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Isolation and Identification of Indigenous Bacteria for the Degradation of Polycyclic Aromatic Hydrocarbons Found in Refinery Wastewater

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ABSTRACT: Polycyclic aromatic hydrocarbon (PAH) degrading bacteria were screened and isolated from contaminated soil taken from Tema Oil Refinery wastewater effluent channels. Refinery wastewater was found to contain a low amount of inhibitory heavy metals but the presence PAHs were detected and identified. The PAH-degrading bacteria isolated and identified were *Pseudomonas stutzeri. Acinetobacter junii, Pseudomonas putida and Stetonotrophomonas nititireducens*. All four bacteria showed high tolerance to a PAH combination of Naphthalene and Anthracene (1:1) of concentration as high as 5mg/ml. The identification of *S. nititreducens* as an indigenous bacterium is very significant since it is one of the very few bacteria known to be able to degrade high molecular weight PAHs.

KEYWORDS: Bacteria, Isolation, Identification, Indigenous, Polycyclic aromatic hydrocarbons.

1.0 INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are a group of several hundred chemically related, environmentally persistent and pervasive compounds with various structures and toxicity [1]. They are made of two or more fused aromatic rings produced through thermal decomposition, incomplete combustion and pyrolysis of different kinds of organic substances [2]. Though PAHs can be released into the environment through natural means such as volcanoes, anthropogenic activities from the petrochemical industry also release PAHs into the environment [3]

The presence of PAHs in wastewater and soil is of great concern since most of them are carcinogenic, mutagenic, immunotoxic and teratogenic. The impact of wastewater on the microbial ecosystems of water bodies, in this case the ocean is important. This is because such impacts can be negative which include a decrease in biodiversity, decrease in oxygen levels, release of toxins and promoting harmful algae growth [4]. On a macro scale, the introduction of PAHs into wastewater can cause a reduction in water quality which will damage aquatic life and cause a public health risk [5]. Industrial activities especially in the petrochemical industry produce copious amounts of solid, liquid and gaseous waste which all contain different types and concentrations of PAHs. Because petroleum refineries consume a lot of water, they produce a lot of wastewater, which needs to be treated to remove pollutants like PAHs before being released, mostly into aquatic bodies [6]. The usual treatment methods of wastewater include physical, chemical, biological or a combination of any of these three methods [7,8].

Several studies have shown that local microbes such as bacteria, fungi, algae and archaea present in wastewater and soil sediment which have been exposed to PAHs have developed the ability to degrade PAHs through either aerobic or anaerobic means by using enzymes such as peroxidase, mono-oxygenase, di-oxygenase and laccase [9].

A number of bacteria have been identified to be able to degrade PAHs, especially in the low molecular weight (LMW) spectrum. The most effective bacteria community found to be able to degrade efficiently is the gram-negative bacteria community. These include *Pseudomonas putida, Stenothrophomonas maltophilia, Acinetobacter calcoaceticus and Bacillus thuringiensis* [10,11]. Even though a lot of success has been achieved in identifying PAH-degrading bacteria and their degradation pathways, enzymes and specific genes responsible for the process [12], very few microbes have been found to withstand high concentrations of PAHs and also degrade High Molecular Weight (HMW) PAHs [13].

This paper seeks to screen, isolate, identify and classify various indigenous bacteria capable of degrading PAHs after such bacteria have been exposed to wastewater for several years from the local refinery. The isolated bacteria are also evaluated for their tolerance to high concentrations of PAHs.

2.0 MATERIAL AND METHODS

2.1 Sample Collection

Wastewater samples were collected at the wastewater effluent channel of Tema Oil Refinery, Heavy Industrial Area, Tema using the grab method as described by the American Public Health Association standard methods [14]. The effluent wastewater gets discharged into the ocean through the Tema municipal sewage system. Composite soil samples were taken from three different sites along the wastewater effluent channel, at about 50-meter intervals between the sites.

2.2 Physicochemical analysis

A properly calibrated multiparameter water analyzer (Hanna H19289) was used to measure the physicochemical properties of the refinery wastewater. The measurement was made at the point of high velocity (rapid section) of the effluent at a depth of 0.50 meters. The probe was inserted into the running wastewater once the analyzer had been calibrated. All parameters were taken on the site and in triplicate. The parameters that were measured include pH, temperature, salinity, resistivity, turbidity, atmospheric pressure, oxidation-reduction potential and GPS location.

