Journal of Hazardous Materials 318 (2016) 702–710

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Comparative metagenomic analysis of PAH degradation in soil by a mixed microbial consortium

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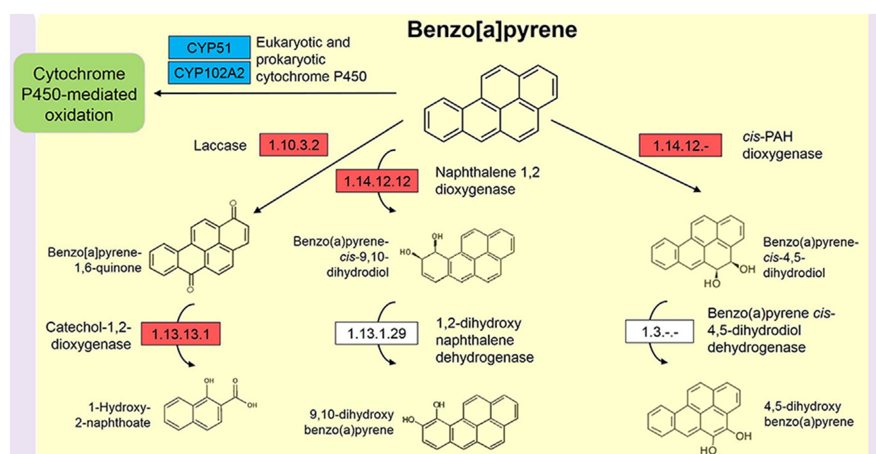
HIGHLIGHTS

- PAH degradation in soil by a microbial consortium studied by metagenomics.
- Consortium induced notable changes in the microbial diversity of polluted soils.
- Consortium shifted soil-native communities in favor of PAH-degrading populations.
- Concomitant degradation pathways taking place for Phe, Pyr and BaP degradation.
- High amounts of PAHs degraded in soils due to increased co-metabolic degradation.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 28 March 2016
Received in revised form 29 June 2016
Accepted 25 July 2016
Available online 26 July 2016

Keywords:
Polycyclic aromatic hydrocarbons (PAHs)
Metagenomics
Soil bioremediation
Aromatic-ring-hydroxylating dioxygenases
Microbial consortium

ABSTRACT

In this study, we used a taxonomic and functional metagenomic approach to analyze some of the effects (e.g. displacement, permanence, disappearance) produced between native microbiota and a previously constructed Polycyclic Aromatic Hydrocarbon (PAH)-degrading microbial consortium during the bioremediation process of a soil polluted with PAHs. Bioaugmentation with a fungal-bacterial consortium and biostimulation of native microbiota using corn stover as texturizer produced appreciable changes in the microbial diversity of polluted soils, shifting native microbial communities in favor of degrading specific populations. Functional metagenomics showed changes in gene abundance suggesting a bias towards aromatic hydrocarbon and intermediary degradation pathways, which greatly favored PAH mineralization. In contrast, pathways favoring the formation of toxic intermediates such as cytochrome P450-mediated reactions were found to be significantly reduced in bioaugmented soils.

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http://dx.doi.org/10.1016/j.jhazmat.2016.07.060
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PAH biodegradation in soil using the microbial consortium was faster and reached higher degradation values (84% after 30 d) as a result of an increased co-metabolic degradation when compared with other mixed microbial consortia. The main differences between inoculated and non-inoculated soils were observed in aromatic ring-hydroxylating dioxygenases, laccase, protocatechuate, salicylate and benzoate-degrading enzyme genes. Based on our results, we propose that several concurrent metabolic pathways are taking place in soils during PAH degradation.

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1. Introduction

Contamination of soils with hydrocarbons has become a worldwide environmental issue because of the potential toxic effects on animals, humans, plants and microorganisms. Continuous contamination with crude oil and its derivatives favor the deposition and accumulation of xenobiotics and toxic compounds in soils. Polycyclic Aromatic Hydrocarbons (PAHs) are considered priority environmental pollutants because of their high toxicity and persistence. PAHs are molecules with physical and chemical characteristics that greatly contribute to their persistence in soil, possessing toxic, mutagenic and teratogenic properties [1]. During the last century there has been an increase in the amount of PAHs released into the environment from anthropogenic sources and atmospheric deposition from natural sources [2]. Microbial degradation is considered the main natural degradation form of hydrocarbons in soils [1,2]. Bioremediation, based on the use of microorganisms to degrade the contaminants, is a promising technology because of its high efficiency and cost-effectiveness. For over three decades it has been shown that microorganisms such as bacteria, fungi and algae possess specific catabolic activities that can be exploited for the remediation of soil and water impacted with low and high molecular weight PAHs [3,4].

Current knowledge about the microbial, functional and metabolic diversity and the impact produced by introduced microbial populations on native communities during the degradation of PAHs in soil is still limited. Metagenomics can be used to monitor microbial communities, providing access to the taxonomic and functional gene composition of soil microbial communities and thus giving information on potentially novel biocatalysts or enzymes and the phylogenetic and functional relationships between them [5]. Recent studies using Next-Generation Sequencing technologies during hydrocarbon bioremediation processes have shown the usefulness of these technologies to identify, monitor and estimate proportions of crude oil [6,7] and diesel degrading populations [8,9] present in soils and bioreactors. Thus, metagenomic sequencing of soil microbial communities involved in PAH degradation can provide insights regarding the microbial populations, functional and metabolic profiles and specific enzymes involved during the bioremediation of soils.

In this study, we used a taxonomic and functional metagenomic approach to analyze the metabolic profiles and main enzymes involved during the bioremediation of a soil polluted with low and high molecular weight PAHs, as well as some of the effects (e.g. displacement, permanence, disappearance) produced between soil native microbiota and a PAH-degrading microbial consortium.

