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# Optimisation of Key Abiotic Factors of PAH (Naphthalene, Phenanthrene and Anthracene) Biodegradation Process by a Bacterial Consortium

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**Abstract** The aim of this work was to determine the optimum values for the biodegradation process of six abiotic factors considered very influential in this process. The optimisation of a polycyclic aromatic hydrocarbon (naphthalene, phenanthrene and anthracene) biodegradation process was carried out with a degrading bacterial consortium C2PL05. The optimised factors were the molar ratio of carbon/nitrogen/phosphorus (C/N/P), the nitrogen source, the iron source, the iron concentration, the pH and the carbon source. Each factor was optimised applying three different treatments during 168 h, analysing cell density by spectrophotometric absorbance at 600 nm and PAH depletion by HPLC. To determine the optimum values of the factors, an analysis of variance was performed using the cell density increments and biotic degradation constants, calculated for each treatment. The most effective values of each factor were: a C/N/P molar ratio of 100:21:16, $NaNO_3$ as nitrogen source, $Fe_2(SO_4)_3$ as iron source using a concentration of 0.1 mmol $l^{-1}$, a pH of 7.0 and a mixture of glucose and PAHs as carbon source. Therefore, high concentrations of nutrients and soluble forms of nitrogen and iron at neutral pH favour the biodegradation. Also, the addition of glucose to PAHs as carbon source increased the number of total microorganism and enhanced PAH biodegradation due to the augmentation of PAH degrader microorganisms. It is also important to underline that the statistical treatment of data and the combined study of the increments of the cell density and the biotic biodegradation constant have facilitated the accurate interpretation of the optimisation results. For an optimum bioremediation process, it is very important to perform these previous bioassays to decrease the process development time and, therefore, the costs.

**Keywords** PAHs · Microbial consortia · Biodegradation · Optimisation

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## 1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are persistent organic compounds with two or more aromatic rings. They are formed by incomplete combustion of fossil fuels and pyrolysis of organic matter derived from human activities and as a result of natural events like forest fires. The toxic, mutagenic and carcinogenic properties of PAHs have concerned the Unites States

Environmental Protection Agency, proposing some of them as priority pollutants (including naphthalene, phenanthrene and anthracene). In addition, PAH solubility is very low in aqueous medium (Luning Prak and Pritchard 2002), affecting their degradation and biomagnification within the ecosystems. Microbial bioremediation removes or immobilises the pollutants, reducing toxicity with a very low environmental impact. Generally, microbial communities present in PAH-contaminated soils are enriched by microorganisms able to use them as the only carbon source (Heitkamp and Cerniglia 1988; Gallego et al. 2007). However, this process can be affected by a few key environmental factors (Roling-Wilfred et al. 2002) that may be optimised to achieve a more efficient process. The molar ratio of carbon, nitrogen and phosphorus (C/N/P) is very important for the metabolism of the microorganisms and, therefore, for PAH degradation (Bossert and Bartha 1984; Alexander 1994; Kwok and Loh 2003). The molar ratio 100:10:1 is frequently considered optimal for contaminated soils (Bossert and Bartha 1984; Alexander 1994), whilst authors have reported negative or no effects (Chaîneau et al. 2005). According to Leys et al. (2005), these contradictory results are due to the nutrient ratio required by PAH-degrading bacteria, which depends on environmental conditions, type of bacteria and type of hydrocarbon. In addition, the chemical form of those nutrients is also important, the soluble forms being (i.e. iron or nitrogen in form of phosphate, nitrate and ammonium) the most frequent and efficient due to their higher availability for microorganisms. Depending on the microbial community and their abundance, another factor that may improve PAH degradation is the addition of readily assimilated, such as glucose, carbon sources (Zaidi and Imam 1999). Moreover, pH is an important factor that affects the solubility of both PAHs and many chemical species in the cultivation broth as well as the metabolism of the microorganisms, showing an optimal range for bacterial degradation between 5.5 and 7.8 (Bossert and Bartha 1984; Wong et al. 2001).

In general, bioremediation process optimisation may be flawed by the lack of studies showing the simultaneous effect of different environmental factors. Hence, our main goal was to set up the optimum values of six abiotic factors: C/N/P molar ratio, nitrogen source, iron source, iron concentration, pH and carbon source for the biodegradation of three PAHs (naphthalene, phenanthrene and anthracene) at 25°C. In order to achieve the main objective, we analysed the effects of the above factors on the microbial growth and the biotic degradation rate.