2.3 Heavy metals analysis

Using standard methods according to [14], 25 mL of the refinery wastewater was digested in 5 mL of concentrated HNO_3. The digested samples were diluted in HNO_3:$HClO_4$ (10 mL each at a ratio of 1:1 v/v). The digested samples were gently evaporated to dryness and then allowed to cool. The cooled samples were then diluted with 50 mL of deionized water and boiled to expel any chlorine and oxides of nitrogen. The samples were then filtered using glass fibre filters. The filtrates were transferred to 100 mL volumetric flasks where they were diluted to the mark.

The samples were then analyzed for the presence of various heavy metals using inductive coupled plasma–optical emission spectroscopy (PerkinElmer's Avio 560Max ICP-EOS). The wavelengths of the metals analyzed by the ICP-EOS were: Cd(288.80nm), Cu(324.75nm), Ni(232.0nm), Cr(375.9nm) and Zn(213.86nm)

2.4 Extraction of PAH and GC-MS analysis

Wastewater (500 mL) samples were extracted with 20 mL n-hexane (HPLC grade, SRL, India) and dichloromethane (DCM) (HPLC grade,) using a liquid-liquid partition gravimetric extraction procedure according to the USEPA method 5520B [15]. Homogenized wastewater samples were extracted by shaking vigorously in a separating funnel three times, each time using 20 mL n-hexane (HPLC-grade). The solvent layer was collected in a 100 mL amber-coloured bottle after separation from the water phase. In the case of DCM, acidic and basic fractions were collected by extracting the water samples at pH = 2 and pH = 11 respectively. PAHs were extracted from the composite soil sample using accelerated solvent extraction. 100 mL acetone was used as the extraction solvent. The extracts were evaporated to dryness using a rotary evaporator and re-constituted in 2 mL of respective solvents and transferred to GC vials for analysis.

GC-MS analysis of the samples was performed using a PerkinElmer GC Clarus 580 Gas Chromatograph interfaced to a Mass Spectrometer PerkinElmer (Clarus SQ 8 S) equipped with ZB-5HTMS (5% diphenyl/95% dimethyl polysiloxane) fused capillary column (30 × 0.25 μm ID × 0.25 μm DF). Helium gas was used as a carrier gas at a constant flow rate of 1ml/min, and an injection volume of 1μl was employed. The injector temperature was maintained at 250°C, the ion-source temperature was 220 °C. The mass detector used in this analysis was Turbo-Mass, and the software adopted to handle mass spectra and chromatograms was Turbo-Mass ver-6.1.0. Interpretation of mass-spectrum GC-MS was conducted using the database of the National Institute of Standard and Technology (NIST) having more than 62,000 patterns.

2.5 Bacteria enumeration and screening of PAH-degrading bacteria

Bacteria populations were enumerated by plating a serial dilution of the soil samples on nutrient agar. Samples from each site were serially diluted using 1X Phosphate Buffer Saline (PBS) and plated from the stock solution to achieve 10^{-4} dilution. Plates were then incubated for 24hrs at 27°C. Plates with bacteria colonies that exhibited between 30 and 300 colonies were selected to be purified. Colonies with different morphology were selected from dilution 10^{-4} plates, plated in freshly prepared nutrient agar plates, and then incubated at 27°C for 24hrs [16].

PAH-degrading bacteria were screened for through aerobic enrichment using a minimum salt agar (MSA) supplemented with 4 mg of Anthracene per plate as the sole carbon and energy source. The MSA contained 1g $(NH_4)_2SO_4$, 0.1g $MgSO_4$, 0.5g $Na_3C_6H_5O_7$, 2g

KH_2PO_4, 7g K_2HPO_4 and 14g Agar per litre. The MSA medium was adjusted to a final pH of 7.00 by using 0.1M HCl solution. Each plate was amended with 1mL of 4mg/mL of Anthracene using acetone as solvent. The plates were left open in a sterile biosafety cabinet for 10 minutes for the acetone to evaporate leaving 4mg of Anthracene on each plate. Each isolate was picked and streaked on an MSA plate amended with Anthracene, this was repeated for all the pure isolates. The plates were incubated at 30ºC for 8 days in the dark.

