# Evaluation of the Removal of Pyrene and Fluoranthene by *Ochrobactrum anthropi, Fusarium* sp. and Their Coculture

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**Abstract** Fluoranthene and pyrene are polycyclic aromatic hydrocarbons of high molecular weight that are recalcitrant and toxic to humans; therefore, their removal from the environment is crucial. From hydrocarbon-contaminated soil, 25 bacteria and 12 filamentous fungi capable of growth on pyrene and fluoranthene as the sole carbon and energy source were isolated. From these isolates, *Ochrobactrum anthropi* BPyF3 and *Fusarium* sp. FPyF1 were selected and identified because they grew quickly and abundantly in both hydrocarbons. Furthermore, *O. anthropi* BPyF3 and *Fusarium* sp. FPyF1 were most efficient at removing pyrene (50.39 and 51.32 %, respectively) and fluoranthene (49.85 and 49.36 %, respectively) from an initial concentration of 50 mg $L^{-1}$ after 7 days of incubation. Based on this and on the fact that there was no antagonism between the two microorganisms, a coculture composed of *O. anthropi* BPyF3 and *Fusarium* sp. FPyF1 was formed to remove fluoranthene and pyrene at an initial concentration of 100 mg $L^{-1}$ in a removal kinetic assay during 21 days. Fluoranthene removal by the coculture was higher (87.95 %) compared with removal from the individual cultures (68.95 % for *Fusarium* sp. FPyF1 and 64.59 % for *O. anthropi* BPyF3). In contrast, pyrene

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removal by the coculture (99.68 %) was similar to that obtained by the pure culture of *Fusarium* sp. FPyF1 (99.75 %). The kinetics of removal for both compounds was adjusted to a first-order model. This work demonstrates that the coculture formed by *Fusarium* sp. FPyF1 and *O. anthropi* BPyF3 has greater potential to remove fluoranthene than individual cultures; however, pyrene can be removed efficiently by *Fusarium* sp. FPyF1 alone.

**Keywords**  Removal · Pyrene · Fluoranthene · Coculture · *Fusarium* sp. · *Ochrobactrum anthropi*

## Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a group of pollutants whose elimination is of high priority according to the US Environmental Protection Agency (EPA), because they are toxic and mutagenic to plants, animals and microorganisms; moreover, these compounds are carcinogenic to humans [1]. The common source of PAHs in the environment includes natural sources, such as volcanic eruptions, forest fires and oil leaks, among others, and anthropogenic sources, such as the incineration of solid waste, oil spills or from products of some industrial processes [2]. PAHs are classified into two groups: high molecular weight PAHs (HMW-PAHs) and low molecular weight PAHs (LMW-PAHs). HMW-PAHs are more recalcitrant to degradation than LMW-PAHs and persist in the environment due to their low water solubility and high affinity for organic matter [3].

Recently, biological methods have been proposed as a good alternative for eliminating or transforming PAHs to less toxic compounds in the environment [4]. Some studies have shown the potential of bacteria and fungi in pure cultures for using PAHs as the sole carbon and energy source [5]. Bacteria capable of degrading HMW-PAHs from soils or sediments contaminated with hydrocarbons have been isolated, including *Rhodococcus* sp. [6] and *Mycobacterium vanbaalenii* PYR-1 [7], which degrade pyrene, naphthalene and anthracene, whereas *Pseudomonas fluorescens* and *Haemophilus* sp. are capable of degrading acenaphthalene, fluorene, phenanthrene, anthracene and pyrene [8].

Moreover, fungi have aroused great interest because they have non-specific enzymes that can oxidise and degrade a wide range of pollutants [9]. Such is the case for *Irpex lactues*, which belongs to the group of white rot fungi that are able to degrade anthracene, phenanthrene, pyrene and fluoranthene [10]. However, the use of these fungi has limitations, including their slow growth, high oxygen requirements and reduced ability to remove pollutants when mixed with bacterial cultures [11]. Thus, non-ligninolytic fungi, which are less sensitive and have a greater capacity to adapt to different environmental conditions, are a better choice. Consequently, non-ligninolytic fungi capable of degrading PAHs have been isolated from various sources, including marine sediments [3] and petroleum-contaminated soils [12]. For example, *Fusarium solani* and *Penicillium variabile* grow and degrade pyrene [3, 12].

In addition, some studies have shown an increase in the PAHs removal rate when mixed cultures of fungi and bacteria are used [13, 14]. In fact, biodegradation with a pure culture does not completely represent the environmental conditions of the microorganisms during bioremediation of hydrocarbon-contaminated soils due to the complex metabolic relationships that occur between the various groups of microorganisms present in the soil that are not present in pure cultures [14]. In particular, in the removal of PAHs by cocultures of bacteria and fungi, it is proposed that fungal extracellular enzymes initiate the process, which breaks molecules, generating a partial oxidation of PAHs [15]. The initial oxidation of the benzene ring increases the potential for degradation and mineralisation by bacteria because the metabolites produced are more soluble in water and have higher chemical

reactivity. Thus, the oxidation is a key initial step in the degradation of PAHs by bacteria, which prevents the accumulation of toxic metabolites produced by fungi that may have an inhibitory effect on degradation [16].

Fluoranthene and pyrene are HMW-PAHs, and their removal is complicated in pure cultures of bacteria or fungi; therefore, the use of a coculture composed of a bacterium and a fungus may improve the efficiency of removing these compounds. For this reason, bacteria and fungi capable of degrading pyrene and fluoranthene from soil contaminated with hydrocarbons were isolated, selected and identified in this study. The removal ability of individual cultures was evaluated to select and integrate a defined coculture of bacteria and fungi that does not have antagonistic effects among its members and is efficient in removing pyrene and fluoranthene.

