i corresponds to each sample or sampling time. The increments were normalized by the initial absorbance measurements to correct the effect of the inoculum dilution.

2.5 PAH Extraction and Analysis

At the end of each experiment (159 h), PAH were extracted with dichloromethane and the residue

precipitated was dissolved in 1 ml of acetonitrile for high-performance liquid chromatography (HPLC) analysis, using a ProStar 230 HPLC system (Varian, Palo Alto, CA, USA) with a reversed-phase C18 column following the method previously described (Bautista et al. 2009). The residual concentration of each PAH was calculated from a standard curve based on peak area at a wavelength of 254 nm. The average percentage of phenanthrene, pyrene, and dibenzofuran and average percentage of total PAH degradation (PD) for each treatment is shown in Table 2.

2.6 Statistical Analyses

The effect of the individual parameters on the CDI and on the PD was analyzed by a parametric one-way analysis of variance. The variances were checked for homogeneity by the Cochran's test. Student–Newman–Keuls test was used to discriminate among different variables after significant F test. When data were not strictly parametric, Kruskal–Wallis test and Tukey-type multiple comparison test were used.

The orthogonal design to determine the optimal conditions for PAH biodegradation is an alternative to the full factorial test which is impractical when many factors are considered simultaneously (Chen et al. 2008). However, the orthogonal test allows a much lower combination of factors and levels to test the effect of interacting factors.

Table 2 Final degradation of phenanthrene (Phe), pyrene (Pyr) and dibenzofuran (Dib) and total degradation (Total PD) for each treatment

	Degradation (wt%)			
Treatment[a]	Phe	Pyr	Dib	Total PD
1	96.5	88.3	86.4	90.4
2	96.9	95.0	83.3	91.7
3	96.6	89.5	84.5	90.2
4	97.2	91.5	92.1	87.2
5	96.9	90.4	95.0	88.2
6	98.2	93.5	99.5	85.2
7	96.4	88.3	85.9	90.2
8	97.7	95.3	96.4	82.3
9	97.6	93.6	98.4	82.5
10	97.0	91.0	89.5	92.5
11	97.9	96.8	98.6	88.8
12	96.6	88.9	92.0	85.0
13	97.8	93.0	99.3	83.5
14	96.6	89.7	94.3	87.1
15	96.3	88.1	89.8	91.4
16	96.3	88.6	95.1	86.7
17	97.7	95.4	98.6	86.1
18	97.6	93.0	96.7	91.5

[a] Treatment conditions are listed in Table 1

3 Results and Discussion

The consortium C2PL05 degrades phenanthrene, pyrene, and dibenfuran efficiently in 159 h (Table 2) as well as other PAH such as naphthalene and anthracene (Molina et al. 2009). The study of the influence of each factor on the total PD (Fig. 1) showed that only the carbon source influenced this parameter significantly (Table 3). Results concerning carbon source showed that PD were higher when PAH were added as the only carbon source (100 % PAH). The reason why the PD did not show statistical significance between treatments, except for the relative concentration of PAH glucose, may be due to significant changes produced in PD at earlier times when PAH were still present in the cultivation medium. However, the carbon source, incubation temperature and inoculum dilution were factors that significantly influenced CDI (Table 3, Fig. 2).

The temperature range considered in the present study might not affect the biodegradation process since it is considered narrow by some authors (Wong et al. 2000). Nevertheless, we observed significant differences in the process at different temperatures, showing an optimum at 25°C for our microbial consortium growth (Fig. 2), whereas when consortium was incubated at 20 and 30°C, microorganisms remained in lag phase. These results were in agreement with the fact that respiration increases exponentially with temperature (Q_{10} relationship; Lloyd and Taylor 1994) but increasing or decreasing temperature beyond the optimal value will cause a reduction in microbial respiration. We suggest that moderate fluctuation of temperatures affect microbial growth rate but not degradation rates because degrading population is able to degrade PAH efficiently in a temperature range between 20 and 30°C (Sartoros et al. 2005).