2.6 Tolerance of bacteria to polycyclic aromatic hydrocarbons

Using Naphthalene and Anthracene, bacteria isolates were tested for their tolerance to PAH by a modified version of plate assay [3]. Sterile filter discs of 6mm diameter were impregnated with a PAH mixture of Naphthalene and Anthracene (1:1) containing final concentrations of 0.5, 1, 2, 3, 4 and 5mg/disc. 100µl of each bacterial culture (OD 0.14) were spread onto an MSA plate with 2% glucose and the discs of each PAH concentration were placed onto the plate. Plates were then incubated at 37ºC for 72hrs and radii of the growth inhibition zones were measured. Discs with acetone (without PAHs) were used as a control.

2.7 Bacteria identification and classification

Fresh cultures were prepared for gram staining on nutrient agar. Using a modified version of the technique by [17], all the PAH-tolerant bacteria were gram stained. The slides were observed under a compound microscope at a magnification of 1000X to determine the gram type, shape and arrangement. All PAH-tolerant bacteria isolates were identified using Matrix-Assisted Ionization Time of Flight (MALDI-TOF). 1µl of 70% formic acid and 1µl of extraction matrix were used to prepare the bacteria samples on a MALDI_TOF plate. The inoculated plate was then placed in the Bruker Biotyper and analyzed using MALDI-TOF to obtain organism profiles.

3.0 RESULTS AND DISCUSSION

3.1 Physicochemical and Heavy Metal Analysis of Wastewater

The physicochemical parameters and heavy metal analysis are shown in Table 1 and Figure 1 and Figure 3. The results from these two analyses were compared with World Health Organization (WHO) standards [18] and similar studies conducted by other researchers.

Table 1 Physicochemical properties of Tema Oil Refinery wastewater

	STUDY	OSUOHA	WHO
Temperature (ºC)	30.34±0.017	26.45±0.19	30
pH	7.64±0.021	7. 82 ± 0.02	6.5-9.5
Turbidity (NTU)	5.64±0.001	3.49 ± 0.01	5.82
Total Suspended Solids (mg/L)	19.17±0.001	15.09±0.18	30
Salinity (mg/L)	24.21±0.027	29.36±0.87	N/A
Dissolved Oxygen (mg/L)	1.89±0.015	4.27±0.18	4-5
Conductivity (µS/cm)	191.70	140	500

The physicochemical parameters were found to be either below the WHO limits or within the range given by WHO. It was also comparable to another oil refinery wastewater analysis study conducted [19]

This study revealed through ICP-OES analysis that the Tema Oil Refinery effluent wastewater is contaminated with heavy metals. The heavy metals include Copper (Cu), Nickle (Ni), Chromium (Cr), Cadmium (Cd) and Zinc (Zn). The concentration of these heavy metals Cu, Ni, Cr, Cd and Zn is 0.272mg/L, 0.0059mg/L, 0.003mg/L, 0.002mg/L and 0.0665mg/L respectively. The presence of heavy metals may be inhibitory to bacteria growth [20] and also toxic to humans, animals and aquatic organisms [21]. In comparison to the World Health Organization (WHO), standards the concentration of these heavy metals fell way below the recommended upper limit set by WHO as shown in Figure 2. Similarly heavy metal presence was also detected by [8,22] when they investigated the effluent channels and vicinity of the Barzian Oil Refinery, Iraq and the Muthura Oil Refinery, India respectively.

The concentration of heavy metals in the current study is much lower than that of the other two studies as well (Figure 2) probably because the refinery was not in active operation at the time the samples were taken. The type of crude processed at each of these refineries may also be different. PAH-degrading bacteria found in places with high concentrations in most cases have resistance to heavy metal inhibition [20]. Heavy metals seep into agricultural land and aquatic bodies causing an accumulation of these metals in humans and resulting in heavy metal poisoning [23].