2. Materials and methods

2.1. Soil samples

Uncontaminated soil samples obtained from the Xalostoc region in Tlaxcala, Mexico (19°24′08″N 98°02′54″W, 18 °C annual average temperature) were used in this study. Homogeneous samples were obtained at 30 cm depth in a simple random sampling, according to procedures described by US-EPA [10]. The soil sample composition was sandy loam with 2.4% organic matter, 1.4% total organic carbon, 0.063% nitrogen, 0.0023% phosphorous and a pH of 8.41. Soil samples were dried, homogenized and separated with a 2 mm test sieve. These soil samples were then spiked with 2500 mg kg^{-1} of a mixture of Phenanthrene (Phe), Pyrene (Pyr) and Benzo[a]pyrene (BaP) (1:1:1 ratio) using acetone as organic solvent and evaporated, as described by Ulla et al. [11].

2.2. PAH-degrading consortium and inoculum preparation

A microbial PAH-degrading consortium (C1), composed by four fungal (*Aspergillus flavus* H6, *Aspergillus nomius* H7, *Rhizomucor variabilis* H9, *Trichoderma asperellum* H15) and five bacterial native strains (*Klebsiella pneumoniae* B1, *Bacillus cereus* B4, *Pseudomonas aeruginosa* B6, *Klebsiella* sp. B10, *Stenotrophomonas maltophilia* B14), was used for the bioaugmentation of PAH-contaminated soils [12]. Individual strains composing the consortium were maintained aerobically at 30 °C in Potato Dextrose Agar (PDA) plates containing 0.1% Maya crude oil (fungi) or liquid Basal Saline Medium (g l^{-1}: NaCl, 0.3; $(NH_4)_2SO_4$, 0.6; K_2HPO_4, 0.75; KH_2PO_4; 0.25; $MgSO_4{\cdot}7H_2O$, 0.15; KNO_3, 0.6; yeast extract, 0.125) using 0.1% Maya crude oil as main carbon source (bacteria), as previously described [12]. Production of fungal spores was carried out in 250-mL flasks containing 30 ml of PDA medium, individually inoculated with each of the fungal strains and incubated at 30 °C. Spores were collected on day 4 with the addition of 20 ml of 0.1% Tween 80 solution, sterile glass beads and the flasks gently shaken for 2 min. The spore suspension concentration was quantified in a Neubauer haematocytometer chamber using an optical microscope. On the other hand, each of the bacterial strains was grown individually in 5 ml of liquid BSM at 30 °C with agitation at 200 rpm until cultures reached an optical density of 0.14 at 600 nm (comparable to a MacFarland standard No. 0.5; approx. cell density 1.5×10^7 CFU/ml). From these cultures, 10 μl of each strain (approximately 1.5×10^6 CFU) were sampled, mixed with the others (to give a final volume of 50 μl) and further used as inoculum in contaminated soil.

2.3. Microcosm treatability tests

Treatability assays were performed in microcosm solid culture systems using sterile corn stover (35.7% carbon, 0.465% nitrogen, 0.000031% phosphorous) for fungal growth support, texturizing and as a biostimulation agent. Corn stover (0.35 g dry weight) was placed in 100 ml glass flasks, moistened with 3 ml Czapeck medium (g l^{-1}: sucrose, 30; sodium nitrate, 3; dipotassium phosphate, 1; magnesium sulfate, 0.5; potassium chloride, 0.5; ferrous sulfate, 0.01; pH 7.3) to reach 30% moisture content and to promote germination of fungal spores and obtain the inoculum. Therefore, the flasks were inoculated with 2×10^6 spores g^{-1} of each fungal strain, hermetically sealed with sterile rubber caps and aluminum seal, then incubated for 5 d at 30 °C. Pre-inoculated corn stover was then mixed with 6.65 g of PAH-contaminated soil, inoculated with 2×10^6 CFU g^{-1} of each bacterial strain and sealed hermetically again. Controls consisted of non-inoculated systems (only

bioestimulation of native microbiota with corn stover), which were used to compare the effect of the bioaugmentation with the C1 consortium on PAH degradation, the taxonomic composition and functional profiles. Treatment (biostimulated and bioaugmented with C1) and control microcosms (only biostimulated) were incubated at 30 °C for 30 days, flushing headspaces every 48 h for 10 min with sterile and moistened air to preserve aerobic conditions and avoid carbon dioxide accumulation. All treatments were sampled at days 0 (post-inoculation), 16 and 30 for PAH quantitation and DNA extraction for metagenomic analysis. All assays were carried out in triplicate.

2.4. Microcosm heterotrophic activity and PAH measurements

Heterotrophic activity in microcosms was measured every 48 h for 30 days by means of CO_2 production, using an Agilent 6890 series Gas Chromatograph (Agilent Technologies, Germany) equipped with a thermal conductivity detector and a GS-CarbonPLOT column. CO_2 was reported as mg of CO_2 per g of initial dry matter (IDM). Residual PAHs from soils were extracted from 1 g of IDM (soil plus corn stover) with the addition of 25 ml of a dichloromethane-acetone solution (7:3 ratio) using an Anton Paar Multiwave 3000 SOLV apparatus (Anton Paar, Germany) for 20 min, according to the EPA 3546 method [13]. The resulting extracts were evaporated, suspended in 2 ml of acetonitrile and analyzed in an HP Agilent 1100 HPLC system (Agilent Technologies, Germany) equipped with a C18 reverse-phase column, with UV absorbance detector set at 245–360 nm under isocratic conditions in acetonitrile:water (90:10) and a flow rate of 1 ml min^{-1}.