## 2 Materials and Methods

### 2.1 Chemicals and Media

Naphthalene, phenanthrene and anthracene (all >99% purity) were purchased from Sigma-Aldrich (Steinheim, Germany) and Fluka (Steinheim, Germany). The consortium C2PL05 was not able to degrade PAHs significantly without the addition of surfactants (data not shown). Therefore, surfactant Tween-80 (Sigma-Aldrich) was selected as the most efficient, biodegradable and non-toxic surfactant (Bautista et al. 2009) for the consortium C2PL05. Bushnell–Haas Broth medium (BHB) was purchased from Panreac (Barcelona, Spain), and its composition is: 0.2 g $l^{-1}$ $MgSO_4 \cdot 7H_2O$, 0.02 g $l^{-1}$ $CaCl_2 \cdot 2H_2O$, 1 g $l^{-1}$ $KHPO_4$, 1 g $l^{-1}$ $K_2HPO_4$, 1 g $l^{-1}$ $NH_4NO_3$, 0.05 g $l^{-1}$ $FeCl_3$. This base composition was modified in each experiment as required.

### 2.2 PAH Degrader Consortium C2PL05

The consortium C2PL05 was obtained from a soil sample in a petrochemical complex in Puertollano, Spain. To obtain the consortium, 1 g of soil (<2 mm) was resuspended in 10 ml of phosphate-buffered saline and incubated for 12 h in an orbital shaker (Innova 40, New Brunswick Scientific, Edison, NJ, USA) at 150 rpm and 25°C under dark conditions. After that, 1.5 ml of the supernatant was inoculated in 50 ml of BHB broth (pH 7.0), 1 wt.% Tween-80 as surfactant and naphthalene, phenanthrene and anthracene (each at 500 mg $l^{-1}$) as carbon source. The culture was incubated at 150 rpm and 25°C under dark conditions until the exponential phase was completed. This was confirmed by monitoring the cell density by absorbance at 600 nm in a spectrophotometer (Spectronic Genesys™, UK). Then, the consortium was stored at 4°C to stop its growth. At the beginning of each experiment, 500 µl of the stored consortium was inoculated into the fermentation flasks.

To identify the microbial consortium C2PL05, colonies from aliquots of the soil extract were isolated in BHB agar plates with PAHs as the only carbon source to confirm that these colonies were PAH degraders. Eight colonies were isolated and transferred

onto LB–glucose agar plates in order to increase microbial biomass for DNA extraction. Total DNA of the colonies was extracted using microbial DNA isolation kit (MoBio Laboratories). Amplification of the 16S rRNA coding region of the DNA was performed as described by Viñas et al. (2005) using the primers 16F27 and 16R1488. Sequences were edited and assembled using BioEdit 4.8.7 software (Hall 1999). All isolated strains of the consortium C2PL05 were γ-proteobacteria, and the genera present were *Enterobacter*, *Pseudomonas* and *Stenotrophomonas*.

In addition, non-culture-dependent molecular techniques as denaturant gradient gel electrophoresis were performed to know the total biodiversity of the microbial consortium C2PL05. 16S rRNA gen was amplified using the primers 341F-GC and 907R (GC clamp: 5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCC CCG CCC-3′; Muyzer et al. 1995). About 6% of polyacrylamide (37.5:1 acrylamide/bisacrylamide) gels with a 30–60% urea–formamide denaturant gradient and 0.75 mm were used in 1× TAE buffer at 200 V for 4 h at 60°C. The bands were excised and reamplificated to identify the DNA. The two genera identified coincided with genera *Pseudomonas* and *Stenotrophomonas* identified by culture-dependent techniques (more details in Molina et al. 2009).

## 2.3 Experimental Design

A total of six abiotic factors were evaluated. To obtain an optimum value, three treatments, each in triplicate, were performed for each factor. The replicates were carried out in 100-ml Erlenmeyer flasks with 50 ml of BHB medium (pH 7.0), Tween-80 (1 wt.%), naphthalene, phenanthrene and anthracene (each at 500 mg $l^{-1}$) and 500 μl of the C2PL05 consortium. The concentration of the inoculum was $3.15\times10^6$ cells $ml^{-1}$ of the heterotrophic microorganism and $6.95\times10^5$ cells $ml^{-1}$ of the PAH-degrading microorganism. The number of microorganisms capable of degrading any carbon source present in the medium (heterotrophic microorganisms) and microorganisms capable of degrading PAHs as sole carbon source (PAH-degrading bacteria) were measured by the most probable number method (Wrenn and Venosa 1996). LB–glucose broth and BHB medium were used to determine heterotrophic microorganism and PAH-degrading microorganism, respectively. To maintain the same initial number of cells in each experiment, the absorbance of the inoculum was measured and diluted if necessary before inoculation to reach an optical density of 1.6 AU. The replicates were incubated in an orbital shaker (Innova 40, New Brunswick Scientific) at 150 rpm and 25°C under dark conditions. Previous to inoculating the consortium, the Erlenmeyer flasks were shacked overnight to solubilize most of the PAHs. Samples were withdrawn at 0, 15, 24, 39, 48, 64, 72, 159 and 168 h to monitor PAH depletion and cell growth.

