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Dynamics of PAH-Degrading Bacteria and Corresponding Marker Genes in Different Petroleum Hydrocarbon-Contaminated Soils in Almadina Almunawarah, KSA

W. S. El-Sayed[1], A. A. Al-Fraidi[2], N. S. Al-Kaff[2], M. M. Elbadry[3]

[1]*Microbiology Department, Faculty of Science, Ain Shams University, Cairo, Egypt.*
[2]*Biology Department, Faculty of Science, Taibah University, Almadina Almunawarah, Kingdom of Saudi Arabia.*
[3]*Agricultural Microbiology Department, Faculty of Agriculture, Fayoum University, Fayoum, Egypt.*

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Correspondence

W. S. El-Sayed

E-mail

waelsamir@sci.asu.edu.eg,
waelsme@yahoo.com

ABSTRACT

Soil contamination with petroleum derivatives, including polycyclic aromatic hydrocarbons (PAHs), is an increasing problem in various parts of the world. Dynamics of bacterial communities were examined in different petroleum-contaminated soils to assess patterns of microbial responses to PAH contamination. Bacterial community fingerprints were determined using denaturing gradient gel electrophoresis (DGGE) of 16S rRNA gene as a molecular marker. Sequence-based identification of dominant DGGE bands in petroleum hydrocarbon-contaminated soils revealed the presence of *Pseudomonas* sp., *P. pseudoalcaligenes*, *P. nitroreducens*, *P. toyotomiensis,* and uncultured *Pseudomonas* clones as the dominant bacterial groups. In a laboratory scale microcosm, prolonged incubation of contaminated soils resulted in marked deterioration of bacterial communities. DGGE fingerprints of PAH-amended microcosms indicated the presence of distinct bands corresponding to hydrocarbon-degrading bacteria, such as *P. mendocina, Cellulosimicrobium* sp., *Stenotrophomonas* sp., and *Sphingobacterium* sp., which were barely detected in unamended soils. Bacterial community structure in amended soils remained stable even after prolonged incubation up to 6 months. Catabolic genes for upper aromatic hydrocarbon metabolism, such as those encoding naphthalene dioxygenase, toluene dioxygenase (*todC1*), and Rieske-type proteins of dioxygenases and lower pathway genes encoding catechol 1,2-dioxygenase and catechol 2,3-dioxygenase (C23O and *xylE*) were used as biomarkers for monitoring PAH biodegradation in constructed microcosms. Among all tested primer sets, those for C23O and naphthalene dioxygenase allowed efficient amplification of extradiol ring cleavage dioxygenase and naphthalene dioxygenase, respectively, from soil samples amended with naphthalene as a model PAH compound. The results indicated the dominance of ring cleavage dioxygenases of the *meta* pathway and naphthalene dioxygenase in contaminated soils of Almadina Almunawarah, Yanbu city, Saudi Arabia, and their possible use as biomarkers for screening PAH biodegradation in petroleum hydrocarbon-contaminated soils.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are hydrophobic aromatic compounds characterized by two or more fused phenyl rings in different arrangements. PAHs are produced from petroleum refining, waste incineration and coal gasification. Because of their ubiquitous nature, PAHs are found in soil [1], air [2], and water [3]. They have been discovered in tobacco smoke, motor engine emissions, and in foods such as smoked fish and meats, and leafy vegetables [4]. They are considered potentially carcinogenic and mutagenic to humans if they enter the food chain and therefore being listed as priority pollutants by the United States Environmental Protection Agency [5].

Biodegradation of PAH-contaminated sites is dependent on either the presence of indigenous PAH-degrading bacteria [6,7] or the introduction of selected microorganisms with potential catabolic properties [8]. Studying the *in-situ* response of microorganisms to various contaminants has led to the identification of specific microbial communities with potential uses in biological treatment protocols [9-11].

PAH-degrading bacteria from ecologically diverse soils have been studied to identify promising strains for application in bioremediation protocols [12,13]. However, determination of the efficacy of bioremediation via isolation of hydrocarbon-degrading microorganisms from contaminated soils may limit and underestimate the real bioremediation power of indigenous microbial populations due to the presence of unculturable organisms. Recent advances in molecular techniques have extended our ability to profile microbial communities in natural environments using culture-independent methods [14,15]. Culture-independent approaches are particularly valuable given that more than 99% of microorganisms cannot be grown in the laboratory [16,17].

Studying microbial communities at the molecular level provides the opportunity to clearly identify microbial community diversity and adaptive responses to environmental contamination [18-20]. Community analysis using specific catabolic genes can be used to study microbial community dynamics that could potentially be responsible for PAH degradation [21-25].

Functional genes have been used as biomarkers to monitor the biodegradation potential of natural microbial communities. However, because of the variation in sources and types of contamination, investigating the distribution of PAH-degrading genes in the environment is still required [26]. Functional gene biomarkers used as indicators of PAH degradation include those encoding naphthalene biodegradation in *Pseudomonas* spp. (*nah*-genes of the NAH plasmid). The action of dioxygenases on PAHs to incorporate atoms of oxygen at the benzene ring is often the first step in bacterial PAH degradation. The result of this reaction is the formation of cis-dihydrodiols, followed by some dihydroxylated intermediates that enter the tricarboxylic acid cycle [27]. Naphthalene dioxygenase genes have been found in diverse microbial communities in different contaminated environments. Therefore, microbes carrying NAH plasmids possess the potential to degrade complex substrates like PAHs [28,29]. There is no existing reliable method to monitor and detect the diversity of genes involved in PAH degradation. The use of the NAH genes as molecular markers to identify PAH-degrading bacteria is a powerful tool to detect these species' diverse communities [21]. Understanding PAH-degrading bacteria in local environments is important; and degradation abilities could be a focal point for future studies and bioremediation applications in the Kingdom of Saudi Arabia. Reports for isolation and characterization of hydrocarbon-degrading microorganisms from the area of the Arabian Gulf and Saudi Arabia are available [30-33]. However, monitoring catabolic genes and microbial population changes in hydrocarbon-contaminated soils in Saudi Arabia has not been yet performed and therefore, information regarding the distribution and identity of microbial populations and corresponding catabolic genes in these areas remain unknown. Therefore, the aim of this study was to monitor microbial community structure and population dynamics in response to PAH contamination for future biostimulation protocols and to define certain functional PAH-catabolic genes to be used as bioremediation biomarkers in petroleum hydrocarbon-contaminated soils in the city of Almadina Almunawarah, Kingdom of Saudi Arabia, and similar contaminated sites.