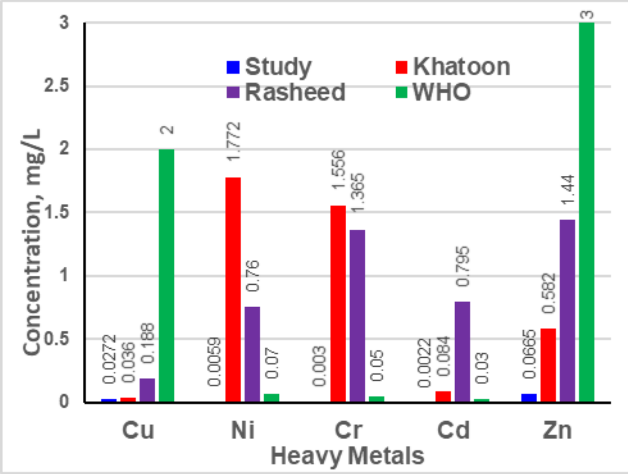

Figure 1: Heavy metal concentration in mg/L compared to WHO and other studies.

3.2 Extraction of PAH and GC-MS analysis

Due to its sensitivity, rapidity, versatility, affordability, and capacity to distinguish between a variety of compounds in a combination, GC-MS is one of the technologies that can be used to identify organic chemicals in environmental samples. Reference [24] which in this case is refinery wastewater. The mass spectra from the GC-MS analysis of the extracted organic compounds from the refinery wastewater at particular retention times were compared to the National Institute of Standards and Technology (NIST) library [25]. The analysis showed several aliphatic and aromatic organic compounds. Some of these compounds found were acids, esters, phenols, PAHs, phthalates and others as documented in Table 2. This was in tune with other studies conducted by researchers on refinery wastewater. Khatoon and Malik [8] found several aliphatic and aromatic compounds such as naphthalene, xylene, tetradecane methyl-tetra butyl ether among others. Other studies [26,27] also found similar compounds in treated refinery wastewater which included both aliphatic and aromatic compounds such as m-Cresol, cyclohexanone, 2-Penton 2-Hexanone, 3-tert-butyl phenol among others.

Table 2 Compounds found in the water samples

EXTRACTION SOLVENT	COMPOUNDS IDENTIFIED
N-HEXANE (Water Sample)	Naphthalene Dodecane Tridecane 1-methyl-Naphthalene 1-ethylidene-1H-Indene Tetradecane 2,6,10,14-tetramethyl-Heptadecane Pentadecane 1-Hexadecanol Hexadecane 1-Nonadecene

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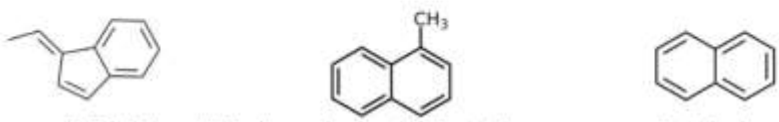
	Cyclic Octa atomic Sulphur 1-Decosene Stigmasterol
DICHOROMETHANE - Acidic Fraction (Water Sample)	P-Xylene Acetophenone Naphthalene Dodecane Tridecane Cyclic Octaatomic Sulfur 9-hexyl-Heptadecane Octadecamethyl-Cyclononasiloxane Tetratrecontane 1,4-Benzenedicarboxylic acid (3a)-Lup-20(29)-en-3-ol acetate 3-ethyl-5-(2-ethylbutyl)-Octadecane Docosonoic acid
DICHOROMETHANE - Basic Fraction (Water Sample)	Acetophenone Undecane Dodecane Tridecane Hexadecane Cyclic Octaatomic Sulfur Heneicosane 1-ethylidene-1H-Indene 2-methyl-2-phenyl-Oxirane 2,3-dimethyl-Phenol 2,4,6-trimethyl Iodine Pyrylium
N-HEXANE (Soil Sample)	Octadecamethyl-Cyclononasiloxane Squalene Hexanedioic acid Phthalic acid Bis(2-ethylhexyl) phthalate Cis-1,3-Dioxan-5-ol Prednisolone Hemisuccinate a-Amyrin

In this study, the PAHs that were identified in treated refinery waste water were Naphthalene, 1-methy-Napthalene and 1-Ethylidene-1H-Indene as shown in figures 2a, 2b and 2c. The low number of PAHs identified as compared to similar studies may be attributed to the nature of the crude processed, the complexity of refinery processes and the fact that the refinery was not in active operation at the time taking the samples. It is also possible that some of these compounds, even if present, might have already been degraded by the bacteria present.