## Materials and Methods

### Enrichment Cultures and Microbial Isolation

Three soil samples were collected in a hydrocarbon-contaminated area located at 21° 20′ 21.08″ N, 97° 48′ 44.13″ E in Tamalín, Veracruz, Mexico. The samples were transferred to polyethylene bags and stored at 4 °C. A composite sample was formed by mixing uniform weight subsamples from each soil sample. One gramme of the composite sample was added to 100 mL of mineral medium (MM) with the following composition per litre: $KH_2PO_4$ (0.68 g), $Na_2HPO_4$ (1.79 g), $MgSO_4 \cdot 7H_2O$ (0.35 g), $NH_4NO_3$ (1 g), $CaCl_2$ (0.8 g), $FeSO_4 \cdot 7H_2O$ (0.4 mg) and 0.1 mL of a solution of 100 mg $L^{-1}$ of $H_3BO_3$, $MnSO_4$, $ZnSO_4$, $CuSO_4$ and $CoCl_2$ [17], using fluoranthene or pyrene at 50 mg $L^{-1}$ as the sole carbon source (Sigma-Aldrich, St. Louis, MO, USA). The incubation was performed at 30 °C and 120 rpm until the culture reached an optical density at 600 nm ($OD_{600 \, nm}$) ranging from 0.25 to 0.30. Subsequently, 10 mL of the culture was transferred to 100 mL of fresh culture medium, and the incubation was carried out under the conditions described above; this procedure was repeated three more times.

Finally, serial decimal dilutions to $10^{-6}$ were prepared from the last enrichment culture, and the microorganisms were isolated by spreading 0.1 mL of the dilutions onto potato dextrose agar (PDA) plates for fungi isolation or onto nutrient agar plates for bacteria isolation (BD, Mexico, DF, Mexico), with the single addition of 50 mg $L^{-1}$ of fluoranthene or pyrene. The culture media were incubated at 28 °C until the onset of microbial growth, which occurred 1 to 2 weeks later. The isolates were grouped according to their colonial and microscopic morphologies.

### Removal of Fluoranthene and Pyrene on Solid Medium

With the aim of selecting microorganisms with an increased ability to grow and tolerate the evaluated PAHs (fluoranthene and pyrene) individually, mineral medium plates containing 100 mg $L^{-1}$ of fluoranthene or pyrene were prepared according to a technique described previously [18], which consists of using a double agar layer with the addition of any of the tested PAHs in the top layer to obtain a homogeneous distribution. Previously, bacterial cells and fungal spores were washed with 0.85 % saline solution. The fungal inoculum consisted of a suspension of $1 \times 10^4$ spores mL$^{-1}$, and the bacterial inoculum consisted of a suspension with an $OD_{600 \, nm}$ of 0.1. The plates were incubated for 5 days at 30 °C. Medium containing acetone solvent and medium free of carbon sources were used as controls.

Molecular Identification of Microorganisms

Fungus DNA was extracted using the method described by Birnboim and Doly [19] from mycelium and spores washed with saline solution. Subsequently, the intergenic ITS1-5.8S-ITS2 region of the ribosomal RNA (rRNA) gene was amplified by PCR with primers ITS1 (5′-TCCGTAGGTGAACCTGCGC-3′) and ITS4 (5′-TCCTCCGCTTTATTGATATGC-3′) [20] in the following reaction mixture: 50 ng template DNA, 2.5 μL of 10× reaction buffer, 50 mM MgCl$_2$, 2.5 mM of each dNTP, 10 pM of each primer, 1 U of Taq DNA polymerase (Invitrogen, Waltham, MA, USA), adjusted to 25 μL with water for injection. The PCR conditions were 1 cycle at 95 °C for 10 min, followed by 35 cycles at 95 °C for 1 min, 60 °C for 1 min and 72 °C for 2 min and finally, 1 cycle at 72 °C for 7 min.

DNA extraction from bacteria was performed according to a technique previously described [21]. The 16S rRNA gene was amplified by PCR with primers 8 (5′-CCG CGG GCG CCG CTG GAT AGT TTG CAG ATC CTG GCT CAG-3′) and 1492 (5′-GGC TCG AGC GGC CGC CCG GGT TAC CTT GTT ACG ACT T-3′) [22]. The reaction mixture and the PCR conditions were similar to those described above, except that the annealing temperature was 55 °C. The amplified fragments were purified using the Zymoclean™ Gel DNA recovery kit (Zymo Research, Irvine, USA), following the manufacturer's instructions, and the integrity was confirmed by electrophoresis. The sequencing of the amplified DNA fragments was performed on an ABI PRISM 310 Genetic Analyser PE (Applied Biosystems, California, USA). The nucleotide sequences obtained were subjected to BLAST [23]. A collection of taxonomically related sequences obtained from the National Centre for Biotechnology Information (NCBI) Taxonomy Homepage (http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html/) was included in the multiple alignment analyses with CLUSTAL X [24] and was manually edited using SEAVIEW software [25]. Similitude analysis was estimated using nucleotide sequences with MEGA [26]. Phylogenetic affiliation was based on the limits proposed by Rosselló-Mora and Amann [27], where <95, 95–97.5 and >97.5 % define the taxonomic levels of family, genus and species, respectively. The sequences of the 16S rRNA gene and ITS1-5.8S-ITS2 regions reported in this paper have been deposited in the GenBank database, under accession numbers from KJ782406 to KJ782411.

Removal of Fluoranthene and Pyrene in Liquid Medium

For the selection of microorganisms with a high potential to remove fluoranthene or pyrene, a 7-day removal assay was performed. In this test, the removal efficiency of fluoranthene and pyrene at 50 mg L$^{-1}$ was evaluated for each microorganism selected using the methodology described by Machín-Ramírez et al. [28]. Briefly, 1.5 mL of a stock solution of pyrene or fluoranthene at 1000 mg L$^{-1}$ dissolved in acetone was added to 125-mL flasks, the acetone was allowed to evaporate, and 27 mL of sterile MM was subsequently added followed by the addition of 3 mL of the bacterial inoculum with an OD$_{600\ nm}$ of 0.1 or 0.1 g of fungal biomass filtered and resuspended in 3 mL of sterile MM. The cultures were incubated at 30 °C and 120 rpm in darkness for 7 days.

The concentration of pyrene and fluoranthene was quantified at the beginning and end of the incubation period. Experiments without biomass and with biomass inactivated by sterilisation were used as abiotic controls to evaluate pyrene and fluoranthene losses by photolysis and adsorption to glass and cells. All experiments were performed in triplicate.