2.5. Metagenomic sequencing and bioinformatic analysis

Metagenomic DNA was isolated from 2 g IDM (soil plus corn stover) using the PowerSoil DNA Isolation Kit (MoBio Laboratories Inc., USA) following the manufacturer's instructions. Extracted DNA preparations were quantified and quality checked using a Nanodrop 1000 Spectrophotometer (Thermo Scientific). Paired-end whole genome shotgun sequencing was performed using an Illumina MiSeq 2 × 250 platform. After this step, we obtained six datasets as follows: C1d0: 806,947 sequences of 343 bp (on average) for a total of 277 Mbp; C1d16: 738,035 sequences of 328 bp (on average) for a total of 242 Mbp; C1d30: 780,405 sequences of 331 bp (on average) for a total of 259 Mbp; Controld0: 2,034,720 sequences of 266 bp (on average) for a total of 541 Mbp; Controld16: 2,202,675 sequences of 280 bp (on average) for a total of 618 Mbp; and Controld30: 2,197,197 sequences of 268 bp (on average) for a total of 589 Mbp. All these datasets are publicly available in the MG-RAST server [14] under the MG-RAST IDs 4600198.3, 4600199.3, 4600200.3, 4658539.3, 4658538.3 and 4658540.3 respectively, in the static link http://metagenomics.anl.gov/linkin.cgi?project=11296. The unassembled metagenomic reads were quality checked with the FastQC v0.10.1 program [15], then compared and annotated using the MG-RAST server. The quality check included sequence quality filtering, length filtering and dereplication steps. Taxonomic analysis was made using the M5nr protein database [16] with a maximum e-value of $1e^{-5}$ and a minimum identity cutoff and alignment length of 60% and 15 bp, respectively. SEED subsystems annotation and KEGG orthology were used for functional hierarchical classification using a maximum e-value of $1e^{-5}$, a minimum identity cutoff of 60% and an alignment length cutoff of 15 bp. Metagenomic data were also analyzed with standalone BLASTX v2.2.29 [17] using a custom database containing sequences from ring hydroxylating dioxygenases, monooxygenases, ligninolytic peroxidases and laccases, and subsequently annotated with MEGAN5 [18]. Results from taxonomic and functional analysis were visualized with the MG-RAST server [14], MEGAN5 and STAMP version v2.0.3 [19].

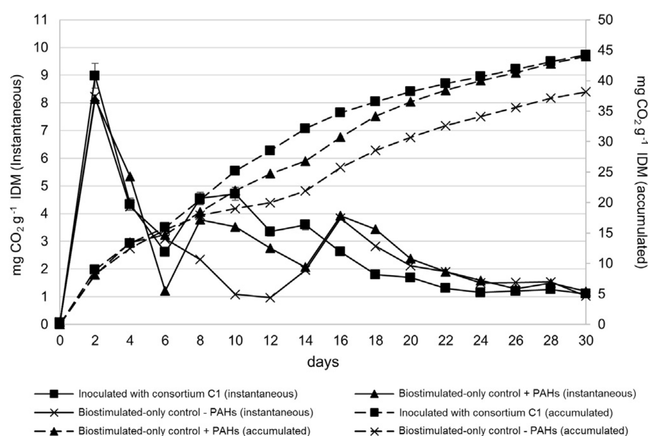

Fig. 1. Heterotrophic activity (CO_2 production accumulated and instantaneous) of microcosms with inoculated soil (bioaugmented with C1/bioestimulated) and non-inoculated soil (biostimulated-only).

2.6. Statistical analysis

Data from CO_2 and PAH measurements were analyzed by Analysis of Variance (ANOVA) followed by a multiple comparison test (LSD) with SPSS Statistics Software version 19 (IBM), considering statistically significant differences those with a p value < 0.05. Statistical analysis of metagenomic profiles was performed with the STAMP v2.0.3 program, using the two-sided Welch's t-test for group to group comparisons, the G test plus Fisher's exact tests for sample to sample comparisons, and the Bonferroni test for p value corrections. Those differences with a p value < 0.05 were considered statistically significant.

3. Results and discussion

3.1. Heterotrophic activity in soil microcosms

Soil microcosms inoculated with consortium C1 presented a rapid initial increase in CO_2 production by day 2, with a slightly lower production by biostimulated-only microcosms. Inoculated microcosms produced more CO_2 than biostimulated-only controls during the first two weeks, but the tendency reverted from day 16 and higher instantaneous CO_2 production was observed for biostimulated-only microcosms, even though accumulated CO_2 levels by day 30 were almost identical (Fig. 1). In general, CO_2 production through the biodegradation process in soil was higher when compared with reported values of similar mixed consortia in microcosms, where maximum accumulated production only reached 1 mg CO_2 gr^{-1} IDM after 60 d growth [20,24]. As expected, inoculation of soils promoted an increase in respiratory levels, especially taking into account that the spiking of soils with high amounts of PAHs could greatly affect the quantity and diversity of native soil populations, which could explain the differences in CO_2 production observed during the first two weeks. Regardless of soil inoculation, biostimulation with corn stover promoted an increase in CO_2 levels in contaminated soils.

3.2. PAH biodegradation in soil

Our results showed that Phe, Pyr and BaP degradation was significantly higher in soils inoculated with consortium C1 than biostimulated-only controls by day 16 (p = 0.011, 0.021 and 0.035, respectively) and 30 (p = 0.007, 0.019, and 0.009, respectively). As