a: 1-Ethylidene-1H-Indene *b: 1-methy-Napthalene* *c: Naphthalene*

Figure 2: PAHs identified

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3.3 Bacteria enumeration and screening of PAH-degrading bacteria

A total of 60 isolates were purified from all three sites, some of which are shown in Figure 3. Using 4g/mL of Anthracene per plate, thirty-five (35) PAH bacteria were isolated after 8 days in the dark at 30ºC with some of the isolates shown in Figure 3.

Figure 3: Pure culture from site one obtained after the second subculture from serial dilution (10^4) cultures

3.4 Tolerance of bacteria to polycyclic aromatic hydrocarbons

Due to the slow growth of some bacteria isolates only sixteen (16) bacteria isolates were used for the PAH tolerance tests. These were the only isolates that grew over 0.14 OD after 7 days of incubation under shaking conditions as shown in Table 3. The concentrations of the PAHs tested were 0.5 mg/disc, 1 mg/disc, 2 mg/disc, 3 mg/disc, 4 mg/disc, and 5 mg/disc.

Table 3. Optical densities of bacteria isolates at 600nm.

SITE	ISOLATES	OPTICAL DENSITY
SITE 1	UBS 1	0.006
	UBS 2	0.547
	UBS 4	0.018
	UBS 5	0.007
	UBS 6	0.014
	UBS 7	0.005
	UBS 8	0.01
	UBS 10	0.295
	UBS 11	0.304
	UBS 14	1.16
	UBS 16	0.303
	UBS 17	0.743
	UBS 20	0.007
SITE 2	UBS 22	0.113
	UBS 23	0.023
	UBS 24	0.365
	UBS 25	0.051
	UBS 27	0.483
	UBS 31	0.284
	UBS 34	0.004
	UBS 35	0.341
	UBS 36	0.128
SITE 3	UBS 41	0.335
	UBS 42	0.122
	UBS 43	0.271
	UBS 46	0.279

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	UBS 48	0.005
	UBS 49	1.697
	UBS 52	1.079
	UBS 53	0.001
	UBS 54	0.005
	UBS 55	0.479
	UBS 56	0.009
	UBS 58	0.003
	UBS 60	0.002

Figure 4: Screening Cultures of Site 3 after 8 days of incubation in the dark using Anthracene as the only carbon source

As shown in Figure 4, there was no measurable zone of inhibition for any of the bacteria isolates at any of the concentrations (0.5, 1, 2. 3, 4, and 5mg/disc). This shows that in this particular study, the isolated bacteria have a much higher tolerance to a PAH combination of Naphthalene and Anthracene even at a high concentrations of up to 5mg of PAH mixture per milliliter. From the control, it can clearly be shown that the acetone used as the PAH carrier had no effect on the growth of the bacteria isolates.
Zafra *et al* [3] reported tolerance levels up to 6 mg/mL using a consortium of several isolated bacteria and fungi. PAH-degrading bacterial populations in soil are subjected to organic compounds that are potentially toxic to these bacteria when they use these compounds as the sole carbon source [28]. The lack of these organotolerance PAH-degrading microbes plays a huge role in the persistence of PAHs in the environment, especially in contaminated soil and water. This can also be seen as a consequence of inhibited growth and metabolism, especially when these compounds have a high concentration of contamination [3].
Usually a mixture of four, five and six ring PAHs are used in this type of test since hydrocarbon contaminated soil contains a complex mixture of low molecular weight and high molecular weight PAHs [3]. This might also explains why no ring of inhibition were not found around any of the discs.