2. Materials and Methods

2.1 Site, sampling, and soil analysis

Soils contaminated with petroleum oil derivatives were collected from areas surrounding local garages, gas stations, lubricating oil car facilities and highway roads in Almadina

Almunawarah, along with other soil samples from corresponding Yanbu city Table 1. These areas were clearly contaminated with different petroleum products such as crude oil, diesel and lubricant oil. Contaminated soils were sampled from 5–10 cm depth; the soils were very wet and oily and had different colors and texture according to the level of contamination. Collected soil samples were placed into sterile bottles and stored at 4°C for future use. Three representative soil samples were collected from each site and then pooled prior to further analysis. Physicochemical characterization of soil samples considered in this study was carried out by AEML (Alamanah Environmental Management Laboratories, Almadinah, KSA, https://medina-lab.com/en/).

2.2 Establishment of enrichment microcosms

Bacterial enrichment microcosms were established in 250 ml sterile Duran bottles containing 20% (w/v) soil in basal mineral medium [34] supplemented with 500 mg/L naphthalene as a model PAH compound. The basal mineral medium used in this study contained (per liter): K_2HPO_4, 4.36 g; NaH_2PO_4, 3.45 g; $(NH_4)_2SO_4$, 1.26 g; $MgSO_4.6H_2O$, 0.91 g; trace salt solution, 1 ml. Trace salt solution contained (per 100 ml): $CaCl_2.2H_2O$, 4.77 g; $FeSO_4$ $.7H_2O$, 0.37 g; $CoCl_2.6H_2O$, 0.37 g; $MnCl_2$, 0.1 g; $Na_2MoO_4.2H_2O$, 0.02 g; pH 7. All microcosms were incubated at 30°C without shacking for 6 months prior to DNA extraction. Unamended microcosms were prepared and incubated as above. Three replicates' microcosms for each soil were prepared. For further DGGE analysis, only one microcosm was used (most active one).

2.3 Gas Chromatography (GC) and UV/Vis Spectra

Gas chromatography was used to monitor the degradation of naphthalene model compound in established microcosms after extraction with methylene chloride. Soil slurry from microcosm was extracted three times with methylene chloride. Extracts were combined in a clean tube and centrifuged at 10,000 rpm for 10 minutes. Samples (1 µl) from the supernatant were injected into an Agilent 7890B GC gas chromatographic system equipped with an FID (flame ionization detector). The column was an Agilent J&W HP-1 (100% Dimethylpolysiloxane, 60 m × 0.25 mm id, 0.25 µm film thickness) and the carrier gas helium was used at flow rate of 0.8 ml/min. The injector temperature was 250°C and the oven temperature was held at 70°C for 2 min then increased to 300°C at 20°C /min, thereafter, held at 300°C for 8 min. UV/Vis absorbance spectra of intermediate compounds were obtained from microcosms after removal of soil debris and cells by centrifugation and filtration through 0.2 µm Millipore filters. Filtrates were scanned using UV-visible spectrophotometer (Varian Cary 100, USA). Spectra were recorded between 200 and 600 nm.

2.4 Extraction and purification of genomic DNA

Soil samples for DNA extraction were collected from each microcosm and genomic DNA was extracted using the Ultra Clean Soil DNA Purification Kit (Mo Bio Laboratories, Solana Beach, CA, USA) according to the manufacturers' instructions. Extracted DNA was analyzed by electrophoresis on 1% (w/v) agarose gels, stained with ethidium bromide and imaged with a UV gel documentation system (Bio-Rad Laboratories Inc., CA, USA).

2.5 PCR- DGGE

For Denaturing Gradient Gel Electrophoresis (DGGE) analysis, GC-clamp primers (EUB341FGC) (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGC CTACGGGAGGC AGCAGCAG-3') and (EUB517R) (5' ATTACCGCGGCTGCTGG-3') corresponding to positions 341 and 517 in *Escherichia coli* were used to amplify the 16S rRNA gene [35]. The amplification reaction mixture (25 µl) consisted of 0.5 U of *Taq* DNA polymerase (Invitrogen, USA), 1 µl of total DNA, 2 µl of 10× PCR buffer, each primer at a concentration of 0.25 µM, and a mixture containing each deoxynucleoside triphosphate (dNTPs) at a concentration of 100 µM. PCR was performed on a 2720 thermal cycler (Applied Biosystems, USA). A touchdown PCR program was implemented as follows: initial denaturation step at 95°C for 5 min; 5 cycles of 94°C for 40 s, annealing at 65°C for 40 s, and extension at 72°C for 40 s; 5 cycles of 94°C for 40 s, annealing at 60°C for 40 s, and extension at 72°C for 40 s; 10 cycles of 94°C for 40 s, annealing at 55°C for 40 s, and extension at 72°C for 40 s; 10 cycles of 94°C for 40 s, annealing at 50°C for 40 s, and extension at 72°C for 40 s were performed, followed by a final hold at 72°C for 7 min. Amplicons were analyzed by electrophoresis on 1% (w/v) agarose gels with size markers (1 Kb DNA Ladder; Invitrogen) and visualized using ethidium bromide. DGGE was performed using the Dcode Mutation Detection System (Bio-Rad Laboratories Ltd., Hertfordshire, UK). PCR products were electrophoresed with 0.5× TAE buffer (1× TAE buffer is 0.04 M Tri s base, 0.02 M sodium acetate,

and 10 mM EDTA [pH 7.4]) on 8% acrylamide gel containing 25 to 50% denaturing gradient of formamide and urea. DGGE was conducted at 60°C for 5 h at 200 V. The gel was stained with SYBR Green I Nucleic acid gel stain (Cambrex Bio Science, Rockland, USA), photographed and analyzed for DGGE band profiles with a UV gel documentation system (Bio-Rad Laboratories, USA).