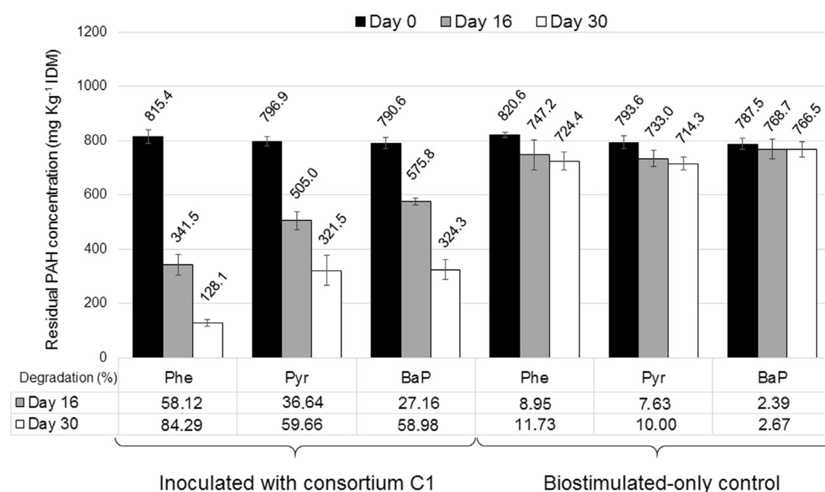

Fig. 2. PAH degradation by C1-inoculated and biostimulated-only soil microcosms with non-sterile soil spiked with 2500 mg kg^{-1} of Phe, Pyr and BaP.

observed in Fig. 2, Phe was degraded at a higher rate during the first 16 days than both Pyr and BaP by consortium C1 (58.12%, 36.64% and 27.16%, respectively) but interestingly Pyr, and especially BaP, were degraded at a higher rate after day 16 until day 30 reaching 59.66% and 58.98%, respectively. These results are in accordance with our previous report using a similar native degrading consortium, where High Molecular Weight (HMW)-PAH degradation in sterile soils was relatively low during the initial stages, but significantly increased during later stages [12]. In fact, an increased co-metabolic degradation of HMW-PAHs such as Pyr and BaP in a mixture with Low Molecular Weight (LMW)-PAHs can be achieved in response to the induction of LMW-PAH degradation pathways [2,21]. PAH biodegradation with consortium C1 was also faster and showed higher degradation values when compared with other mixed microbial consortia in soil [22–25]. This was more evident for Phe, reaching 84.29% degradation by day 30, even though HMW-PAH degradation was also higher than in previous reports [22,23]. Even when the PAH soil concentrations used in this study were at least five times higher than those used in previous studies evaluating mixed microbial consortia in soils, degradation was nearly twice as fast for both LMW- and HMW-PAHs [24,25].

Even though microbial growth rates are not necessarily related to the degree of pollutant degradation [26], a higher growth rate along with effective PAH biodegradation is suggestive of their utilization as a carbon source by microbial populations, as evidenced by CO_2 production differences between biostimulated-only controls in the presence or absence of PAHs (Fig. 1). Moreover, a decrease in the microbial growth rate could be indicative of microorganisms reaching a stationary phase and secondary metabolism, producing hydrocarbon-degrading enzymes which may positively influence the oxidation of PAHs [27,28]. Control microcosms where only corn stover was added, presented significantly lower degradation rates of Phe (11.73%), Pyr (10.00%) and BaP (2.67%) (approximately corresponding to 6% of degradation of the total mixture of PAHs) by day 30 in comparison to C1-inoculated soils that showed degradation rates above 65% of initial mixture of PAHs. In fact, most of the degradation occurred during the first stage of the process in a growth-dependent PAH degradation triggered by the presence of culture media and corn stover at day 0.

3.3. Taxonomic analysis of soil bioremediation

The composition of different taxa based on full sequence datasets from soil microcosms showed an overall predominance of bacterial and a lesser relative proportion of eukaryotic and archaeal organisms, regardless of their treatment or sampling day (Fig. 3A). Archaeal communities remained stable throughout the bioremediation process, showing only slight differences in community composition with the exception of control microcosms at day 30 (Fig. 3B). In contrast, soil inoculation with our microbial consortium resulted in appreciable shifts in bacterial populations. For example, treatment with consortium C1 produced a marked increase over time of Firmicutes, Actinobacteria, and Bacteroidetes taxa to the detriment of Proteobacteria (Fig. 3C). However, the relative proportion of Proteobacteria was visibly higher in inoculated microcosms than in biostimulated-only controls, and the opposite was observed for Firmicutes as control microcosms contained a higher proportion of this taxa than inoculated systems. As the microbial consortium contained members of Firmicutes (*Bacillus cereus* H4) and Proteobacteria (*K. pneumoniae* H1, *P. aeruginosa* H6, *Klebsiella* sp. H10 and *S. maltophilia* B14), this displacement could indicate a better adaptation of Proteobacteria and a positive effect over other communities, particularly in the Bacteroidetes/Chlorobi group, whose proportion in control microcosms was lower. Within Proteobacteria, Alpha and Gammaproteobacteria were the taxa with higher proportions (Fig. 3D), both containing known species of PAH degraders [29–31], and Actinobacteria populations as well. Similar results have been reported previously in microcosms containing heavy crude oil-contaminated soils, where members of Gammaproteobacteria were initially dominant in the contaminated microcosms but after 90 days of incubation were superseded by Firmicutes and Alphaproteobacteria [32]. Fungal populations remained relatively stable over time, with Ascomycota being the most abundant taxa with a minor proportion of sequences belonging to Basidiomycota and incertae sedis (Fig. 3E). Within the Ascomycota phylum, Pezizomycotina was the most prominent group (Fig. 3F). This is not unexpected as Pezizomycotina contains all fungal strains included in the degrading consortium, except for *R. variabilis* H9.

Alpha diversity measurements (Shanon's diversity index) of metagenomes showed that the addition of consortium C1 into contaminated soil contributed to an overall increase in the species richness over time, changing from 45.1 at day 0, to 67.7 at day 16 and 421.1 at day 30. We hypothesize this may be a consequence of a decrease in the toxicity of soils due to the disappearance of PAHs by consortium C1, and as a result, microbial diversity in soil increases with decreasing PAH concentrations. Besides, population dynamics of bacterial and fungal species comprising the degrading consortium showed important shifts across time, exhibiting marked differences compared to control microcosms.