## 2.4 Treatment Conditions

The composition of BHB base was 0.2 g $l^{-1}$ $MgSO_4{\cdot}7H_2O$, 0.02 g $l^{-1}$ $CaCl_2{\cdot}2H_2O$, 1 g $l^{-1}$ $KHPO_4$, 1 g $l^{-1}$ $K_2HPO_4$, 1 g $l^{-1}$ $NH_4NO_3$, 0.05 g $l^{-1}$ $FeCl_3$. The compounds $MgSO_4$ and $CaCl_2$ and their concentration were constant for all treatments, and $KHPO_4$ and $K_2HPO_4$ were modified only in concentration. The other components were modified, both the concentration and compounds, according to the requirements of the optimised factors. PAHs at 1,500 mg $l^{-1}$ (500 mg $l^{-1}$ of naphthalene, phenanthrene and anthracene) were used as carbon source for all treatments, except for those in which the carbon source was optimised and PAHs were mixed with glucose in a proportion of 50% PAHs–glucose or only glucose was added. In all cases, an overall carbon concentration of 0.1176 mmol $l^{-1}$ was used. Once a factor was optimised, its optimum value was kept for the subsequent factor optimisation.

The levels of each factor studied were selected as described below. For the C/N/P molar ratio, the values employed were 100:10:1, frequently described as optimal (Bossert and Bartha 1984), 100:5:0.5 and 100:21:16. To optimise the nitrogen source, $NaNO_3$, $NH_4(NO_3)$ and $(NH_4)_2SO_3$ were used. The optimal iron source was selected amongst $FeCl_3$, $Fe(NO_3)_3$ and $Fe_2(SO_4)_3$. The concentration levels of the optimal iron form were 0.05, 0.1 and 0.2 mmol $l^{-1}$, and three different pH values were also tested, 5.0, 7.0 and 8.0. The effect of the carbon source was determined by adding PAHs as the only carbon source, PAHs and glucose (50% of carbon atoms from each source), or glucose as the only carbon source.

## 2.5 Bacterial Growth

Bacterial growth during the PAH degradation process was monitored at 0, 15, 24, 39, 48, 64, 72, 159 and

168 h by spectrophotometric absorbance of the culture media at 600 nm in a UV–Vis spectrophotometer (Spectronic Genesys™). From the above optical density data, the average of the cell density increments (CDI) was calculated by applying the following equation:

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

where $A$ is the absorbance at 600 nm, $t$ is the time elapsed in hours, and the subscript $i$ corresponds to each sample or sampling time.

### 2.6 Kinetic Degradation

Naphthalene, phenanthrene and anthracene concentrations in the culture media were analysed using a ProStar 230 HPLC system (Varian, Palo Alto, CA, USA) with a reverse phase C18 column following the method described in Bautista et al. (2009). The concentration of each PAH was calculated from a standard curve based on peak area using the absorbance at 254 nm. The depletion rate of each PAH ($-r_i$) during the experiments was fitted to a first-order kinetic model Eq. 1:

$$-r_i = -\frac{dC_i}{dt} = k_{Ai} \times C_i + k_{Bi} \times C_i \quad (2)$$

where $C$ is the concentration of the corresponding PAH, $k_A$ is the apparent first-order kinetic constant due to abiotic processes, $k_B$ is the apparent first-order kinetic constant due to biological processes, $t$ is the time elapsed, and the subscript $i$ corresponds to each PAH. Degradation caused by abiotic processes was determined by control experiments carried out in triplicate in 100-ml Erlenmeyer flasks with 50 ml BHB medium (pH 7.0), Tween-80 (1 wt.%), naphthalene, phenanthrene and anthracene (each at 500 mg $l^{-1}$) without any microbial inoculum in an orbital shaker (Innova 40, New Brunswick Scientific) at 150 rpm and 25°C under dark conditions. PAH concentrations in the control experiment were analysed using the HPLC system described previously. The values of $k_A$ for each PAH were calculated by applying Eq. 2, considering $k_B \approx 0$ since no bacterial consortium was inoculated.

### 2.7 Statistical Analysis

In order to evaluate the effects of the treatments on the kinetic biodegradation constant ($k_B$) and CDI, bifactorial analysis of variance (ANOVA) was used. The variances were checked for homogeneity by applying Cochran's test. When indicated, data were transformed to homogenise variances. Student–Newman–Keuls (SNK) test was used to discriminate amongst different treatments after significant $F$ test. All tests were performed with the software Statistica 6.0 for Windows.

## 3 Results

Control experiments (Fig. 1) show that phenanthrene and anthracene concentration was not affected by any abiotic process since no depletion was observed along the experiment, so that $k_A \approx 0$ $h^{-1}$. However, in the case of naphthalene, some degree of abiotic depletion was measured during the controls, yielding an apparent first-order abiotic rate constant of $2.7 \times 10^{-3} \pm 7 \times 10^{-5}$ $h^{-1}$. This value was accounted for the calculation of the biodegradation rate constant ($k_B$) for naphthalene in the optimisation experiments.