2.6 Sequencing of DGGE bands

Selected DNA bands of the DGGE of total community DNA were cut from the gels with a sterile scalpel and placed in Eppendorf tubes, soaked in TE buffer, boiled for 5 min, and used as template for PCR amplification. PCR of excised DNA fragments was performed using the same bacterial primers as described above but without the GC clamp. Amplification was proved by electrophoresis on 1% (w/v) agarose gel. Amplicons were directly sequenced using BigDye terminator cycle sequencing [36] at the Genoscreen sequencing facility (Genoscreen, Lille, France).

2.7 Sequence and phylogenetic analysis

16S rDNA sequences were analyzed by *Genetyx-Win* MFC application software version 4.0. The reference 16S rRNA gene sequences were retrieved from the GenBank database (National Center for Biotechnology Information, National Library of Medicine, USA). Sequences were compared with their closest matches in GenBank with nucleotide-nucleotide BLAST to obtain the nearest phylogenetic neighbors (www.ncbi.nlm.nih.gov/BLAST/). Multi-sequence alignments were performed by *ClustalW*, and phylogenetic trees were constructed with *MEGA 6* software (The Biodesign Institute) (http://www.megasoftware.net/) [37] using evolutionary distance and neighbor-joining method. Bootstrapping was performed on 1,000 replications of the alignments [38].

2.8 Numerical analysis of DGGE fingerprints

The DGGE fingerprints were analyzed using Quantity One 1D software (Bio-Rad, UK). Bacterial diversity was assessed using the Shannon–Weaver diversity index (H') [$H' = -\Sigma P_i (\ln P_i)$, where $P_i = n_i/N_i$] [39]. P_i is the relative intensity of DNA bands in the fingerprint, n_i is densitometrically measured intensity of individual DNA bands, and N_i is the total amount of DNA in the fingerprint.

Table 1 Local samples sites and soil contamination type of the collected samples from area around Almadina Almunawarah in the KSA

Area	No.	Sampling site	Location (coordinates)	Description
Al Meqat	1	Omar Ibn Al-Khatab road	24°25′29″ N, 39°32′49″ E	Lubricating oil-contaminated
	2	Omar Ibn Al-Khatab road	24°25′23″ N, 39°32′43″ E	Lubricating oil-contaminated
Yanbu road	3	Yanbu road	24°21′56″ N, 39°31′54″ E	Petroleum-contaminated soil
	4	Yanbu road	24°21′55″ N, 39°312′50″	Petroleum-contaminated soil
Al Azizia	5	Alemam Albukhari	24°26′56″ N, 39°31′51″ E	Tar-contaminated soil
Industrial area	6	Al-Hijrah road	24°23′28″ N, 39°29′56″ E	Diesel oil-contaminated soil
	7	Al-Hijrah road	24°23′25″ N, 39°29′59″ E	Diesel oil-contaminated soil
	8	Al-Hijrah road	24°23′16″ N, 39°30′7″ E	Lubricating oil-contaminated
	9	Al-Hijrah road	24°23′5″ N, 39°30′15″ E	Lubricating oil-contaminated
Yanbu city	10	Yanbu	23°53′42″ N, 38°23′21″ E	Petroleum-contaminated soil
	11	Yanbu	23°59′17″ N, 38°15′56″ E	Petroleum-contaminated soil
Tabok road	12	Tabok road	24°30′53″ N, 39°31′38″ E	Tar-contaminated soil
	13	Tabok road	24°30′54″ N, 39°31′13″ E	Tar-contaminated soil
	14	Tabok road	24°30′57″ N, 39°30′46″ E	Tar-contaminated soil
Al Barkah	15	Prince Naif Ibn Abdulaziz	24°31′1″ N, 39°33′59″ E	Tar-contaminated soil
	16	Prince Naif Ibn Abdulaziz	24°31′34″ N, 39°35′35″ E	Tar-contaminated soil
	17	Prince Naif Ibn Abdulaziz	24°31′35″ N, 39°35′40″ E	Tar-contaminated soil

2.9 PCR amplification of aromatic oxygenase genes

PCR was carried out using oligonucleotide primers specific for key oxygenase genes involved in both aromatic hydrocarbon and PAH biodegradation. Amplifications of catabolic genes were performed in 50 µl total reaction volumes containing: 5 µl of 10× *Taq* buffer (100 mM Tris-HCl, pH 8), 1.25 mM $MgCl_2$, 200 µMdNTPs (Invitrogen), 1.2 µM of both forward and reverse primers (Invitrogen), 1 U *Taq* polymerase (Invitrogen), and about 5 ng of template DNA. PCR was Performed with the Applied Biosystems 2720 thermal cycler. Primer sequences and PCR parameters used to amplify aromatic oxygenase genes are summarized in Table 2. Amplified genes were electrophoresed on a 1% (w/v) agarose gel with size markers (DNA 1 Kb Ladder; Promega, USA) and visualized using ethidium bromide.

2.10 Nucleotide sequence accession number

The 16S rDNA sequences identified in this study were deposited in the GenBank database under the accession numbers: AB936786 to AB936796.

Table 2 Primer sequences and PCR parameter used to amplify aromatic oxygenase genes in this study

Gene	Primers[d]	Primer sequence (5` - 3`)	Nucleotide length	Fragment size (bp)	Thermal profile[e]	Cycles	Reference
Catechol 1,2-dioxygenase	Cl2Of	GCCAACGTCGACGTCTGGCA	20	282	94°C (60s), 56°C (30s), 72°C (30s)	35	[40]
	Cl2Or	CGCCTTCAAAGTTGATCTGCGTGGT	25				
Catechol 2,3-dioxygenase	C23Of	AAGAGGCATGGGGGCGCACCGGTTCGATCA	30	380	94°C (60s), 56°C (30s), 72°C (30s)	35	[40]
	C23Or	CCCAGCAAACACCTCGTTGCGGTTGCC	26				
Catechol 2,3-dioxygenase-mt2[a]	*xylE*f	GTNYTNGGNTTYTAYYTNGCNGAR	24	280	92°C (40s), 53°C (50s), 72°C (60s)	35	[41]
	*xylE*r	NCKRTTNCCNSWNGGRTCRAA	21				
Toluene dioxygenase[b]	*todC*1f	CGGGTGGGCTTACGACACCGCCGGCAATCT	30	560	92°C (40s), 53°C (50s), 72°C (60s)	35	[41]
	*todC*1r	TCGAGCCGCGCTCCACGCTACCCAGACGTT	30				
Naphthalene dioxygenase	NAHf	CAAAA(A/G)CACCTGATT(C/T)ATGG	20	377	95°C (60s), 49°C (60s), 72°C (60s)	35	[42]
	NAHr	A(C/T)(A/G)CG(A/G)G(C/G)GACTTCTTTCAA	16				
Rieske iron sulfur center[c]	Rieske-f	AGGGATCCCCANCCRTGRTANSWRCA	26	78	94°C (30s), 48°C (30s), 72°C (30s)	35	[9]
	Rieske-r	GGAATTCTGYMGNCAYMGNGG	21				