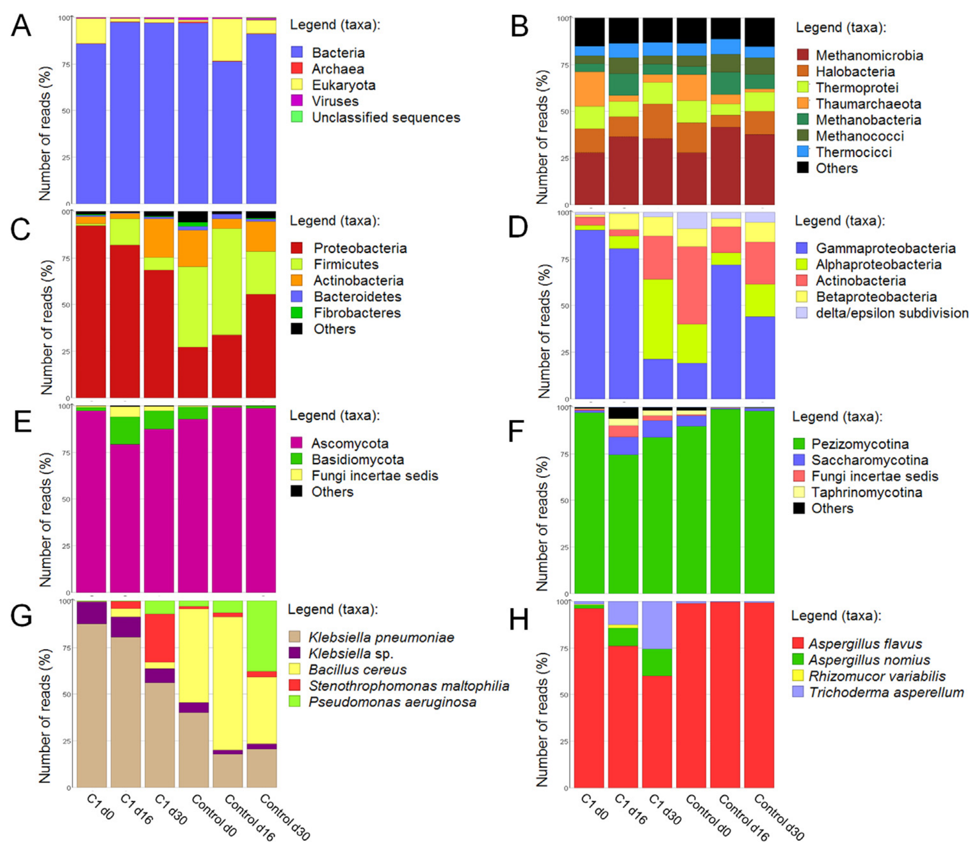

Fig. 3. Composition of different taxa based on full sequence datasets from soil microcosms. (A) domain level; (B) Archaeal phyla level; (c) Bacterial phyla level; (D) Proteobacteria and Actinobacteria orders; (E) Fungal phylum; (F) Ascomycota subphylum; (G) and (H), Bacterial and fungal taxa included in the degrading consortium.

Stenotrophomonas maltophilia populations were found to be the most prominent bacteria in the inoculated microcosms with a constant increase in their relative proportion, concomitant with PAH degradation, while *Klebsiella* populations showed a tendency to decrease over time (Fig. 3G). A similar pattern was observed in the fungal species, where *Aspergillus flavus* predominated in soils inoculated with consortium C1 and *Trichoderma asperellum* and *Aspergillus nomius* proportions increased over time (Fig. 3H). *Rhizomucor variabilis* populations were not detectable after day 16, suggesting a poor adaptation to soil and presumably a displacement by other microorganisms. This is consistent with previous studies reporting poor tolerance levels to PAHs for several *Rhizomucor* species, even though they are shown to remove small quantities of PAHs in surface culture [33,34]. With the exception of *Rhizomucor variabilis*, the microbial species comprising the degrading consortium remained relatively stable across time in the control microcosms, with a higher proportion of *Bacillus cereus, Klebsiella pneumoniae* and *Aspergillus flavus*.

3.4. Functional and metabolic analyses of PAH bioremediation

SEED functional subsystems annotation showed a predominance of genes belonging to metabolic pathways in soils, especially for carbohydrate, amino acid and energy metabolism (Fig. 4A). Even though aromatic degradation pathways do not show appreciable differences in soils at SEED level 1, a deeper look focusing on degradation pathways for aromatic compounds revealed a dominance of peripheral pathways for aromatic degradation genes in both inoculated and non-inoculated soils, while metabolism of central aromatic intermediaries were also found at relatively high levels (Fig. 4B). Interestingly, gene abundance of aromatic anaerobic degradation pathways was higher, although not significantly different, in biostimulated-only controls. As all treatments used corn stover as a texturizing agent, this difference could be directly attributed to the presence of members of the degrading consortium, which could potentially lead to a higher proportion of hydrocarbon aerobic-degradation genes in inoculated soils.

Although our approach was limited to analyzing the functional potential of metagenomes, we observed a relation between the abundance of several genes, the presence of the consortium C1 and a high degradation of PAHs in contaminated soils. These results are in accordance with recent studies, which also showed a relation between the presence of PAHs in soil and a high gene abundance of several oxidoreductases involved in PAH metabolism, as a reflection of the PAH degradation potential by native or introduced soil microorganisms. For example, aromatic-ring-hydroxylating dioxygenase family genes have been found to be more abundant in soils and sediments contaminated with PAHs than in non-contaminated soils [35–37]; Meier [38] also reported a higher abundance of salicylate oxygenase family genes in PAH-polluted soils by the substrate-induced gene expression (SIGEX) metagenomic method. Our results indicate that the inoculation of soils promoted a significant increase in the abundance of genes involved in benzoate degradation and metabolism, protocatechuate degradation and chloroaromatic compounds degradation, as well as non-significant increments in the abundance of gentisate degradation, salicylate and gentisate catabolism genes (Fig. 5). A similar set of enzyme-encoding genes has been found to play an important role in the degradation of aromatic compounds, producing key metabolic intermediates including salicylate, 3-chlorobenzoate and hydroquinone that act as strong inducers of PAH degradative enzymes [39]. A well-studied molecule is salicylate, which induces naphthalene dioxygenase in bacteria and also stimulates the degradation of HMW-PAHs by bacteria [40,41]. The observed differences