Cell density increments of the consortium for three different treatments of C/N/P molar ratio are shown in Fig. 2a. According to the statistical analysis of CDI, there were significant differences between the C/N/P molar ratio ($F_{2,6}$=23.8, $p$<0.01; Table 1), and SNK showed that treatments with molar ratios of 100:10:1 and 100:21:16 reached larger increases. With regard to the kinetic biodegradation constant ($k_B$), the interaction between $k_B$ of the treatments with each hydrocarbon was significant ($F_{4,18}$=57, $p$<0.001; Table 1). The SNK test (Fig. 2b) showed that the treatment 100:21:16 with naphthalene yielded the highest value,

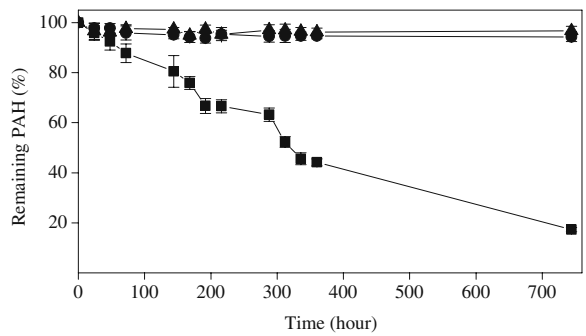


**Fig. 1** Naphthalene (*filled square*), phenanthrene (*filled circle*) and anthracene (*filled triangle*) depletion due to abiotic processes in control experiments (programme used Origin 6.1)

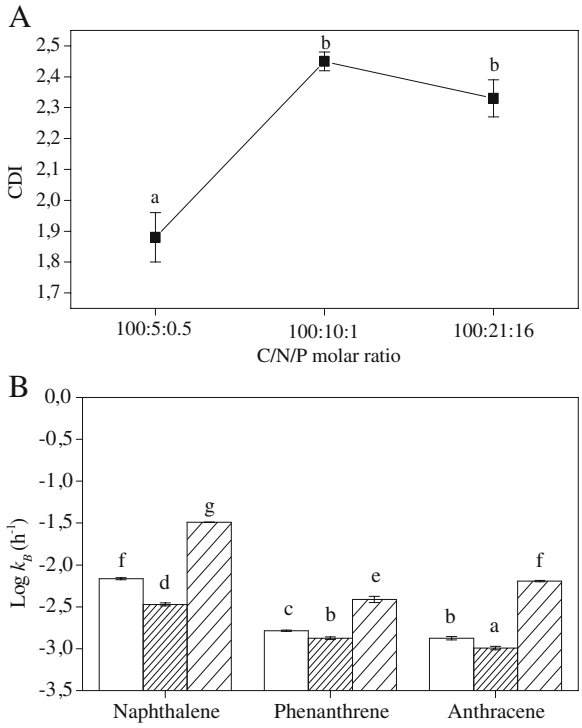


**Fig. 2** **a** Cell density increments of the consortium C2PL05 with the treatments 100:5:0.5, 100:10:1 and 100:21:16. *Error bars* show the standard error. **b** Differences between treatments (100:10:1 ▭, 100:5:0.5 ▨ and 100:21:16 ▨) and PAHs in the biodegradation kinetic constant ($k_B$). The *letters* show differences between groups ($p<0.05$, SNK) and the *error bars* the standard deviation (programme used Origin 6.1)

whereas the lowest were achieved with 100:5:0.5 and 100:10:1 for anthracene and phenanthrene. In addition, within each PAH group, the highest values were observed with 100:21:16 molar ratio. Therefore, although there are no differences for CDI between ratios 100:10:1 and 100:21:16, 100:21:16 molar ratio is the most effective for PAH degradation, so that this ratio was considered as the optimal.

Figure 3a shows that the three different nitrogen sources added had significant effects on CDI ($F_{2,6}$= 23.4, $p<0.01$: Table 1). The SNK test shows that the addition of $NaNO_3$ significantly improved CDI. The interaction between PAHs and the nitrogen sources was significant ($F_{4,18}$=113, $p<0.001$; Table 1), and the highest $k_B$ values were achieved with $NaNO_3$ for naphthalene, phenanthrene and anthracene (Fig. 3b). According to these results, $NaNO_3$ is considered as the best form to supply the nitrogen source for both PAH degradation and growth of the C2PL05 consortium.