From the results of the evaluation of individual removal of PAHs by microorganisms, a fungus and a bacterium were selected for antagonism tests [29]. From these evaluations, a

bacterium-fungus coculture was formed, which was evaluated for its ability to remove fluoranthene or pyrene from 0.1 g of fungal biomass and 3 mL of bacterial suspension adjusted to 0.1 $OD_{600 \ nm}$ in 30 mL of MM supplemented with 100 mg $L^{-1}$ of any of the hydrocarbons to be evaluated. In addition, pure cultures of the microorganisms were tested to determine the individual removal efficiency. Additionally, inactivated biomass of each organism and biomass-free controls were included. The kinetics of PAH removal were measured for 21 days, and experiments were performed in triplicate. Samples were taken every 3 days, and the concentration of residual hydrocarbon, dry cell weight and colony-forming units (CFU) per millilitre were quantified. All chemical analyses were repeated three times, and the data were statistically analysed using a one-way ANOVA with Tukey's test.

## Analytical Methods

### Determination of Cell Growth

Bacterial growth was determined by CFU. Serial decimal dilutions were made out to $10^{-4}$, and 100 μL from the dilutions was spread onto nutrient broth agar plates (BD, Mexico, DF, Mexico). The plates were incubated at 30 °C for 48 h, after which time the colonies were counted [30].

Fungal cell growth was quantified by dry weight, for which the samples were filtered through previously weighed Whatman no. 42 membranes (2.5 μm). Subsequently, membranes were dried at 70 °C for 24 h and brought to a constant weight. Eventually, cell concentration was obtained by weight difference. For coculture samples, the fungal biomass was washed extensively with 0.85 % saline solution to remove the bacterial biomass and residual culture medium.

### Determination of Residual PAH

Biomass was separated from the culture by filtration, and the supernatant was mixed with 10 mL of dichloromethane ($CH_2Cl_2$) in a separatory funnel. The mixture was shaken vigorously for 10 min and allowed to stand to allow phase separation. The organic phase was collected, and the above procedure was repeated twice more. The residual water was removed with $Na_2SO_4$. The $CH_2Cl_2$ was evaporated using air. Finally, the residue was dissolved in 5 mL of methanol and analysed by high-performance liquid chromatography (HPLC) with an ultraviolet detector (UV) using a Discovery C18 column (25 cm×4.6 mm, ID 5 μm). The mobile phase used was methanol–water (85:15, $v/v$), and the flow rate was 1 mL $min^{-1}$ [31]. Pyrene and fluoranthene standards (99.9 % purity; Sigma-Aldrich, St. Louis, MO, USA) were used to build the calibration curves.

## Criteria for Evaluating the Performance of Microorganisms

The following kinetic parameters were calculated

(a)   Maximum specific growth rate: $\mu_{\max} = \frac{\ln Xf - \ln Xi}{tf - ti}$
(b)   Duplication time: $t_d = \frac{\ln 2}{\mu_{\max}}$
(c)   PAH removal efficiency: $E_R = \frac{Ci - Cf}{Ci} \times 100$
(d)   Overall removal rate of PAH: $V_R = \frac{Ci - Cf}{Tf - Ti}$

where,

$Xi$=Biomass concentration at the beginning of the exponential phase
$Xf$=Biomass concentration at the end of the exponential phase
$Ci$=Initial hydrocarbon concentration
$Cf$=Final hydrocarbon concentration
$Ti$=Initial time of incubation
$Tf$=Final time of incubation
$ti$=Time at the beginning of the exponential phase
$tf$=Time at the end of the exponential phase

Kinetic Models

To evaluate the kinetics of removal, the following kinetic models were applied:

Zero order $C_t = -K_o t + C_o$  $T_{1/2} = \dfrac{C_o}{2K_o}$

First order $\ln C_t = -K_1 t + \ln C_o$  $T_{1/2} = \dfrac{\ln 2}{K_1}$

Second order $\dfrac{1}{C_t} = \dfrac{1}{C_o} + K_2 t$  $T_{1/2} = \dfrac{1}{K_2 C_o}$

where,

$C_t$=Hydrocarbon concentration at time $t=t$
$K$=Apparent rate constant or removal rate constant
$t$=Time
$C_o$=Initial concentration of PAH
$T_{1/2}$=Half-life period

## Results and Discussion

Isolation and Identification of Microorganisms Capable of Degrading Fluoranthene and Pyrene

Previous studies have shown that sediments and soils contaminated with oil-refining products are a potential source for the isolation of microbial species able to metabolise PAHs containing four aromatic rings, such as pyrene and fluoranthene [12]. Therefore, in this study, a hydrocarbon-contaminated soil was used for the isolation of bacteria and fungi capable of removing HMW-PAHs through subsequent enrichment cultures. Twenty-five bacteria and 12 fungi were isolated, which were grouped into 11 and 7 morphotypes, respectively (Table 1). Isolated fungi were classified according to their colonial and microscopic morphology within the genera *Penicillium*, *Fusarium* and *Aspergillus*, which are members of the division Deuteromycota and class Hyphomycetes and are the most abundant fungi in sediments and oil-contaminated soils [12].

Subsequently, the potential of the isolated microorganisms to grow individually on solid media at a concentration of 100 mg L$^{-1}$ of pyrene or fluoranthene was evaluated; the results are shown in Table 1. One criterion considered for the selection of microorganisms with the potential to degrade fluoranthene and pyrene was grow in both compounds, and for the case of fungi, a colony diameter of 3 to 4 mm. Most isolates (9/18, 50 %) grew using pyrene and fluoranthene as the sole carbon and energy source, and 33.3 % (6/18) of them only grew in one of the HMW-PAHs tested. Additionally, it was observed that some of the fungal isolates that grew on both PAHs had a larger colony growth (diameter of 3 to 4 mm; FPyF1, FPyF2 and