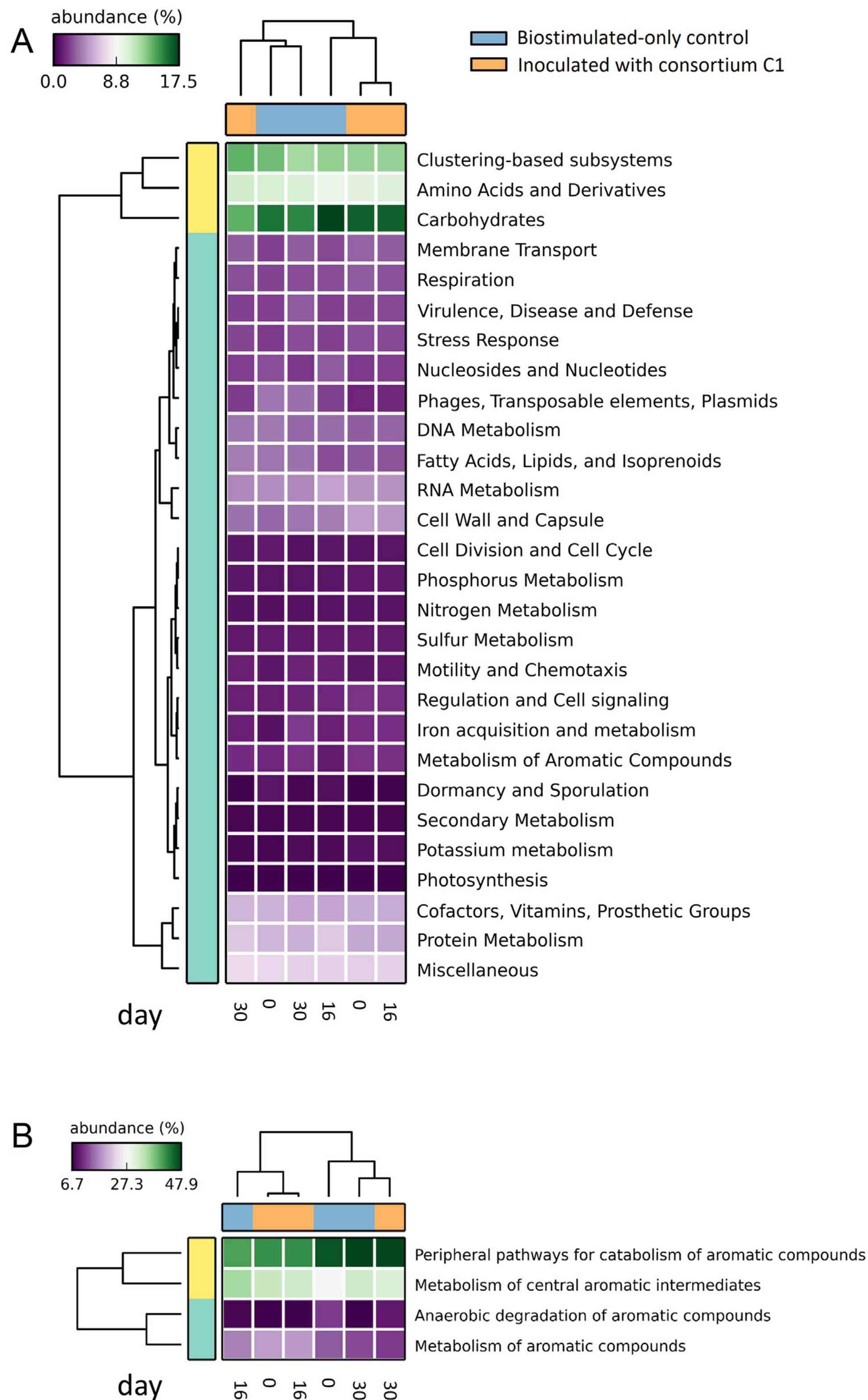

Fig. 4. SEED subsystems functional heatmap of metagenomes from inoculated and non-inoculated microcosms during the bioremediation treatment. A) Differences at level 1 subsystems; B) Differences in subsystems related to metabolism of aromatic compounds.

in gene abundance indicate an overall increase in aerobic pathways involved in the degradation of aromatic compounds into products that can enter the tricarboxylic acid cycle, which could greatly favor the mineralization of PAHs. This was also corroborated when aerobic specific ring hydroxylating cleavage enzymes such as naphthalene 1,2-dioxygenase, extradiol dioxygenase, ring hydroxylating dioxygenase, benzoate/toluate-1,2 dioxygenase, catechol 1,2-dioxygenase, catechol 2,3-dioxygenase, protocatechuate 3,4-dioxygenase, protocatechuate 4,5-dioxygenase and 1,2-dihydroxynaphthalene dioxygenase were found at significantly higher levels ($p = 0.0001$) in inoculated microcosms as compared to biostimulated-only controls (Fig. 6). Such enzymes have also been

Biostimulated-only control
Inoculated with consortium C1
95% confidence intervals