CDI of the treatments performed with three different iron sources (Fig. 4a) were significantly different ($F_{2,6}$=5.1, $p<0.05$; Table 1). Although no significant differences between adding $Fe_2(SO_4)_3$ or $Fe(NO_3)_3$ were observed, the addition of $Fe_2(SO_4)_3$ contributes more to CDI than $FeCl_3$. The $k_B$ (Fig. 4b) showed significant differences in the interaction between PAHs and the different iron sources ($F_{4,18}$= 43, $p<0.001$; Table 1). The highest $k_B$ values were observed with $Fe_2(SO_4)_3$ for the degradation of phenanthrene, followed by $FeCl_3$ degrading naphthalene and phenanthrene. The lowest values of $k_B$ were observed with $Fe(NO_3)_3$ degrading naphthalene and anthracene. Nevertheless, the most recalcitrant PAHs (phenanthrene and anthracene) showed the highest $k_B$ values with $Fe_2(SO_4)_3$, in agreement with the highest CDI values, also obtained with $Fe_2(SO_4)_3$.

**Table 1** ANOVA summary for the CDI and the biotic degradation constant ($k_B$)

| Factor | CDI | | | | $k_B$ | | | |
|---|---|---|---|---|---|---|---|---|
| | *df* | M.S. | *F* value | *p* value | *df* | M.S. | *F* value | *p* value |
| C/N/P ratio* | 2 | $2.7 \times 10^{-1}$ | 23.8 | 0.01 | 4 | $5.7 \times 10^{-2}$ | 56.6 | 0.001 |
| Error | 6 | $1.1 \times 10^{-2}$ | | | 18 | $1.0 \times 10^{-3}$ | | |
| N source | 2 | $2.1 \times 10^{-1}$ | 23.4 | 0.01 | 4 | $9.0 \times 10^{-6}$ | 113 | 0.001 |
| Error | 6 | $1.0 \times 10^{-2}$ | | | 18 | $7.0 \times 10^{-7}$ | | |
| Fe source | 2 | $1.8 \times 10^{-2}$ | 5.1 | 0.05 | 4 | $3.0 \times 10^{-6}$ | 43 | 0.001 |
| Error | 6 | $3.6 \times 10^{-3}$ | | | 18 | $7.0 \times 10^{-8}$ | | |
| Fe concentration | 2 | $4.5 \times 10^{-1}$ | 34.8 | 0.06 | 4 | $3.0 \times 10^{-6}$ | 38 | 0.0001 |
| Error | 6 | $9.5 \times 10^{-2}$ | | | 18 | $1.0 \times 10^{-7}$ | | |
| pH | 2 | $3.0 \times 10^{-2}$ | 11.03 | 0.01 | 4 | $1.5 \times 10^{-4}$ | 5 | 0.05 |
| Error | 6 | $2.7 \times 10^{-3}$ | | | 18 | $3.3 \times 10^{-5}$ | | |
| Glucose/PAHs | 2 | $5.4 \times 10^{-1}$ | 454.01 | 0.001 | 2 | $6.6 \times 10^{-4}$ | 7 | 0.05 |
| Error | 6 | $1.2 \times 10^{-3}$ | | | 12 | $9.3 \times 10^{-5}$ | | |

* Logarithmically transformed kB data to achieve homogeneity of variance

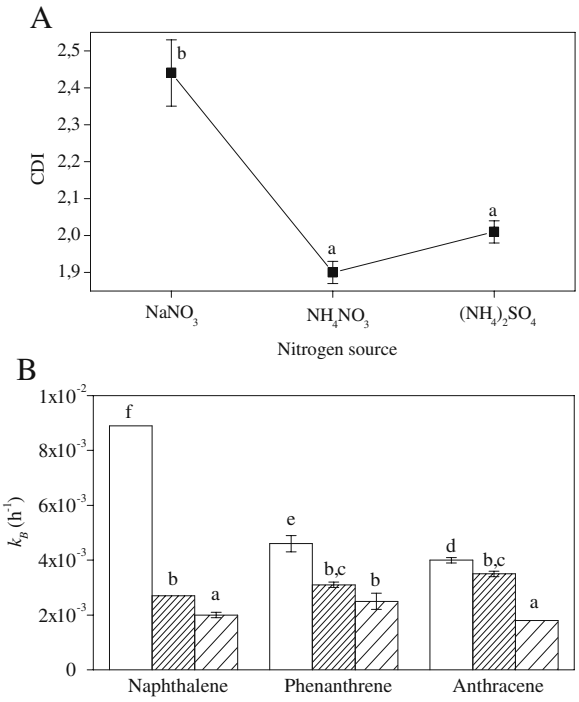


**Fig. 3** **a** Cell density increments of the consortium C2PL05 with the treatments $NaNO_3$, $NH_4NO_3$ and $(NH_4)_2SO_4$. *Error bars* show the standard error. **b** Differences between treatments ($NaNO_3$ ▭, $NH_4NO_3$ ▨ and $(NH_4)_2SO_4$ ▨) and PAHs in the biodegradation kinetic constant ($k_B$). The *letters* show differences between groups ($p<0.05$, SNK) and the *error bars* the standard deviation (programme used Origin 6.1)