Nutrient requirements for microorganisms increase during the biodegradation process since low C/N/P molar ratio could limit the metabolic activity of the degrader

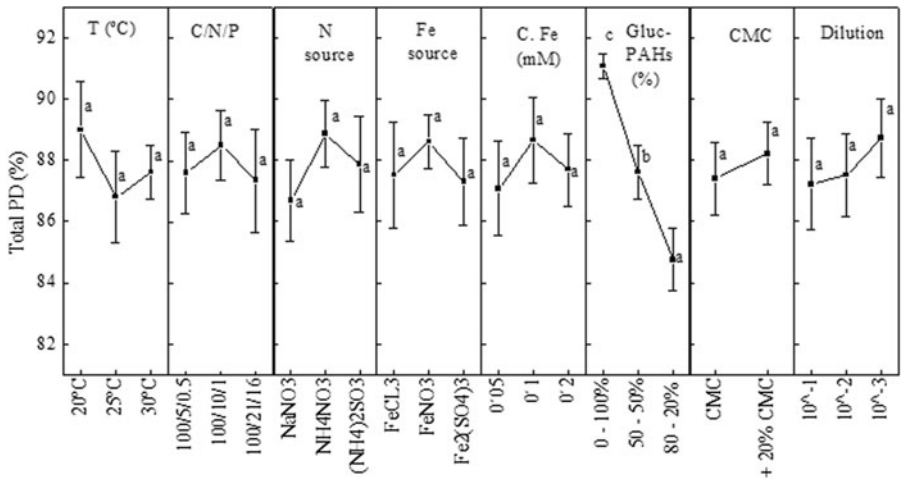

Fig. 1 Graphical analysis of average values of total degradation (PD) under different treatments and levels of the factors. Letters *a*, *b*, and *c* represent significant differences between groups

microorganisms and thus the biodegradation rate (Leys et al. 2004). According to this author, C/N/P ratios above 100/10/1 provide enough nutrients to metabolize the pollutants. However, our results showed that the C/N/P ratios supplied to the cultures, even the ratio 100/5/0.5, did not affect CDI and total PD. This results indicate that the consortium C2PL05 is able to degrade PAH even under low nutrients conditions due to its high adaptation to the hard conditions of a chronically contaminated soil. The results concerning the addition of different nitrogen and iron sources did not show significant differences in CDI and total PD. Other works (Santos et al. 2008; Schlessinger 1991) have suggested that the addition of nitrogen in form of nitrates (Schlessinger

Table 3 Analysis of variance (ANOVA) for the increments of cell density (CDI) and total degradation (PD) of each factor

	ANOVA of CDI				ANOVA of total PD			
Factor	*df*	Mean of squares	*F* value	*p* value	*df*	Mean of squares	*F* value	*p* value
T (°C) error	2	0.56	18.89	**	2	22	1.83	n.s
	51	0.02			51	12		
Molar ratio C/N/P error	2	0.03	0.69	n.s	2	22	1.83	n.s
	51	0.05			51	12		
N source error	2	0.01	0.07	n.s	2	21.4	1.77	n.s
	51	0.05			51	12.1		
Fe source error	2	0.03	0.66	n.s	2	8.9	071	n.s
	51	0.05			51	12.6		
Fe concentration error	2	0.07	1.46	n.s	2	11.8	0.95	n.s
	51	0.05			51	12.4		
% Glucose-PAH error	2	0.24	5.84	*	2	180.2	30.85	**
	51	0.04			51	3.95.8		
CMC error	1	0.01	0.27	n.s	1	8.9	0.71	n.s
	52	0.05			52	12.5		
Inoculum dilution error	2		33.1[a]	**	2	11.3	0.91	n.s
	54		66.14		51	12.5		

[a] H *value* obtained from Kruskal–Wallis test used for nonparametric data. Chi-square=28, overall median=0.44

*p value<0.01

**p value<0.001

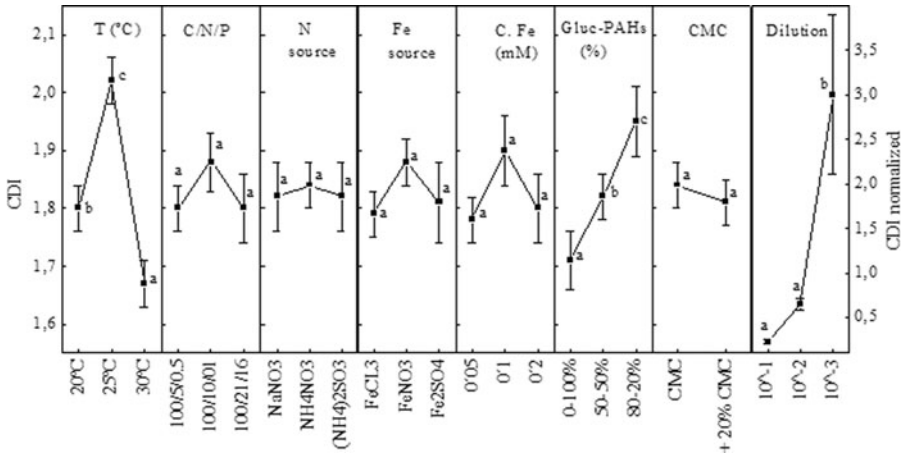

Fig.2 Graphical analysis of average values of cell density increments (CDI) and normalized cell density increments (CDI normalized) of different treatments and levels of the factors. Letters *a*, *b*, and *c* represent significant differences between groups

1991) and the iron in form of sulfates or chlorides (Santos et al. 2008), is more effective due to their high solubility.