Pathway	p-value (corrected)
Benzoate degradation	0.028
Benzoate catabolism	0.044
Protocatechuate branch of beta-ketoadipate pathway	0.081
Chlorobenzoate degradation	0.120
Toluene 4-monooxygenase (T4MO)	0.189
Gentisare degradation	0.200
Salicylate and gentisate catabolism	0.233
Phenylacetyl-CoA catabolic pathway (core)	0.246
Naphtalene and antracene degradation	0.271
4-Hydroxyphenylacetic acid catabolic pathway	0.272
Hydroxyaromatic decarboxylase family	0.274
p-Hydroxybenzoate degradation	0.308
Aromatic Amin Catabolism	0.351
Chloroaromatic degradation pathway	0.371
N-heterocyclic aromatic compound degradation	0.388
Phenylpropanoid compound degradation	0.390
Central meta-cleavage pathway of aromatic compound degradation	0.390
p-cymene degradation	0.527
Toluene degradation	0.585
Salicylate ester degradation	0.586
Homogentisate pathway of aromatic compound degradation	0.596
Catechol branch of beta-ketoadipate pathway	0.604
Biphenyl Degradation	0.622
carbazol degradation cluster	0.637
Acetophenone carboxylase 1	0.713
Phenol hydroxylase	0.797
Quinate degradation	0.814
Anaerobic toluene and ethylbenzene degradation	0.817
Anaerobic benzoate metabolism	0.856
Cresol degradation	0.940
Benzoate transport and degradation cluster	0.958

0.0 0.1
Mean proportion (%)
−0.20 −0.10 0.00 0.10
Difference in mean proportions (%)

Fig. 5. SEED extended error bar plot of genes from soil metagenomes belonging to the metabolism of aromatic compounds subsystem. Pairwise comparisons were made with a two-sided Welchís *t*-test.

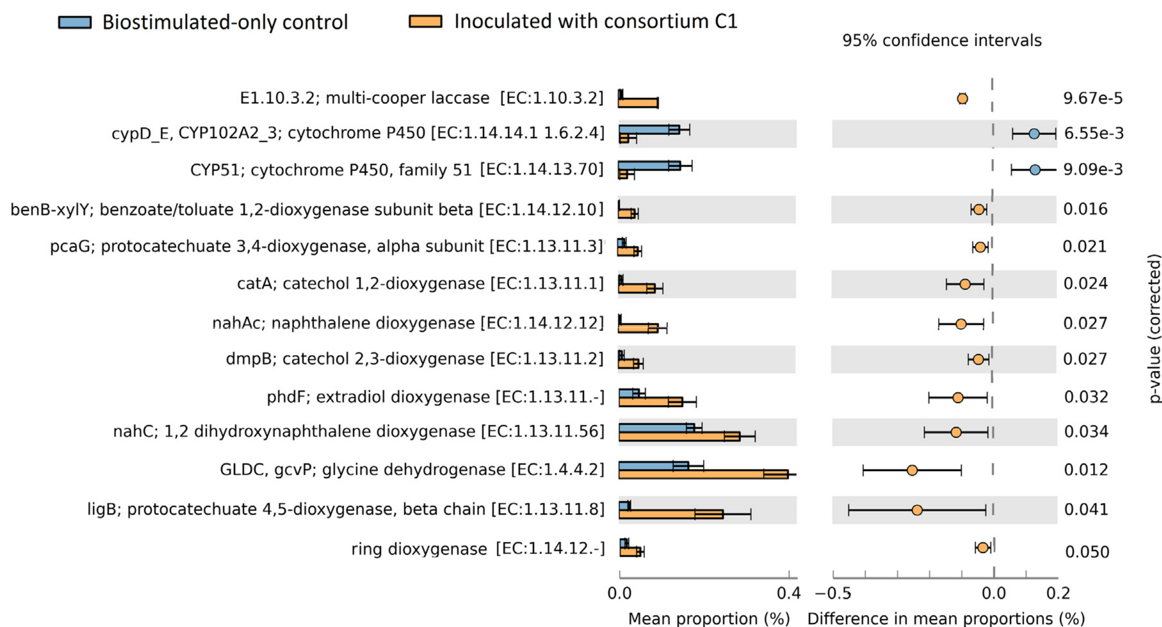

Fig. 6. Extended error bar plot of genes from soil metagenomes where two-sided Welchís *t*-test produced a *p*-value ≤ 0.05. All genes shown are restricted to aromatic and intermediate degradation subsystems.