Concerning the effect of the iron concentration (Fig. 5), supplied in the form of the optimal $Fe_2(SO_4)_3$, no significant differences in CDI were found for all three concentrations used ($F_{2,6}=34.8$, $p=0.06$; Table 1 and Fig. 5a). However, the interaction between iron concentration and $k_B$ of three PAHs was significant ($F_{4,18}=38$, $p<0.001$; Table 1), reaching the highest values for $k_B$ by using an iron concentration of 0.1 mmol$l^{-1}$ degrading naphthalene and phenanthrene (Fig. 5b). The lowest values of $k_B$ were observed with 0.05 and 0.2 mmol $l^{-1}$ degrading phenanthrene and anthracene (Fig. 4b). Since each PAH showed the highest $k_B$ with 0.1 mmol $l^{-1}$, this iron concentration was considered as the most efficient for the PAH biodegradation process.

With reference to pH, Fig. 6a and statistical analysis ($F_{2,6}=11.03$, $p<0.01$; Table 1) clearly show that the neutral pH of the medium favour the CDI of the consortium. The $k_B$ of the three different treatments (Fig. 6b) also showed significant differences in the interaction ($F_{4,9}=5$, $p<0.05$; Table 1). The highest value of $k_B$ was observed for anthracene degradation at neutral pH (Fig. 6b). The other two PAHs, naphthalene and phenanthrene, did not show significant differences between any treatments. Therefore, given that the highest values of both parameters (CDI and $k_B$) were observed at pH 7, this value will be considered as the most efficient for the PAH biodegradation process.

The last factor analysed was the addition of an easily assimilated carbon source (Fig. 7). Regarding CDI values (Fig. 7a), there were significant differences between treatments ($F_{2,6}=454{,}01$, $p<0.001$; Table 1). The addition of glucose as the only carbon source significantly improved CDI. Figure 7b only shows the $k_B$ of the treatments with PAHs (100% or 50% of PAHs); therefore, the treatment with glucose as the only carbon source was not included in the ANOVA. The interaction between PAHs and type of

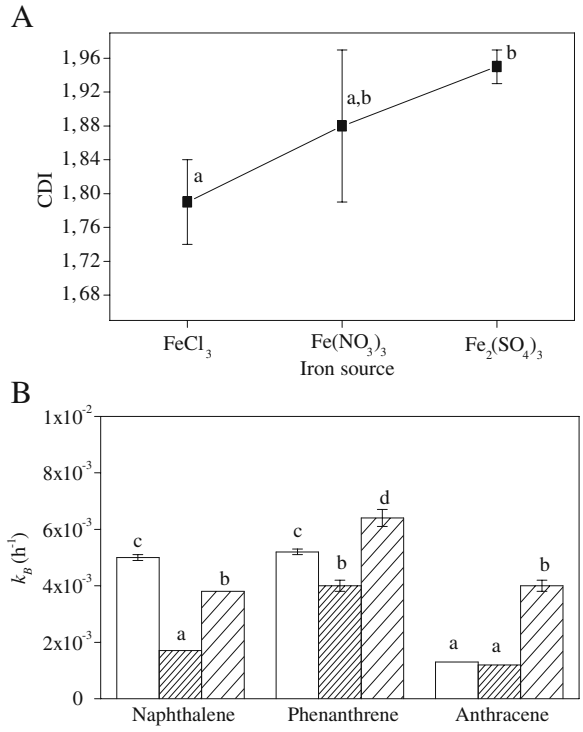


**Fig. 4** **a** Cell density increments of the consortium C2PL05 with the treatments $FeCl_3$, $Fe(NO_3)_3$, and $Fe_2(SO_4)_3$. *Error bars* show the standard error. **b** Differences between treatments ($FeCl_3$ ▭, $Fe(NO_3)_3$ ▨ and $Fe_2(SO_4)_3$ ▨) and PAHs in the biodegradation kinetic constant ($k_B$). The *letters* show differences between groups ($p<0.05$, SNK) and the *error bars* the standard deviation (programme used Origin 6.1)