[a] catechol 2,3-dioxygenase-mt2[a] encoding gene (*xylE*) from *P. putida* mt-2
[b] Toluene dioxygenase encoding gene (*todC1*) from *P. putida* F1
[c] Rieske iron sulfur center common to all PAH dioxygenase enzymes
[d] Forward (f) and reverse (r) primers are indicated
[e] Thermal profiles for PCR are presented as denaturation, annealing, and extension temperatures.

3. Results and Discussion

3.1 Physicochemical Properties of Soil

Significant differences in soil properties were found between hydrocarbon-contaminated soils and uncontaminated control soil. Physicochemical characteristics of contaminated soils in comparison with uncontaminated control soil were shown in Table 3. All soils had a pH ranging from 6.5 to 7. Four hydrocarbon-contaminated soils (S4, S5, S7 and S8) had higher total dissolved solids (TDS) than the control soil. Contaminated soils also showed higher amounts of nitrate, potassium, calcium, magnesium and chlorine compared with uncontaminated soil. On the other hand, control soil had higher phosphate content than contaminated soils. Total organic carbon ranged from 13 to 21% in contaminated soils. These results indicate that some basic soil properties might have been changed by different hydrocarbon contamination. **Peng *et al.*** [43] demonstrated that total nitrogen, total phosphorus and organic matter were significantly different in oil-contaminated soils exposed to different oil pollution.

3.2 Enrichment microcosms and initial screening

Laboratory-scale microcosms were established for enrichment of indigenous bacteria with the capability to degrade PAHs using naphthalene as a model compound. The success of microcosms in enriching microbial populations involved in the biodegradation process can be revealed by increasing turbidity and color change in microcosms. Biodegradation potential for naphthalene in established microcosms was revealed by the reduction in its concentration with time as monitored by GC/FID analysis Fig. 1 A, B. A change in color to yellow has been considered a preliminary evidence for biodegradation via extradiol *meta*-pathway. Coloration of the medium is usually due to the production of canonical ring cleavage products, hydroxymuconic

semialdehyde, during metabolic activity of endogenous bacteria *via* their oxygenase reactions. It has been reported that extradiol ring cleavage dioxygenases can be easily identified by the presence of such yellow-colored products [44]. Hydroxymuconate semialdehyde is a precursor for downstream compounds that fed eventually to the tricarboxylic acid cycle.

Naphthalene as a model compound was used to enrich PAH-degrading bacteria prior to detection of their catabolic genes. Naphthalene-amended microcosms showed higher turbidity and more intense coloration than unamended microcosms. It is therefore recommended to establish enrichment microcosms prior to PCR detection of catabolic genes as a convenient method for increasing the abundance of the degrading microorganisms and their target genes [45].

Table 3 Physical and chemical characteristics of soils considered in this study

Parameters	Soil samples				
	Control	S 4	S 5	S 7	S 8
Contamination	Uncontaminated	Petroleum-contaminated	Tar-contaminated	Diesel oil-contaminated	Lubricating oil-contaminated
pH	6.8	6.75	6.7	6.65	6.75
TDS (mg/L)	425	10300	10550	11350	11780
NO_3 (mg/L)	116	947.5	883.7	911.4	961.2
PO_4 (mg/L)	325	235	215	224	238
K^+ (mg/L)	39	325	412	303	338
Ca^{2+} (mg/L)	42.8	3120	2867	3026	2637
Mg^{2+} (mg/L)	37.72	211.2	202.4	164.7	197.3
Cl^- (mg/L)	120	5600	3200	3450	4200
Organic carbon (%)	5.27	13.1	17.5	21.3	16.7

TDS , total dissolved solids

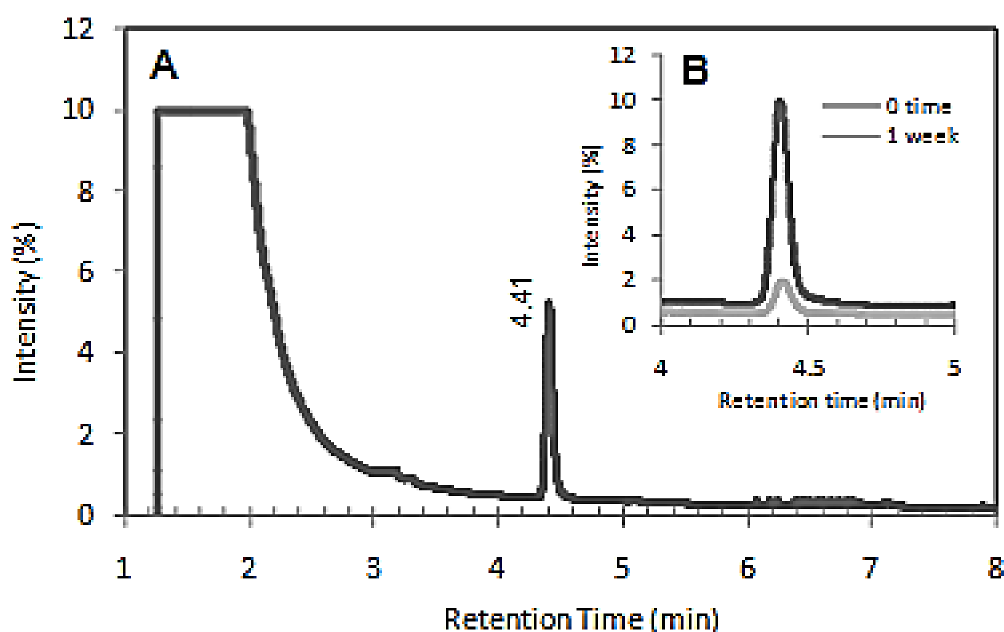

Fig. 1 PAH-biodegradation potential in established microcosm (soil S4). GC/FID chromatogram of authentic naphthalene sample eluted at 4.41 min (A). GC/FID chromatogram showing the time dependent degradation of naphthalene after one week incubation (B)