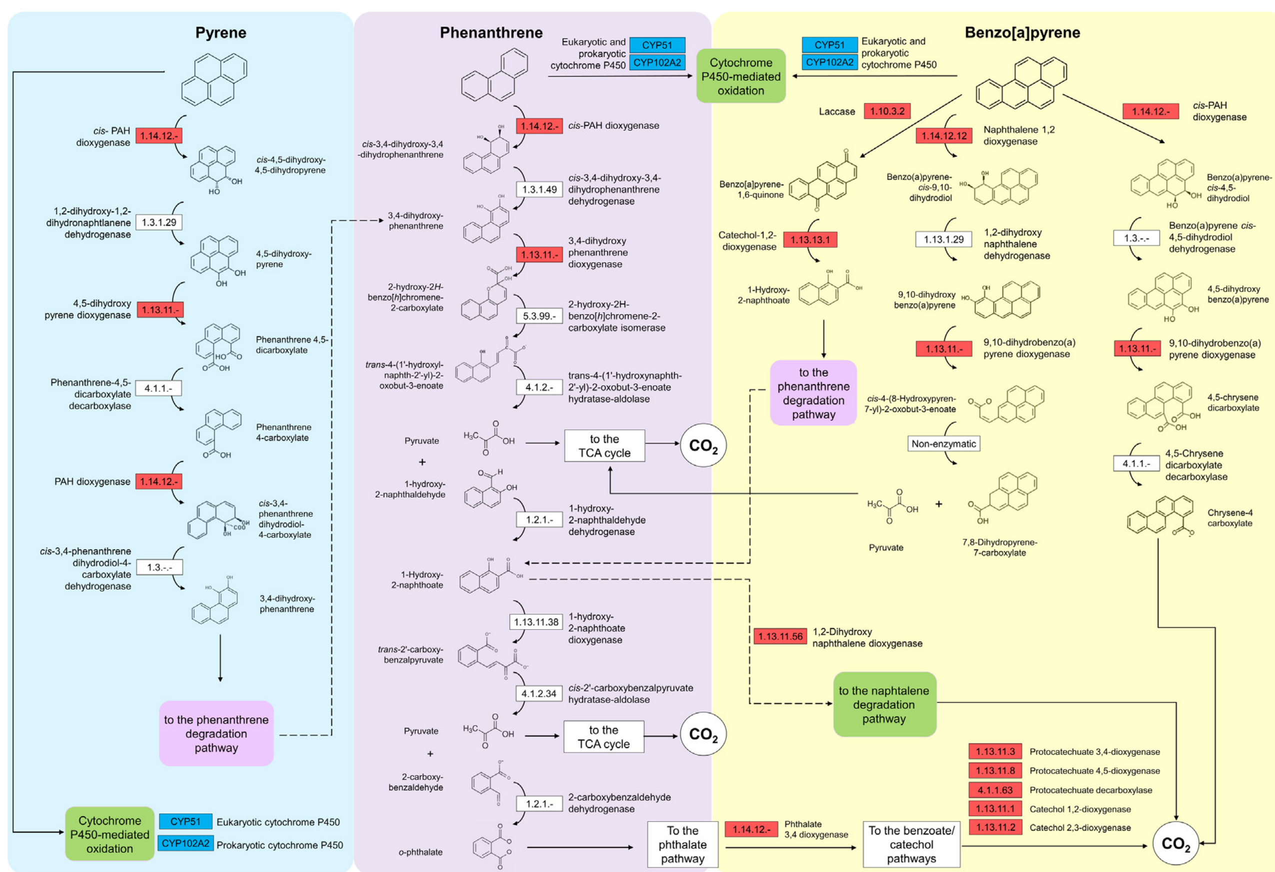

Fig. 7. Proposed metabolic pathways for the degradation of phenanthrene, pyrene and benzo[a]pyrene by the microbial consortium C1 in PAH-polluted soils. Red squares denote the enzyme-encoding genes found to be significantly more abundant in inoculated soils. Blue squares denote the enzyme-encoding genes found to be significantly less abundant in inoculated soils. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

found at elevated levels in metagenomic analyses of the bioremediation of soils and sediments contaminated with diesel and crude oil [9,42,43]. Even though these enzymes have been mainly described in bacteria, several non-ligninolytic fungal species are also known to produce *meta*- and *ortho*-cleavage dioxygenases potentially involved in PAH degradation [44,45]. In addition, laccase gene abundance was also found to be significantly increased in inoculated soils, indicating the involvement of eukaryotic organisms in both the initial oxidation and intermediate steps of PAH degradation. In one particular case, *Trichoderma asperellum* H15 was found to produce both ring-cleavage dioxygenases and laccase in the presence of PAHs [46]. Our results showed a higher abundance of laccase genes concomitant with the survival and permanence of *T. asperellum* H15 in inoculated soils (Fig. 3H). Remarkably, the abundance of bacterial and eukaryotic genes involved in PAH metabolism via cytochrome P450 monooxygenases were found to be significantly reduced when the microbial consortium was inoculated to soils (Fig. 6). This indicates a bias towards PAH mineralization pathways, potentially decreasing the formation of dead-end metabolites, toxic intermediates (e.g. *trans*-dihydrodiols) and conjugates (e.g. *o*-glucuronides, *o*-glucosides and methoxy molecules) responsible for the formation of DNA adducts and subsequent carcinogenesis in mammals [47].

From our functional analysis, we found evidence for several concomitant degradation pathways taking place in inoculated soils for the degradation of Phe, Pyr and BaP (Fig. 7). Initial oxidation steps are driven by laccases and ring hydroxylating dioxygenases, while subsequent steps involve the action of dioxygenases and decarboxylases, promoting the further degradation of PAHs via central metabolic pathways. It is interesting to find significantly higher proportions of several broad-specificity enzyme-encoding genes in inoculated soils, such as naphthalene 1,2-dioxygenase (EC 1.14.12.12), possessing a wide substrate specificity, which eventually permits the *cis*-hydroxilation of several other aromatic compounds including HMW-PAHs [48]. Our results showed that the same enzyme, along with a similar bacterial ring cleavage dioxygenase referred to here as *cis*-PAH dioxygenase (EC 1.14.12.-) plays an important role in the initial and intermediate steps of Phe, Pyr and BaP degradation in inoculated soils. Assuming that both inoculated and native soil microorganisms are able to use broad specificity enzymes and common pathways to metabolize multiple PAHs, the inducers for the degradation of a single PAH could eventually co-stimulate the degradation of a wide range of PAHs, as has been reported [41].

4. Conclusions

Our results demonstrate the efficiency of a mixed microbial consortium to degrade high amounts of PAHs in soils, presumably due to increased co-metabolic degradation. Metagenomic analysis showed that inoculation of a PAH-degrading consortium produced appreciable changes in the microbial diversity of PAH-contaminated soils, shifting native microbial communities in favor of degrading specific populations. Functional metagenomics showed changes in gene abundance indicating a bias towards aromatic hydrocarbons and intermediary degradation pathways, greatly favoring PAH mineralization. The information derived from our metagenomic analysis provided new insights into the metabolic potential of PAH-degrading microorganisms inoculated into soil, and suggest several possible metabolic pathways which

could potentially take place simultaneously during the degradation of LMW and HMW-PAHs in contaminated soils.

Acknowledgments

This research was supported by project SIP20162025 of Instituto Politécnico Nacional and CONACYT grant number 269828.

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