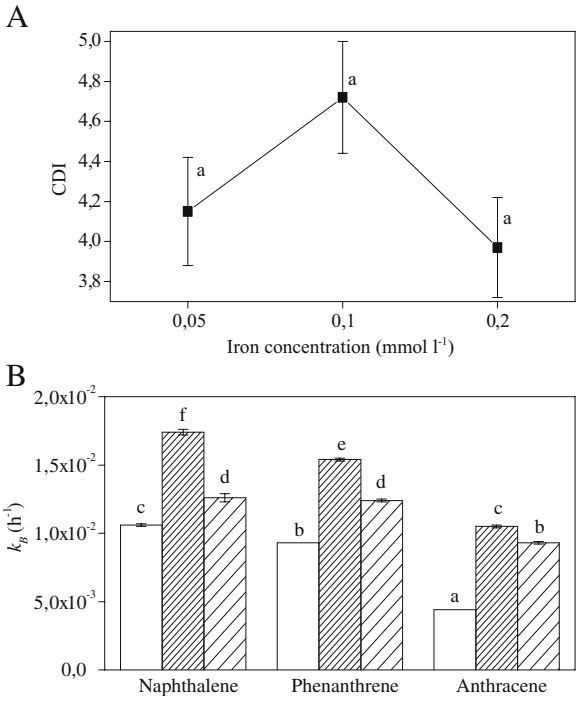


**Fig. 5** **a** Cell density increments of the consortium C2PL05 with the treatments 0.05, 0.1 and 0.2 mmol $l^{-1}$. *Error bars* show the standard error. **b** Differences between treatments (0.05 mmol $l^{-1}$ ▭, 0.1 mmol $l^{-1}$ ▨ and 0.2 mmol $l^{-1}$ ▨) and PAHs in the biodegradation kinetic constant ($k_B$). The *letters* show differences between groups ($p<0.05$, SNK) and the *error bars* the standard deviation (programme used Origin 6.1)

carbon source was significant ($F_{2,12}=7$, $p<0.05$; Table 1). The $k_B$ for the treatment with PAHs and glucose (50:50) was significantly higher for phenanthrene and naphthalene (Fig. 6b), although there were no differences with the treatment for anthracene where PAHs were the only carbon source. Therefore, the addition of glucose with PAHs as the carbon source proved to be positive for CDI and $k_B$ of the consortium.

## 4 Discussion

It is important to highlight that the increments of the cell density is a parameter that brings together all the microbial community, whereas the biotic degradation constant is specific for the PAH-degrading microorganisms. For that reason, when the effect of the factors studied on CDI and $k_B$ yielded opposite results, the latter always prevailed since PAH degradation efficiency is the main goal of the present optimisation study.

With regard to the C/N/P molar ratio, some authors consider that low ratios might limit the bacterial growth (Leys et al. 2005), although others show that high molar ratios such as 100:10:1 are optimum for hydrocarbon-polluted soils (Flathman et al. 1994; Bouchez et al. 1995; Eweis et al. 1998). However, in agreement with Leys et al. (2005), our results confirmed that the most effective molar ratio was the highest (100:21:16). This result suggests that the supply of the inorganic nutrients during the PAH biodegradation process may be needed by the microbial metabolism. In addition, the form used to supply these nutrients can affect the metabolism of the microorganism (Carmichael and Pfaender 1997) and limit the amount of carbon that bacteria can assimilate, limiting in turn the biodegradation extent. Our results showed that nitrate (sodium nitrate) as nitrogen source improved PAH biodegradation as compared to ammonium. This is likely due to the fact that nitrate is more soluble and available for

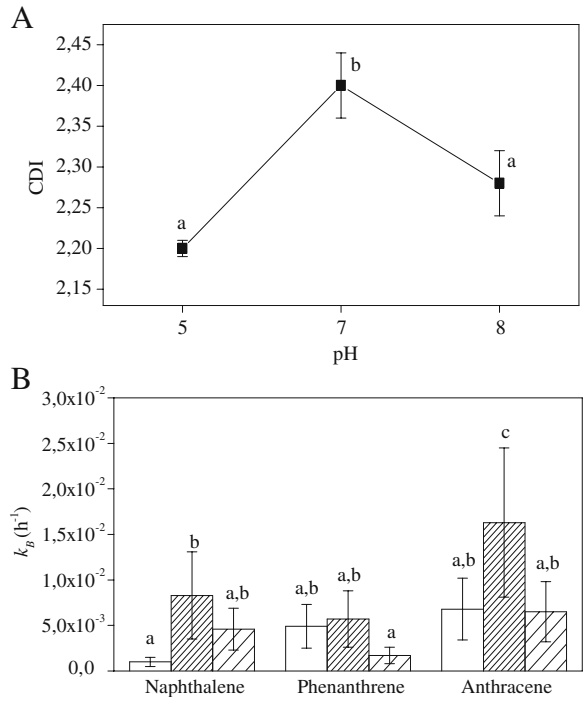


**Fig. 6** **a** Cell density increments of the consortium C2PL05 with the treatments pH 5.0, pH 7.0 and pH 8.0. *Error bars* show the standard error. **b** Differences between treatments (pH 5.0 ▭, pH 7.0 ▨ and pH 8.0 ▨) and PAHs in the biodegradation kinetic constant ($k_B$). The *letters* show differences between groups ($p<0.05$, SNK) and the *error bars* the standard deviation (programme used Origin 6.1)