3.3 Profiling and dynamics of bacterial communities in contaminated soils

Analysis of 16S rRNA genes using DGGE represents a prevailing tool to investigate bacterial populations in native environments and/or laboratory microcosms [35, 46]. It is also possible to profile microbial community structure by constructing and sequencing the clone libraries of specific catabolic genes [47]. The time-dependent change in bacterial community structure for selected unamended soil samples contaminated with different hydrocarbons was analyzed using DGGE of PCR-amplified 16S rRNA genes Fig. 2A. Sequenced DGGE bands were identified after BLAST sequence alignments Table 4. Community fingerprints of the non-PAH-treated soil showed that there were no major changes during the first month of incubation. The bacterial profiles of the original unamended soils revealed the general dominance of *Pseudomonas* spp., *P. pseudoalcaligenes*, *P. nitroreducens*, and *P. toyotomiensis*. In addition to pseudomonads C*ellulosimicrobium* sp. was also detected in unamended soils. Pseudomonads are known for their distribution in petroleum hydrocarbon contaminated soils due to their biodegradation potential. [18]. However, some soils may have different community structures due to biogeographic characteristics. **Peng *et al.*** [43] demonstrated that petroleum-contaminated soils are dominated by *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *chloroflexi*, *Planctomycetes*, and *Proteobacteria*.

The community fingerprints observed for contaminated soil samples were different to those obtained from uncontaminated soils. Microbial community structure of one unpolluted soil in Almadina Almunawarah showed a predominance of *Photobacterium* sp., *Allomonas* sp., *Cellulomonas* sp., and uncultured members as major bacterial groups [48]. Uncontaminated arid soils in Almadina Almunawarah were also found to be dominated by members of *Pseudomonas, Bacillus,* and *Enterobacter* spp. [49]. Therefore, contamination induced a change in the microbial populations, enriching for those with the potential to biodegrade the corresponding contaminant.

Microbial populations in polluted soils or sediments have been studied [50,51]. Degrading populations were frequently affiliated with the genera *Sphingomonas*, *Polaromonas*, *Burkholderia*, *Pseudomonas*, *Mycobacterium*, *Nocardia*, and *Rhodococcus* [52].

The dynamics of bacterial communities in response to PAH pollution were analyzed Fig. 2B. The PAH-treated soils showed no drastic changes over time. In contrast, significant changes over time were observed when comparing aged non-PAH-treated soil with PAH-treated soils. Bacterial communities flourished over a relatively long period. PAH-amended soil samples S4, S5 and S7 were dominated by *Pseudomonas* sp., *P. pseudoalcaligenes*, *P. nitroreducens*, *P. toyotomiensis* and *Cellulosimicrobium* sp. Soil sample S8 was dominated by uncultured *Pseudomonas* sp., *P. mendocina* and *Stenotrophomonas* sp. This suggested that microorganisms could adapt and flourish under the enrichment conditions [53].

Bacterial community structure changes were monitored for soil samples amended with the model PAH naphthalene. After 6 months, some changes in the bacterial populations were observed. The bacterial profile in sample S4 was found to be conserved and almost no changes in the bacterial community structure were observed. A similar situation was found for sample S5, except that one uncultured bacterium appeared over time. Bacterial community structure changes were evident in sample S7 where *P. pseudoalcaligenes*, *P. nitroreducens,* and *Pseudomonas* sp. disappeared from the community and a new bacterial species, *Sphingobacterium* sp. Appeared Fig. 2, Table 4. A previous report has shown the successful recovery of previously non-cultivated bacterial strains after the addition of specific growth supplements to the media [54]. Naphthalene-amended soil sample S8, which was dominated by uncultured *Pseudomonas* sp., showed a remarkable change in its bacterial profile with prolonged incubation time. The bacterial community in Sample S8 after enrichment was dominated by *Pseudomonas* sp., *P. pseudoalcaligenes*, *P. nitroreducens*, and *P. toyotomiensis.*

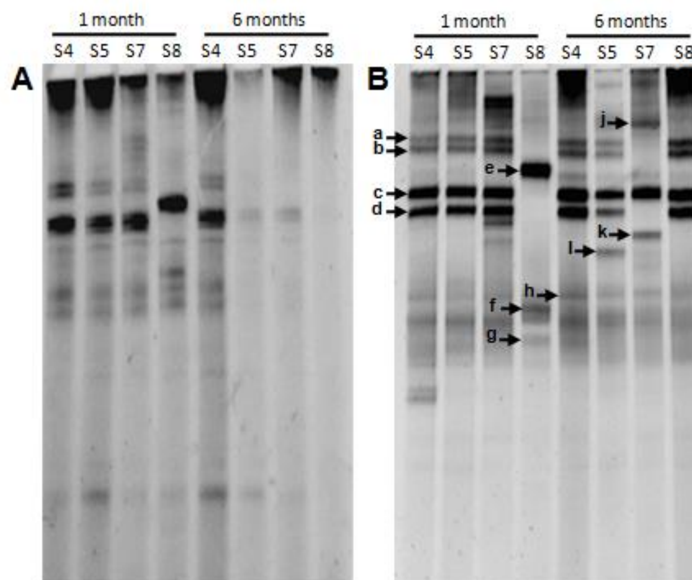

Fig. 2 Denaturing gradient gel electrophoresis of PCR-amplified 16S rRNA genes from unamended (A) and polycyclic aromatic hydrocarbon (PAH)-amended (B) microcosms. The time of sampling (months) is indicated above the lanes

Table 4 Identification of the 16S rDNA sequences from DGGE bands from NCBI database with the highest similarity to each band