**Table 1** Growth of fungi (F) and bacteria (B) on solid media containing pyrene (Py) or fluoranthene (F)

|          | Morphotype | Py  | F   |
|----------|------------|-----|-----|
| Fungi    | FPyF1      | +++ | +++ |
|          | FPyF2      | +++ | +++ |
|          | FPyF3      | +++ | +++ |
|          | FPyF4      | +   | +   |
|          | FPyF5      | +   | +   |
|          | FPyF6      | +   | +   |
|          | FPy7       | +   | −   |
| Bacteria | BPyF1      | +   | +   |
|          | BPyF2      | +   | +   |
|          | BPyF3      | +   | +   |
|          | BPy4       | +   | −   |
|          | BPy5       | +   | −   |
|          | BF6        | −   | +   |
|          | BF7        | −   | +   |
|          | BF8        | −   | +   |
|          | B9         | −   | −   |
|          | B10        | −   | −   |
|          | B11        | −   | −   |

Radial growth: 3-4-mm diameter (+++), 2–3-mm diameter (++), diameter <2 mm (+) and no growth (−). Mineral medium (MM) without addition of hydrocarbons and MM supplemented with acetone were used as controls

FPyF3) compared with fungi with a smaller colony growth (diameter <2 mm; FPyF4, FPyF5 and FPyF6). There was no growth in the controls used (MM without addition of hydrocarbon, and MM supplemented with acetone). The three fungi that grew on both compounds and showed a larger colony growth were identified as *Fusarium* sp., *Fusarium camptoceras* and *Aspergillus terreus* (Table 2).

Among the fungi isolated in this work with the potential ability to degrade fluoranthene and pyrene, there is an isolate identified as *A. terreus*, whose ability to grow on pyrene and benzo(a)pyrene has been previously recognised [32]. *Aspergillus fumigatus*, from the same genus as *A. terreus*, has also been known to grow on anthracene [31]. Both *Aspergillus* species

**Table 2** Molecular identification of isolates with the potential to remove fluoranthene and pyrene

| Isolate | Related sequence (GenBank accession number) | Similarity (%) | Microbial group affiliation |
|---------|---------------------------------------------|----------------|------------------------------|
| FPyF1   | *Fusarium oxysporum* (HQ248198)             | 95.65          | *Fusarium* sp.               |
| FPyF2   | *Aspergillus terreus* (HQ608043)            | 98.11          | *A. terreus*                 |
| FPyF3   | *Fusarium camptoceras* (EU520082)           | 99.22          | *F. camptoceras*             |
| BPyF1   | *Pseudomonas nitroreducens* (NR042435)      | 99.31          | *P. nitroreducens*           |
| BPyF2   | *Bacillus megaterium* (FJ969759)            | 98.11          | *B. megaterium*              |
| BPyF3   | *Ochrobactrum anthropi* (JQ435714)          | 99.00          | *O. anthropi*                |

The genetic affiliation is based on criteria set forth by Rosselló-Mora and Amann [27], who proposed <95, 95–97.5 and >97.5 % as limits to define the taxonomic levels of family, genus and species, respectively

were isolated from petroleum hydrocarbon-contaminated soils. Another isolate obtained in this study was identified as *F. camptoceras*, a species that, until now, has not been associated with the removal of PAHs, even though *F. solani* and *Fusarium oxysporum* within the same genus have been reported to be capable of removing PAHs [33, 34]. In fact, this is the first study demonstrating the ability of *F. camptoceras* to grow on pyrene and fluoranthene.

Furthermore, three bacteria (BPyF1, BPyF2 and BPyF3) that grew on fluoranthene and pyrene at a concentration of 100 mg $L^{-1}$ were identified as *Bacillus megaterium*, *Pseudomonas nitroreducens* and *Ochrobactrum anthropi* (Tables 1 and 2). Previously, it has been reported that *B. megaterium* Py6 and *Ochrobactrum* sp. VA1 have the ability to degrade pyrene [35, 36]. However, to the best of our knowledge, there are no reports on the ability of *P. nitroreducens* to grow on pyrene or fluoranthene.

### Removal of Pyrene and Fluoranthene in Liquid Medium

The three selected fungi and the three selected bacteria were individually subjected to removal testing in liquid culture media with fluoranthene or pyrene added at an initial concentration of 50 mg $L^{-1}$ and incubated for 7 days. The inactivated biomass of both microorganisms showed low adsorption efficiency of fluoranthene and pyrene (0.88–7.1 %), and the fungi reached a greater removal efficiency compared with bacteria (Fig. 1). However, the phenomenon of adsorption was not significant compared with the removal achieved by live microorganisms. The fungus and bacterium that exhibited the highest removal efficiency of fluoranthene and pyrene were *Fusarium* sp. FPyF1 and *O. anthropi* BPyF3 (Fig. 1). *Fusarium* sp. FPyF1 and
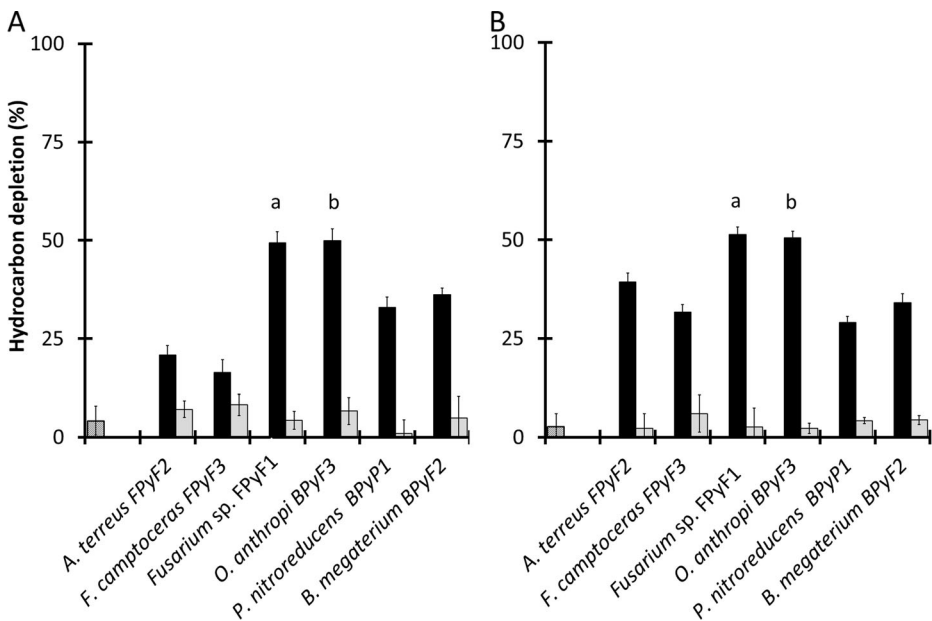