The addition of readily biodegradable carbon source such as glucose to a polluted environment is considered an alternative to promote biodegradation. The easy assimilation of this compound results in an increase in total biomass (heterotrophic and PAH degrader microorganisms) of the microbial population, thereby increasing the degradation capacity of the community. Piruvate is a carbon source that favors the growth of certain degrading strains such as *Pseudomonas putid*a (Lee et al. 2003), whereas salicylate induces the synthesis and activation of degradative enzymes (Chen and Aitken 1999). Similar to previous results observed by Wong et al. (2000), in the present study the addition of glucose to the cultures had significant effects in total PD and CDI (Fig. 1, Fig. 2). Although the consortium C2PL05 showed a significantly better growth with 80 % of glucose, the difference between treatments (0/100, 50/50, 80/20 % of glucose/PAH) showed that PD was higher when PAH were added as the only carbon source. Previously, it has been described that after a change in the type of carbon source supplied to PAH-degrader microorganisms, an adaptation period for the enzymatic system was required, reducing the mineralization rate of pollutants (Maier 2009; Simarro et al. 2010; Wong et al. 2000). As glucose was added as additional carbon source, our results show an increase in CDI although the PD values decrease significantly. This indicated that glucose enhance the overall growth of consortium but decrease the biodegradation rate of PAH-degrader population due to the adaptation of the corresponding enzymatic system. So, in this case, the addition of a readily available carbon source retards biodegradation process. The use of surfactants to the culture media at a concentration above the CMC is essential to increase PAH degradation rate (Pantsyrnaya et al. 2011). However, Yuan et al. (2000) reported negative effects when the surfactant was added at concentrations above the CMC, because the excess of micelles around PAH reduces their bioavailability (Mulligan et al. 2001). Our results showed that PD and CDI were not affected by concentrations far beyond the CMC. Some nonbiodegradable surfactants can be toxic to bacteria and therefore they do not improve the biodegradation process (Bautista et al. 2009). Tween-80 was selected as optimal for the strains of the consortium C2PL05 (Bautista et al. 2009). However, the optimal type of surfactant must be determined by the type of degrading strains involved in the process (Bautista et al. 2009). In addition, it is important to consider the possible use of surfactant as a carbon source, preferentially to PAHs, by the strains which would reduce biodegradation rates (Kim and Weber 2003).

Further dilution of the inoculum represents the practical elimination of minority species which could result in a decrease in the degradation capability of the consortium if the missing species play an important role in the biodegradation process (Szabó et al. 2007). Concerning the inoculum concentration, our results showed that this factor significantly influenced CDI but had no effect on total PD, indicating that the degrading ability of the consortium was not altered by inoculum dilution. In González et al. (2011), the evolution and bacterial succession of the consortium C2PL05 by culture-dependent techniques are described. All of these identified strains were efficient in degradation of PAH (Bautista et al. 2009) but *Enterobacter* sp. was dominant at the beginning of the degradation process,

whereas *Stenotrophomonas* sp. and *Pseudomonas* sp. were less abundant. In addition, DGGE fingerprint pattern studied and described in Molina et al. (2009) showed a low microbial diversity of the consortium C2PL05, typical of an enriched consortium from chronically contaminated soil (Viñas et al. 2005). The results present in this work, suggest that in cultures inoculated with the highest dilution of the consortium (10^{-3}), the less abundant microorganisms were eliminated, reducing the competition for the dominant species which can grow vigorously.

The influence of some environmental factors on the biodegradation of PAH can undermine the effectiveness of the process. In this study, the combination of all factors simultaneously by an orthogonal design allowed to establish, considering the interactions between them, the most influential parameters in biodegradation process. Finally, we conclude that the only significant factor affecting PAH biodegradation by consortium C2PL05 is the carbon source. Although cell growth is affected by temperature, carbon source, and inoculum dilution, these factors do not condition the effectiveness of biodegradation. Therefore, the optimal condition for a more efficient degradation by consortium C2PL05 is the use of PAH as the only carbon source.

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The authors have declared no conflict of interest.

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