Figure 5: Nutrient Agar plates with PAH discs showing no zone of inhibition

3.5 Bacteria Identification and Classification

A total of 16 PAH-tolerant bacteria were isolated from the effluent channel soil of Tema Oil Refinery contaminated with wastewater from the facility. All 16 isolates were found to be gram-negative bacilli or cocci. Using Matrix-Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) spectrometry four distinct bacteria were identified from all three sites the soil samples were taken as shown in Table 5
The two pseudomonads namely *P. putida* and *P. stutzeri* gram-negative, non-spore-forming aerobic bacilli that are flagellate motile and non-fermenting. When cultured on solid media, *P. putida* exhibits a yellowish-green to yellow-brown colour while *P. stutzeri* exhibits a light yellow colour. *Acinetobacter junii* is a gram-negative coccobacillus, aerobic, non-fermenting and non-motile

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exhibiting an off white-colour when cultured on solid media. *Stetonotrophomonas nititireducens* are motile, aerobic gram-negative bacteria.

Table 4. All bacteria isolates identified using MALDI-TOF

SITE	ISOLATE	BACTERIA IDENTIFIED
	UBS 2	*Pseudomonas stutzeri*
SITE1	UBS 10	*Acinetobacter junii*
	UBS 11, UBS14, UBS16, UBS17	*Pseudomonas putida*
SITE 2	UBS 27, UBS31	*Pseudomonas stutzeri*
	UBS 41, UBS52, UBS 55	*Pseudomonas stutzeri*
SITE 3	UBS 43	*Acinetobacter junii*
	UBS 46	*Stetonotrophomonas nititireducens*

These bacteria are considered to be PAH-utilizing bacteria based on a mixture of anthracene and naphthalene being used as the sole carbon source. These species are among the known PAH-degraders previously isolated. Acinetobacter sp. from contaminated groundwater in a coal mining area have been isolated and reported by Avramova *et al* [29], Fernando Bautista *et al* [30] and Shao *et al* [31]. The low level of diversity in bacteria in this study may be attributed to the low levels of contamination from the sites where the samples were taken. The organic loads in the treated waste has been shown to be relatively low [32] and the refinery has not been operational for several months. It is also possible that most of the hydrocarbons (including aromatics) get removed during the refinery wastewater treatment which decreases the chances of isolating the bacteria population that have adapted to tolerate and degrade high concentrations of PAH.

PAH-degrading bacteria which are gram-negative and which belong to the gammaproteobacterial in several studies have been shown to dominate and have improved tolerance in the bacteria population present in contaminated soil [33,34]. *Pseudomonas sp*. in particular, has been found to degrade several types of PAH due to its high resistance to organic solvents [35]. Resistance against organic solvents and aromatic hydrocarbons generally involves turning these into harmless molecules, the rigidity of cell walls, and cell surface impermeability. Other transformation mechanisms include the presence of constitutive and inducible efflux pumps related to resistance, cell division, nodulation, protein stricture modification, and the production of certain specific tolerance proteins [28,35]. Lazaroaie, 2010 [36] found out that this mechanism was more effective in gram-negative bacteria due to the presence of an additional outer wall. These allow speedy changes to occur in lipopolysaccharides and fatty acids composition in gram-negative bacteria.

As a feature, PAH-contaminated soil is made up of a mixture of LMW and HMW PAHs. Stenothrophomonas sp. is capable of solubilizing HMW PAHs by producing surfactants and extracellular products [37]. The use of HMW PAH-degrading bacteria such as *Stenothrophomonas sp*. has been shown to remove considerable amounts of LMW and HMW PAHs in contaminated soil. *Stenothrophomonas sp*. has also been shown to exhibit high tolerance to a mixture of phenanthrene, pyrene, and benzo[a]pyrene (1:1:1) even at a high concentration of 5000mgL^{-1} in a study conducted by [3] Acinetobacter is found to be very effective in degrading LMW PAHs, especially in the presence of glucose and bicarbonate [31]. Additional studies will have to be undertaken to optimize the conditions that maximize PAH degradation by these bacteria. The synergy in mixed culture systems is also to be considered as it has been shown to improve tolerance to these recalcitrant molecules.

4.0 CONCLUSION

In this study, four different species of bacteria capable of degrading PAHs were isolated from the effluent channels of Tema Oil Refinery. The level of heavy metals in these wastewaters was very low compared with the standard and waste from refineries elsewhere. The isolation of *Stenothrophomonas sp*. as an indigenous bacterium was significant since it is one of the very few bacteria known to be able to degrade high molecular weight PAHs. All isolated bacteria showed tolerance levels to a mixture of naphthalene and anthracene up to concentrations of 5 mg/mL.

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