**Fig. 1** Efficiency of fluoranthene (A) and pyrene (B) removal in liquid medium. Living microorganism (■), inactivated microorganism (▨) and biomass-free medium (▧). Fluoranthene or pyrene removal at an initial concentration of 50 mg $L^{-1}$ for 7 days at 30 °C and 120 rpm. The mean and standard error of the mean are indicated, and each point on the graph is the mean of $n=3$. One-way ANOVA was performed using the Tukey's test. *a* $P<0.05$, significant differences of *Fusarium* sp. with other fungi tested. *b* $P<0.05$, significant differences from *Ochrobactrum anthropi* with other bacteria evaluated

*O. anthropi* BPyF3 reached a removal efficiency of 51.32 and 50.39 % for fluoranthene and 49.36 and 49.85 % for pyrene, respectively. There was a significant difference ($P<0.05$) between the removal efficiency of fluoranthene and pyrene reached by *Fusarium* sp. FPyF1 compared with the other two fungal strains and between the removal efficiency of the two PAHs obtained by *O. anthropi* BPyF3 in relation to the two remaining bacteria.

The metabolic potential of *Fusarium* to degrade pyrene and fluoranthene found in this study is supported by several reports suggesting the involvement of this fungal genus in the removal of some PAHs. Among them, *Fusarium* sp. E033 isolated from leaves of *Pterocarpus macrocarpus* Kurz has the ability to degrade benzo[a]pyrene with an efficiency of 65–70 % from an initial concentration of 100 mg L$^{-1}$ over 30 days [37]. In addition, *F. solani* was isolated from a fuel-contaminated soil and exhibited the ability to grow on benzo[a]pyrene as the sole carbon source [38]. In that study, *F. solani* spores germinated in the presence of PAHs and the mycelia mineralised benzo[a]pyrene. Another strain of *F. solani* removed 68 % of pyrene from an initial concentration of 160 mg L$^{-1}$ over 20 days [12].

Moreover, *Ochrobactrum* has been reported as a degrader of 2,4,6-tribromophenol through reductive dehalogenation [39] and to produce glycolipid biosurfactants [40]. Recently, the ability of *Ochrobactrum* sp. VA1 to degrade LMW-PAHs and HMW-PAHs under saline conditions was reported, and such degradation is subject to the availability of nutrients and the concentration of hydrocarbons [36].

Kinetics of Fluoranthene and Pyrene Removal of a Fungus-Bacteria Coculture

To determine whether *Fusarium* sp. FPyF1 and *O. anthropi* BPyF3 could integrate into a fungus-bacterium coculture, both microorganisms were subjected to a test of antagonism. No antagonistic effects were observed among them. Consequently, the coculture was composed of *Fusarium* sp. FPyF1 and *O. anthropi* BPyF3, which previously had the highest removal efficiency of pyrene and fluoranthene (Fig. 1). Subsequently, to evaluate the removal efficiency of pyrene and fluoranthene at an initial concentration of 100 mg L$^{-1}$ by the *Fusarium* sp. FPyF1 and *O. anthropi* BPyF3 coculture, a kinetic study was performed over 21 days. Monocultures of each microorganism were included to control for their individual participation. During the removal kinetics of fluoranthene, three main stages were observed (Fig. 2a): (1) at first, there was a lag phase in which all of the cultures removed a significant amount of fluoranthene ($P>0.05$) (Fig. 2b); (2) the exponential growth phase of the microorganisms occurred, and fluoranthene removal continued; also, *O. anthropi* BPF3 growth was greatly favoured in coculture ($P<0.05$), and the duration of its lag phase in coculture decreased compared with its pure culture; and (3) finally, the death phases of the microorganisms occurred along with the stabilising of fluoranthene removal by individual cultures; however, in the coculture, removal continued, and it was significant compared with the individual cultures ($P<0.05$, Fig. 2b).

The evaluation of the kinetic parameters of cell growth and fluoranthene removal indicated that the maximum specific growth rate of *O. anthropi* BPyF3 in coculture was approximately 77 % higher than in the individual culture of the bacterium, and hence, the duplication time during the exponential phase was lower for the bacterium in coculture versus in its individual culture ($P<0.05$). Additionally, the maximum specific growth rate of *Fusarium* sp. FPyF1 in individual culture and in coculture was similar, and therefore, both microbial cultures exhibited similar duplication times. The most efficient fluoranthene removal was obtained from the coculture, with a value of 87.95 % from an initial concentration of 100 mg L$^{-1}$, which was 27.56–36.2 % higher than the removal efficiency achieved by individual cultures of microorganisms ($P<0.05$). Additionally, the overall removal rate of fluoranthene exhibited by the coculture was superior to that of individual cultures (Table 3).
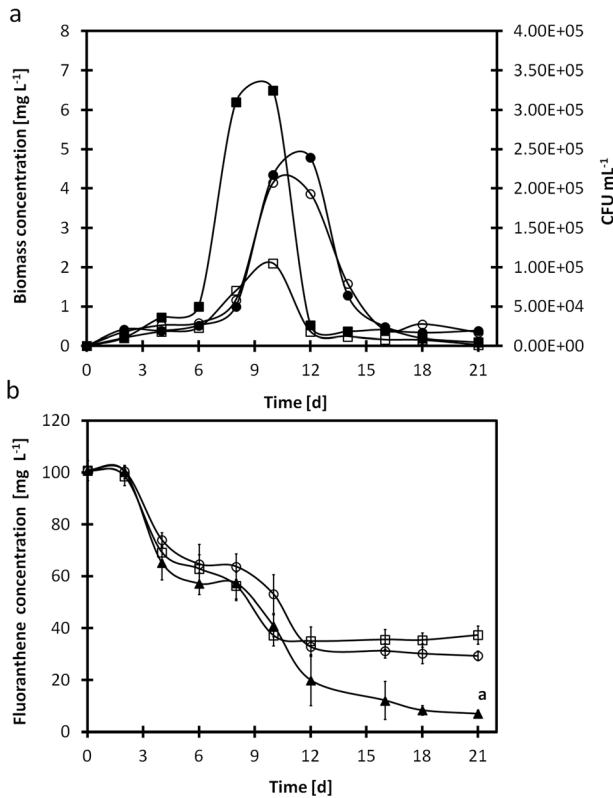
**Fig. 2** Kinetics of cell growth and removal of fluoranthene. **a** Growth of *Ochrobactrum anthropi* BPyF3 (CFU mL$^{-1}$; *empty squares*) and *Fusarium* sp. FPyF1 (mg L$^{-1}$; *empty circles*) in individual culture and in coculture *Ochrobactrum anthropi* BPyF3 (CFU mL$^{-1}$; *filled squares*) and *Fusarium* sp. FPyF1 (mg L$^{-1}$; *filled circles*). **b** Removal of fluoranthene (mg L$^{-1}$): *Fusarium* sp. FPyF1 (*empty circles*), *Ochrobactrum anthropi* BPyF3 (*empty squares*) and coculture (*filled triangles*) for 21 days at 30 °C and 120 rpm. The *bars* indicate the standard error of the mean, and each point on the graph is the mean of $n=3$. One-way ANOVA was performed using the Tukey's test. *a* $P<0.05$, significant difference between coculture with individual cultures from day 12