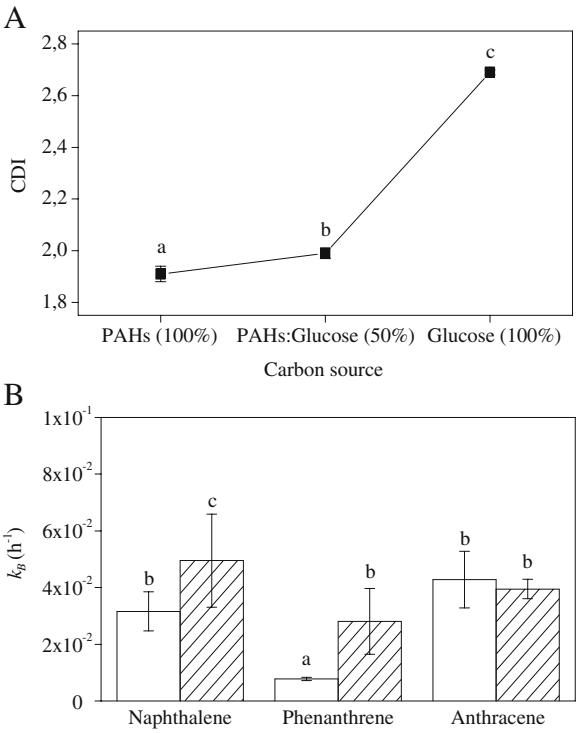


**Fig. 7** **a** Cell density increments of the consortium C2PL05 with the treatments PAHs (100%), PAHs/glucose (50:50%) and glucose (100%). *Error bars* show the standard error. **b** Differences between treatments (PAHs (100%) ▭ and PAHs/glucose (50:50%) ▨) and PAHs in the biodegradation kinetic constant ($k_B$). The *letters* show differences between groups ($p<0.05$, SNK) and the *error bars* the standard deviation (programme used Origin 6.1)

microorganisms than ammonium which has adsorbent properties (Schlessinger 1991). Iron is the other essential compound to stimulate the microbial activity on PAH degradation (Dinkla and Janssen 2003; Santos et al. 2008). On one hand, iron acts as a cofactor of enzymes catalysing PAH oxidation (Dinkla and Janssen 2003), but it is also related with the production of biosurfactants (Santos et al. 2008). These compounds are naturally produced by genera such as *Pseudomonas* and *Bacillus* (Wei et al. 2003), increasing PAH solubility and, therefore, their bioavailability. In agreement with previous works (Dinkla and Janssen 2003; Santos et al. 2008), our results confirmed that the addition of iron in a concentration of 0.1 mmol $l^{-1}$ makes the biodegradation more effective. Santos et al. (2008) stated that there is a limit concentration above which the growth is inhibited due to toxic effects. According to these authors, our results showed lower degradation and growth with the concentration 0.2 mmol $l^{-1}$ since this concentration may be saturating for these microorganisms. However, in contrast to previous works (Dinkla and Janssen 2003; Santos et al. 2008), the most effective iron form was $Fe_2(SO_4)_3$ for the PAH biodegradation, likely due to the higher solubility, which makes it more available for the microorganism.

The addition of easy assimilated carbon forms, such as glucose, for the PAH-degrading process can result in an increment in the total number of bacteria (Wong et al. 2001) because PAH degrader population can use multiple carbon sources simultaneously (Herwijnen et al. 2006). However, this increment in the microbial biomass was previously considered (Wong et al. 2001) because the utilisation of the new carbon source may increase the lag phase, delaying the bacterial growth (Maier 2000). Our results confirmed that PAH degradation was more efficient with the addition of an easy assimilated carbon source, probably because the augmentation of the total heterotrophic population also enhanced the PAH-degrading community. Our consortium showed a longer lag phase during the treatment with glucose than that observed during the treatment with PAHs as the only carbon source (data not shown). These results are consistent with a consortium completely adapted to PAH biodegradation, and its enzymatic system requires some adaptation time to start assimilating the new carbon source (Maier 2000).

Depending on the type of soil and the type of PAHs to degrade, the optimum pH range can be very variable (Dibble and Bartha 1979). Some acid-resistant Gram-positive bacteria, such as *Mycobacterium* sp., show better PAH degradation capabilities under acid condition because low pH seems to render the mycobacteria more permeable to hydrophobic substrates (Kim et al. 2005). However, other microorganisms belonging to *Pseudomonas* genus prefer neutral pH conditions. In agreement with previous works (i.e. Dibble and Bartha 1979), our results confirmed that neutral pH is optimum for the biodegradation of PAHs.

In summary, the current work has shown that the optimisation of environmental parameters may significantly improve the PAH biodegradation process. It is also important to underline that the statistical analysis of data and the combined study of the bacterial

growth and the kinetics of the degradation process provide an accurate interpretation of the optimisation results. In conclusion, for an optimum bioremediation process, it is very important to perform these previous bioassays to decrease the process development time and, therefore, the associated costs.

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