Bands	source	Accession No.	Closest matches		
			Identity	Similarity (%)	Accession No.
A	Petroleum-contaminated soil	AB936786	*Pseudomonas pseudoalcaligenes* XY4	100	KJ174592.1
B	Petroleum-contaminated soil	AB936787	*Pseudomonas nitroreducens* VITWW2	100	KJ146071.1
C	Petroleum-contaminated soil	AB936788	*Pseudomonas toyotomiensis* AO 0020	100	KF984313.1
D	Petroleum-contaminated soil	AB936789	*Pseudomonas* sp. FSGRN7	99	KJ200412.1
E	Lubricating oil-contaminated soil	AB936790	Uncultured *Pseudomonas* sp. HHG10	100	GU565252.1
F	Lubricating oil-contaminated soil	AB936791	*Stenotrophomonas* sp. SO5.1	100	KC859435.1
G	Lubricating oil-contaminated soil	AB936792	*Pseudomonas mendocina* PM2011AH01	99	KJ150296.1
H	Petroleum-contaminated soil	AB936793	*Cellulosimicrobium* sp. H.p10	100	KJ024067.1
I	Tar-contaminated soil	AB936794	Uncultured bacterium clone 7	99	FJ459826.1
J	Diesel oil-contaminated soil	AB936795	*Sphingobacterium* sp. TSAR21	100	KC854376.1
K	Diesel oil-contaminated soil	AB936796	*Pseudomonas* sp. 9-31	100	HF954515.1

3.4 Diversity and phylogenetic analysis of DGGE phylotypes

The Shannon diversity index is an important parameter for estimation of bacterial diversity in environmental samples. The Shannon index (*H`*) was applied as a compound index to compare time-dependent changes in community diversity in unamended and PAH-amended microcosms. In general, species richness is known to be relatively higher in contaminated soils than uncontaminated ones [43].

The bacterial community in unamended microcosms showed a diversity index ranging from 0.99 to 1.9 Fig. 3A, B. Except for one soil sample (S4), extended incubation time resulted in a gradual decrease in the diversity index (1.7 to 0), as revealed by the deterioration of community structure with time. PAH-amended microcosms showed almost no change in diversity with time. The diversity indexes for amended, one month- and six month-incubated microcosms ranged from 2.02 to 0.6 and from 1.7 to 1.27, respectively. These values suggest that PAH amendment might result in a conservation of the community structure for those community members involved in the biodegradation process.

Phylogenetic analysis of the detected bacterial species in contaminated soils was performed. The phylogenetic

tree based on 16S rRNA gene sequences showed the relationship between the detected DGGE phylotypes and closely related representative bacterial members Fig. 4. Phylogenetic studies confirmed the affiliation of DGGE phylotypes a–e and g to the *Pseudomonas* spp. group. Phylotypes i and k were affiliated with uncultured
Pseudomonas spp. Phylotype f was clustered with the genera *Xanthomonas* and *Stenotrophomonas*. Phylotype h was clustered with the same phylogenetic branch as *Actinobacteria*, while phylotype j was clustered with the *Sphingobacterium* group.

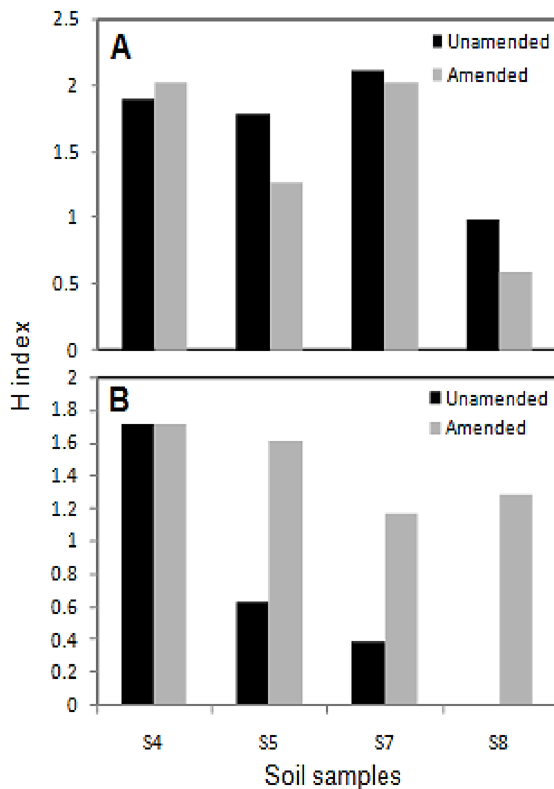

Fig. 3 Shannon diversity index (*H*`) for PAH-amended and unamended microcosms after one month (A) and six months (B) of incubation

3.5 Detection of aromatic oxygenase genes

Adaptation of bacteria to hydrocarbon contamination can be monitored by using specific DNA probes targeting hydrocarbon-catabolic genes. Catabolic genes responsible for the aerobic biodegradation of many hydrocarbons are targeted towards the initial oxidation of hydrocarbons, such as naphthalene dioxygenase or ring cleavage, such as catechol dioxygenase [55]. Generally, the enzymes in the upper pathway hydroxylate the aromatic compounds to corresponding diols via the action of a monooxygenase or dioxygenase [56], resulting in the dihydroxy compound. After that, they are subjected to ring cleavage dioxygenases in the lower pathway forming ring-cleavage products.

In this study, PCR primer sets were selected for amplification of major catabolic genes involved in aromatic metabolism including different aromatic oxygenases. The primers were selected to cover key steps in the upper and lower aromatic pathways. To cover up the upper pathway, naphthalene dioxygenase (NAH), toluene dioxygenase (*todC1*), and Rieske-type clusters encoding fragments common to most dioxygenases were selected. Oxygenase genes have been identified and isolated from a number of polluted soils [57]. Catechol 1,2-dioxygenase (C12O) and catechol 2,3-dioxygenase (C23O, *xylE*) were selected to cover the lower pathway. PCR results showed that the C23O primer set allowed amplification of extradiol ring cleavage dioxygenase genes from all soils amended with naphthalene Fig. 5. In unamended soils, C23O primers were able to amplify dioxygenase gene fragments from some soil samples but not all. Likewise, the NAH primer set amplified naphthalene dioxygenase gene fragments from some amended soils but not all. Naphthalene degradation genes, including *nah, pah, ndo* and *dox* operons, are highly conserved in some soil pesudomoads [58]. Although the NAH gene was regarded as one of the main marker genes used to detect bacteria involved in PAH biodegradation, it was not possible to detect it in unamended soils most properly because they are under the detection limits. When stimulated, it could be detected easily. Moreover, the *xylE* gene, encoding catechol 2,3-dioxygenase of toluene and methyl benzene derivatives, could not be detected in most of the soils in this study, especially when naphthalene was added as sole carbon source.