Next, three mathematical models (zero, first, and second order) were tested to describe the kinetic process of fluoranthene removal; the fit of the experimental data to kinetic models prediction is shown in Fig. 3. In addition, in Table 4, the apparent rate constant ($K$), half-life period ($T_{1/2}$) and the regression equation for each kinetic model tested, along with their corresponding coefficients of determination ($r^2$) are shown. The $r^2$ is higher for the first-order kinetic model, indicating that the kinetics of removal of fluoranthene by *Fusarium* sp. FPyF1, *O. anthropi* BPyF3 and coculture successfully follows the first-order model. This implies that the concentration of fluoranthene decays exponentially with time, and therefore, the removal is a time-dependent process. Additionally, the half-life of the first-order removal reaction is independent of the initial PAH concentration. The removal rate constant $K_1$ is 0.0586, 0.0571 and 0.0963 day$^{-1}$, for *Fusarium* sp. FPyF1, *O. anthropi* BPyF3 and coculture, respectively. It was also observed that the time needed to remove half of the fluoranthene ($T_{1/2}$) is smaller in the coculture (7.197 days) than in individual cultures of *Fusarium* sp. FPyF1 (11.828 days) or *O. anthropi* BPyF3 (12.139 days).

**Table 3** Parameters of cell growth and removal of fluoranthene and pyrene

| Parameter | PAH | Fusarium sp. | Fusarium sp. in coculture | Ochrobactrum anthropi | O. anthropi in coculture | Coculture |
|---|---|---|---|---|---|---|
| Maximum specific growth rate (day$^{-1}$) | F | 0.19±0.02 | 0.18±0.02 | 0.30±0.02 | 0.53±0.01[a] | |
| | Py | 0.53±0.02 | 0.47±0.03 | 0.52±0.02 | 0.53±0.03 | |
| Duplication time (day) | F | 3.65±0.3 | 3.85±0.3 | 2.31±0.2 | 1.31±0.2[a] | |
| | Py | 1.31±0.3 | 1.47±0.2 | 1.33±0.2 | 1.31±0.2 | |
| Removal efficiency (%) | F | 68.95±2.4 | | 64.59±2.1 | | 87.95±2.1[b] |
| | Py | 99.75±1.5 | | 55.46±2.3 | | 99.68±3.1[a] |
| Overall volumetric removal rate (mg L$^{-1}$ day$^{-1}$) | F | 4.34±0.2 | | 4.07±0.2 | | 5.54±0.3[b] |
| | Py | 4.70±0.3 | | 2.64±0.3 [c] | | 4.68±0.3 |

The values were calculated from the kinetics of removal of pyrene (Py) and fluoranthene (F) for 21 days at 30 °C and 120 rpm. Experiments were performed in triplicate

[a] Significant differences between individual culture of *O. anthropi* and *O. anthropi* in coculture

[b] Significant differences between coculture and individual cultures of *Fusarium* sp. and *O. anthropi*

[c] Significant difference between the individual culture of *O. anthropi* and coculture

During the kinetic experiment of pyrene removal, *Fusarium* sp. FPyF1 had an adaptation phase of 6 days either in pure culture or in coculture, while in the individual culture of *O. anthropi* BPyF3, a lag phase was not observed (Fig. 4a). From the earliest days of the kinetics experiment, pyrene removal by *Fusarium* sp. FPyF1 and by the coculture was higher compared with pure culture of *O. anthropi* BPyF3 ($P<0.05$). The higher initial rate of pyrene removal coincided with the lag phase of *Fusarium* sp. FPyF1 in individual culture and in coculture (Fig. 4b). Contrary to what was observed with fluoranthene, the growth of *O. anthropi* BPyF3 in pyrene was higher in pure culture than in coculture (Fig. 4a).

There was no significant difference in the maximum specific growth rate or duplication time in coculture compared with individual cultures of *Fusarium* sp. FPyF1 and *O. anthropi* BPyF3 (Table 3). However, *O. anthropi* BPyF3 in individual culture reached a maximum removal efficiency of pyrene of 55.46 %, while the overall removal efficiency of *Fusarium*
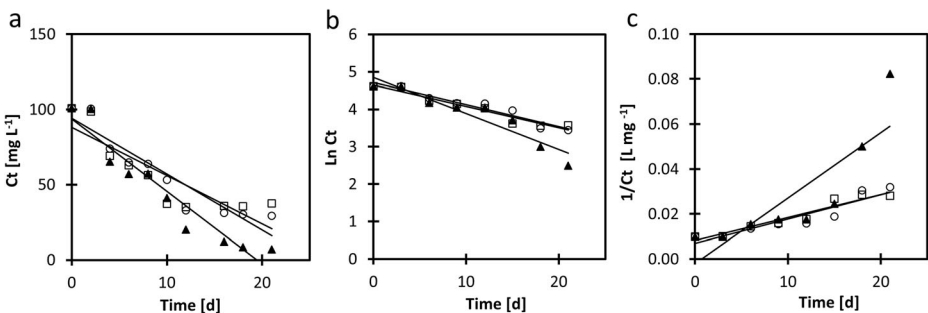


**Fig. 3** Kinetic modelling of fluoranthene removal process. Zero-order (**a**), first-order (**b**) and second-order (**c**) kinetic modelling of fluoranthene removal by *Fusarium* sp. (*empty circles*), *O. anthropi* (*empty squares*) and coculture (*empty triangles*)

**Table 4** Kinetic parameters for fluoranthene removal at an initial concentration of 100 mg L$^{-1}$

| Parameters | Fluoranthene | | |
|---|---|---|---|
| | *Fusarium* sp. | *Ochrobactrum anthropi* | Coculture |
| Zero-order regression equation | $C_t=-3.715t+94.13$ | $C_t=-3.210t+88.05$ | $C_t=-4.835t+93.81$ |
| $K_0$(mg L$^{-1}$ day$^{-1}$) | 3.715 | 3.210 | 4.835 |
| $T_{1/2}$(day) | 13.458 | 15.576 | 10.341 |
| $r^2$ | 0.884 | 0.762 | 0.914 |
| First-order regression equation | $\ln C_t=-0.0586t+4.71$ | $\ln C_t=-0.0571t+4.69$ | $\ln C_t=-0.0963t+4.84$ |
| $K_1$(day$^{-1}$) | 0.0586 | 0.0571 | 0.0963 |
| $T_{1/2}$(day) | 11.828 | 12.139 | 7.197 |
| $r^2$ | 0.936 | 0.944 | 0.934 |
| Second-order regression equation | $\frac{1}{C_t}=0.0011t+0.0069$ | $\frac{1}{C_t}=0.001t+0.0082$ | $\frac{1}{C_t}=0.0029t+0.0022$ |
| $K_2$(L mg$^{-1}$ day$^{-1}$) | 0.0011 | 0.001 | 0.0029 |
| $T_{1/2}$(day) | 9.091 | 10 | 3.448 |
| $r^2$ | 0.866 | 0.927 | 0.716 |

FPyF1 was nearly 100 % (Table 3; Fig. 4b); similarly, the overall removal efficiency of pyrene in coculture was also close to 100 %. The overall volumetric removal rate of pyrene in coculture was 4.68 mg L$^{-1}$ day$^{-1}$, which is approximately 77.3 % higher than what was obtained by the individual *O. anthropi* BPyF3 culture (2.64 mg L$^{-1}$ day$^{-1}$). Controls showed less than 5 % removal of fluoranthene or pyrene adsorption to inactivated biomass or by photolysis effects during kinetic experiments.

Removal data for pyrene were adjusted to different kinetic models (Fig. 5), and the respective removal rate constants were obtained. According to the $r^2$, it was concluded that the removal of pyrene by the individual cultures of *Fusarium* sp. FPyF1 and *O. anthropi* BPyF3 as well as by coculture fit to first-order kinetics, with removal constants of 0.263, 0.040 and 0.275 day$^{-1}$, respectively (Table 5). Likewise, it was observed that the time required to remove half of the pyrene ($T_{1/2}$) is less in coculture (2.520 days) and in the individual culture of *Fusarium* sp. FPyF1 (2.635 days) than in the individual culture of *O. anthropi* BPyF3 (17.328 days). These data suggest that pyrene removal is carried out mainly by *Fusarium* sp. FPyF1 because kinetic degradation profiles by the coculture and by the individual culture of *Fusarium* sp. FPyF1 were quite similar.

Cocultures of fungi and bacteria have been described as efficient hydrocarbon removal systems [14, 41] based on the fact that microbial cultures composed of different species have multiple metabolic capabilities that increase the efficiency of the bioremediation process [14, 42]. This work confirms that the use of a coculture, composed of *Fusarium* sp. FPyF1 and *O. anthropi* BPyF3, had a positive effect on fluoranthene removal. This same effect has been observed in other works, including Machín-Ramírez et al. [28], who isolated bacteria and fungi capable of removing benzo[a]pyrene from soil contaminated with oily sludge. These authors observed that a *Penicillium* sp. and *Serratia marcescens* coculture increased the degradation of benzo[a]pyrene (65 %) compared with the removal by pure cultures by 45 and 50 %, respectively, at an initial concentration of 50 mg L$^{-1}$ after 20 days of incubation. The overall volumetric removal rate of benzo[a]pyrene by the *Penicillium* sp. and *S. marcescens* coculture was 1.62 mg L$^{-1}$ day$^{-1}$, a value that is significantly lower than the rates obtained in this work,
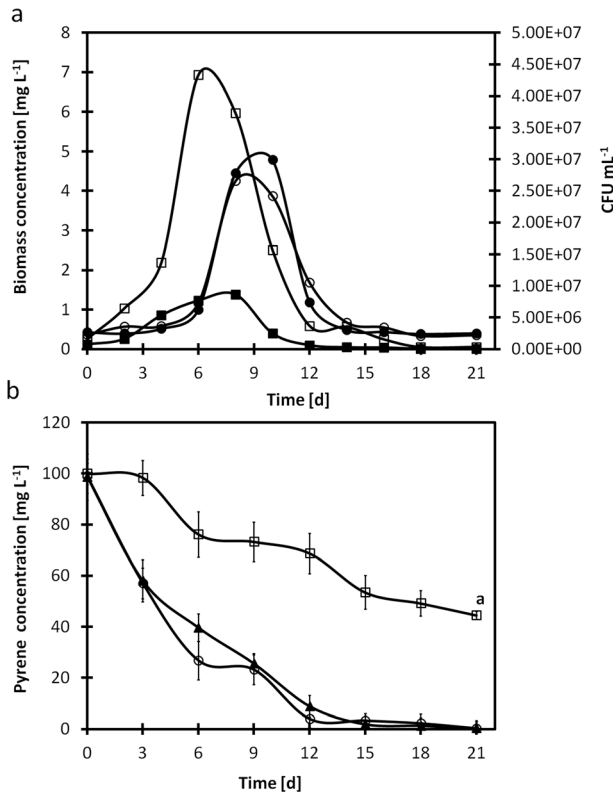
Fig. 4 Kinetics of cell growth and removal of pyrene. **a** Growth of *Ochrobactrum anthropi* BPyF3 (CFU mL$^{-1}$; *empty squares*) and *Fusarium* sp. FPyF1 (mg L$^{-1}$; *empty circles*) in individual culture and in coculture *Ochrobactrum anthropi* BPyF3 (CFU mL$^{-1}$; *filled squares*) and *Fusarium* sp. FPyF1 (mg L$^{-1}$; *filled circles*). **b** Removal of pyrene (mg L$^{-1}$): *Fusarium* sp. FPyF1 (*empty circles*), *Ochrobactrum anthropi* BPyF3 (*empty squares*) and coculture (*filled triangles*) for 21 days at 30 °C and 120 rpm. The *bars* indicate the standard error of the mean, and each point on the graph is the mean of $n=3$. One-way ANOVA was performed using the Tukey's test. *a* $P<0.05$, significant difference from *Ochrobactrum anthropi* BPyF3 with coculture and with *Fusarium* sp. FPyF1
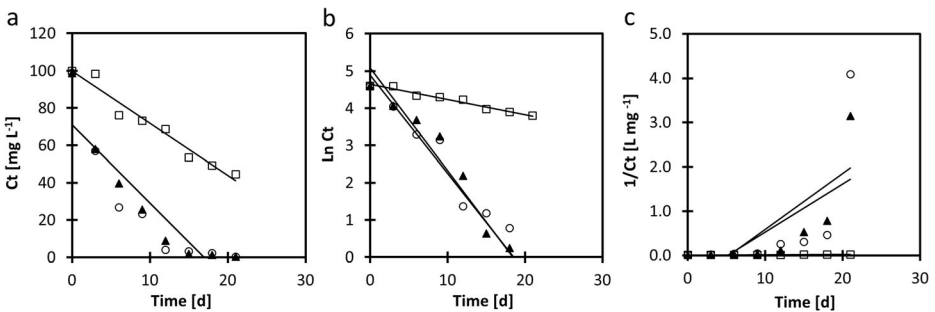