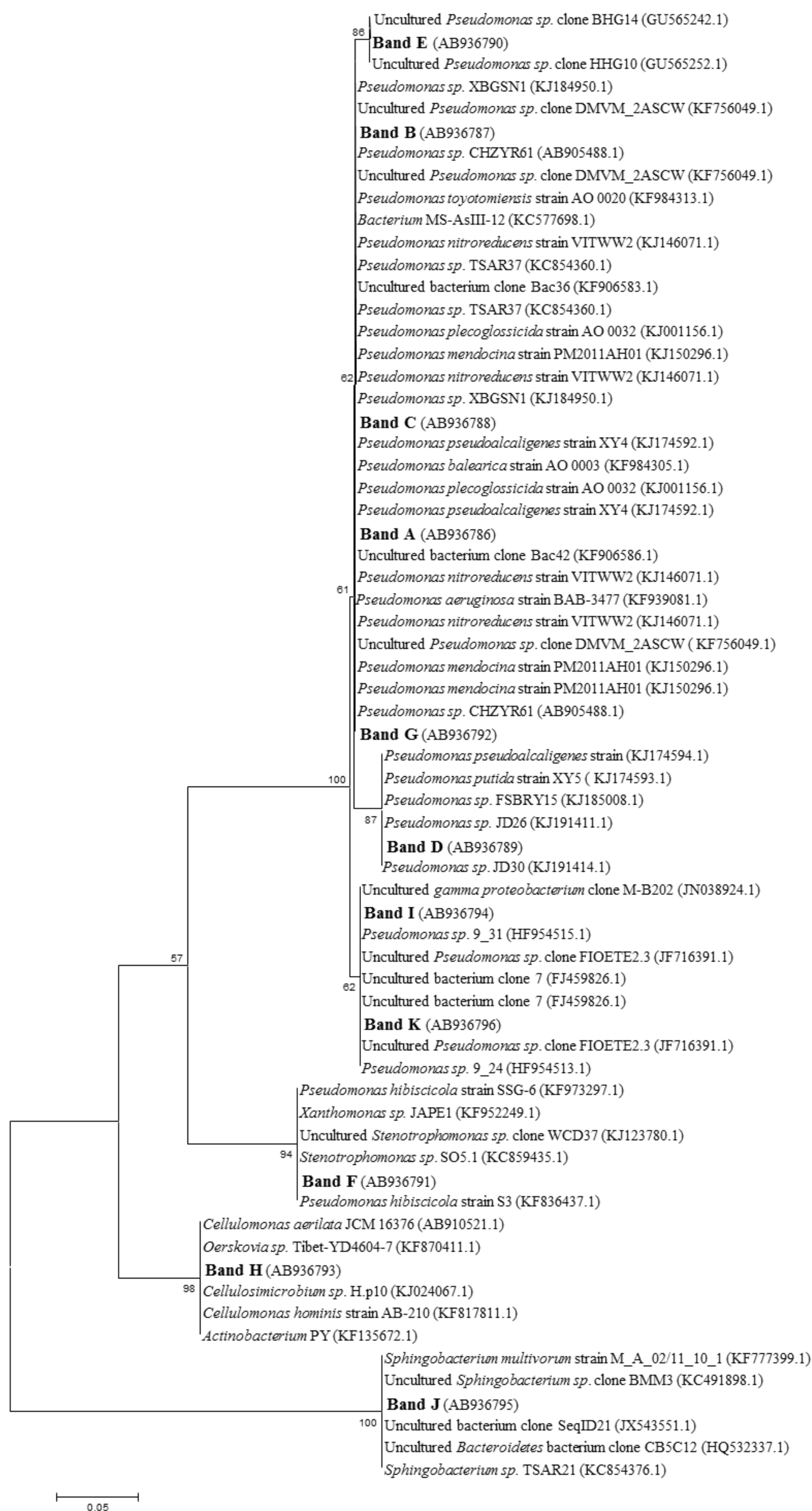

Fig. 4 Neighbor-joining phylogenetic tree showing the relationship between detected bacteria from contaminated soil samples and closest matches from GenBank data base. The bar represents 0.05 substitutions per site. The phylogenetic tree was generated using MEGA 6 software, bootstrap values (*n* = 1000) are displayed

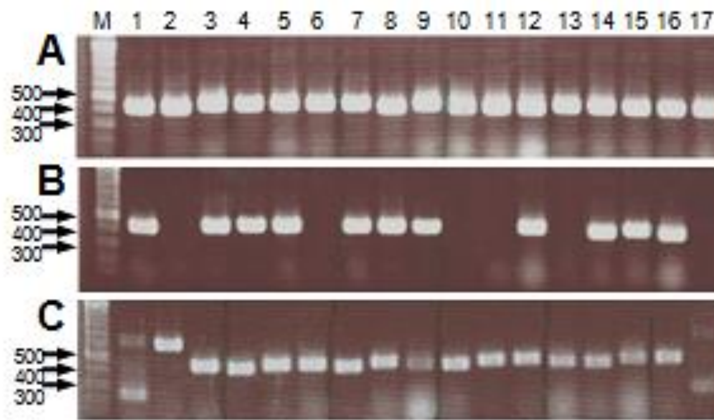

Fig. 5 PCR amplification of catabolic genes from amended and unamended microcosms. PCR amplification of DNA fragments corresponding to naphthalene dioxygenase directly from unamended microcosm (A); PCR amplification of DNA fragments corresponding to catechol 2,3-dioxygenase (C23O) directly from unamended microcosm (B); and after enrichment with naphthalene (C). Lane M represents molecular weight size marker (DNA 1 Kb Ladder). Non-specific amplifications were considered negative