Fig. 5 Kinetic modelling of pyrene removal process. Zero-order (**a**), first-order (**b**) and second-order (**c**) kinetic modelling of pyrene removal by *Fusarium* sp. (*empty circles*), *O. anthropi* (*empty squares*) and coculture (*filled triangles*)

**Table 5** Kinetic parameters for pyrene removal at an initial concentration of 100 mg L$^{-1}$

| Parameters | Pyrene | | |
|---|---|---|---|
| | Fusarium sp. | O. anthropi | Coculture |
| Zero-order regression equation | $C_t=-4.187t+70.90$ | $C_t=-2.80t+99.82$ | $C_t=-4.372t+75.18$ |
| $K_0$(mg L$^{-1}$day$^{-1}$) | 4.187 | 2.80 | 4.372 |
| $T_{1/2}$(day) | 11.941 | 17.857 | 11.441 |
| $r^2$ | 0.777 | 0.956 | 0.845 |
| First-order regression equation | $\ln C_t=-0.263t+4.89$ | $\ln C_t=-0.040t+4.64$ | $\ln C_t=-0.275t+5.07$ |
| $K_1$(day$^{-1}$) | 0.263 | 0.040 | 0.275 |
| $T_{1/2}$(day) | 2.635 | 17.328 | 2.520 |
| $r^2$ | 0.940 | 0.969 | 0.959 |
| Second-order regression equation | $\frac{1}{C_t}=-0.126t+0.672$ | $\frac{1}{C_t}=0.0006t+0.0089$ | $\frac{1}{C_t}=-0.108t+0.557$ |
| $K_2$(L mg$^{-1}$day$^{-1}$) | 0.126 | 0.0006 | 0.108 |
| $T_{1/2}$(day) | 0.079 | 16.666 | 0.092 |
| $r^2$ | 0.439 | 0.958 | 0.551 |

by the *Fusarium* sp. FPyF1 and *O. anthropi* BPyF3 coculture for either of the PAHs evaluated here (Table 3).

Similarly, in another study using a consortium composed of *Mycobacterium fortuitum*, *Bacillus cereus*, *Microbacterium* sp., *Gordonia polyisoprenivorans*, a Microbacteriaceae bacterium and the fungus *F. oxysporum*, higher degradation efficiencies of 99, 99 and 96 % were found from 250, 500 and 1000 mg of anthracene, phenanthrene and pyrene/kg of soil, respectively, after 70 days of incubation compared with their individual cultures [43].

*Fusarium* sp. FPyF1 and *O. anthropi* BPyF3 coculture exhibited interesting advantages in the removal of fluoranthene, including shorter growth times and increased efficiency and rate of fluoranthene removal. These data suggest that this microbial consortium could be applied successfully in bioremediation processes. Furthermore, *Fusarium* sp. FPyF1 could be potentially useful in pyrene removal.

## Conclusions

The results presented in this work show that the *Fusarium* sp. FPyF1 and *O. anthropi* BPyF3 coculture exhibits a greater efficiency (87.95 %) and rate (5.54 mg L$^{-1}$ day$^{-1}$) of fluoranthene removal compared with individual cultures of each microorganism (68.95 % and 4.34 mg L$^{-1}$ day$^{-1}$ for *Fusarium* sp FPyF1 and 64.59 % and 4.07 mg L$^{-1}$ day$^{-1}$ for *O. anthropi* BPyF3) from an initial concentration of 100 mg L$^{-1}$ for 21 days at 30 °C and 120 rpm. Furthermore, these results suggest that *Fusarium* sp. FPyF1 is the microorganism responsible for the removal of pyrene in coculture. The kinetic behaviour of fluoranthene and pyrene removal by *Fusarium* sp. FPyF1, *O. anthropi* BPyF3, and the coculture was adjusted to a first-order model. The results suggest that the coculture, composed of *Fusarium* sp. FPyF1 and *O. anthropi* BPyF3, has great potential for bioremediation of fluoranthene-contaminated environments. Additionally, the pure culture of *Fusarium* sp. FPyF1 is a promising candidate for detoxification of pyrene-contaminated environments.

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