3.6 Evaluation of catabolic genes as markers for PAH biodegradation

Degradation of complex PAH mixtures is naturally mediated by extensive microbial diversity. Molecular markers can be used to profile the degradative activities of microbial communities. **Kappell *et al.*** [15] found that functional genes involved in oil and PAHs degradation were abundant in oil-contaminated sand beach, suggesting that biodegradation was ongoing. Naturally occurring bacterial isolates capable of catabolizing PAHs have received considerable attention because of the environmental persistence and toxicity of PAHs. Microbial populations are dynamic and are therefore not easily characterized. It has been reported that functional genes could be useful not only for detecting certain microbes but also for monitoring changes in community structure of contaminant-degrading bacteria in microcosms [59]. The accuracy and assessment of this step is very important for successful biodegradation. Characterization of aromatic catabolic pathways has led to a selection of catabolic genes as candidate markers to reveal the biodegradation potential of contaminated environments. The amplification potential and dominance of catabolic genes in various contaminated soils was shown in Fig. 6. The PCR amplification potential for catechol dioxygenase [60] and naphthalene dioxygenase [55] catabolic genes in the studied contaminated soils was evaluated. The C23O primer set was able to amplify specific DNA fragments of 380 bp from all the contaminated soils. These primers were designed from conserved regions of the C23O gene from different species of hydrocarbon-degrading bacteria [40]. Detection of C23O genes in test soils revealed the over distribution of corresponding extradiol ring cleavage dioxygenases in the studied petroleum-contaminated soils. The other gene studied was the *xylE* gene, which encodes catechol 2,3-dioxygenase. Specific primer sets for the *xylE* gene were not able to amplify this gene from the soils in this study. This could be explained by the absence of appropriate substrates (toluene, benzene, or methyl benzene derivatives), especially in the enrichment cultures. Therefore, C23O was superior to *xylE* for detecting PAH catabolic potentials. However, negative PCR amplification of C12O indicated the limitation or absence of intradiol ring cleavage dioxygenases in the studied contaminated soils. Genes extensively used as indicators of PAH degradation are those encoding naphthalene biodegradation in *Pseudomonas* spp. (*nah*-genes of the NAH plasmid). Naphthalene dioxygenase genes have been detected in a range of environments especially in polluted sites [28,29]. Abundance and diversity of naphthalene dioxygenase genes in oil-contaminated soils has been studied in some oil fields like the Shengli oil field, China [26]. NAH primers targeting naphthalene dioxygenase were successful in amplifying the corresponding DNA fragments of the expected size in enrichment cultures amended with naphthalene. In contrast, no NAH PCR product was obtained from unamended contaminated soils.

Results indicated that the NAH gene was useful as a marker gene. Enrichment microcosms should be established prior to PCR detection, as the inclusion of naphthalene in enrichment microcosms would lead to an increase in the corresponding naphthalene degraders. It has also been shown that in some PAH-contaminated soils and sediments, expression of naphthalene catabolic genes was positively correlated with naphthalene concentrations [45]. Rieske-type primers amplified target fragments in some amended contaminated soil samples. *XylE* and *todC* were not appropriate for use as marker genes to monitor PAH biodegradation in this study.

These results suggested that C23O and NAH primers could be used as a detection system for PAH biodegradation in petroleum-contaminated soils. Archetypical naphthalene and catechol dioxygenase genes were found in two *Pseudomonas* strains isolated from contaminated sediments, suggesting biodegradation potential in these sediments [7]. It was also concluded that the biodegradation potential of PAHs in contaminated soils occurs through dioxygenases and further metabolism via extradiol *meta* pathways revealed by the accumulation of the canonical, yellow-colored intermediates in established microcosms characteristic for such type of activity.

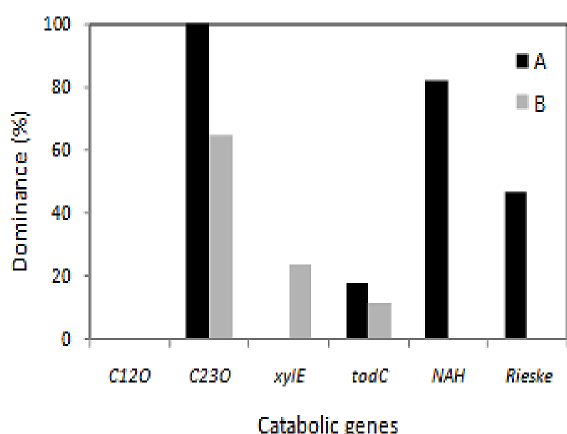

Fig. 6 Evaluation of catabolic genes as markers for PAH biodegradation revealed by PCR detection of major catabolic genes in contaminated soils

4. Conclusion

Saudi Arabia has the largest proven crude oil reserves in the world and therefore incidence of oil pollution is much frequent. Bioremediation is one of the main strategies for treatment of various forms of oil pollution problems in Arabian Gulf area in general and KSA in particular. The present study demonstrated that the soil investigated at Almadina Almunawarah, Yanbu city, KSA harbored hydrocarbon-degrading bacterial populations that could be biostimulated to achieve effective bioremediation of petroleum hydrocarbon contamination. Community fingerprints of contaminated soils in Almadina Almunawarah, Yanbu city, showed the predominance of a variety of *Pseudomonas* spp. along with *Cellulosimicrobium* sp. and *Stenotrophomonas* sp. Soils amended with PAHs showed a conservation and selection of specific microbial communities mainly involved in biodegradation processes. Naphthalene-amendment generally resulted in the predominance of several pseudomonads such as *Pseudomonas* sp., *P. pseudoalcaligenes*, *P. nitroreducens*, and *P. toyotomiensis* and recovery of novel species like *Sphingobacterium* sp. Hydrocarbon-contaminated soils in Almadina Almunawarah, Yanbu city harbored the archetypical naphthalene dioxygenase (NAH) and catechol 2,3-dioxygenase (C23O) encoding genes. These catabolic functional genes were proved to be useful markers for monitoring PAH biodegradation at petroleum-contaminated.

5. Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6. Acknowledgments

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