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Hydrocarbon Degrading Microflora in a Tropical fuel-Contaminated Aquifer: Assessing the Feasibility of PAH Bioremediation

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ABSTRACT: An aquifer located within a petroleum processing plant in Moín, Costa Rica, suffers hydrocarbon pollution. This study aimed to determine the ability of indigenous microorganisms from this site to degrade polycyclic aromatic hydrocarbons (PAHs) to evaluate the feasibility of an eventual bioremediation process. Aerobic conditions were found in the aquifer, while microbial analyses of the groundwater indicated the presence of important hydrocarbon-degrading populations. Sixteen PAH-degrading strains were isolated with the ability to grow on naphthalene (5 strains), phenanthrene (3), fluorene (6) and pyrene (2). Most of the identified isolates belonged to the genus *Pseudomonas*, although, *Comamonas*, *Sphingomonas* *Stenotrophomonas* and *Delftia* were also found. A mixture of selected strains was evaluated by its performance of PAH degradation in soil-slurry systems, where efficiency of removal was naphthalene > fluorene > phenanthrene > pyrene. This study is an initial approach to evaluate the feasibility of applying a bioremediation process in the contaminated site.

Key words: Groundwater, Hydrocarbon, Bioremediation, Polycyclic aromatic hydrocarbon, Bioslurry

INTRODUCTION

Groundwater contamination by toxic pollutants, such as petroleum hydrocarbons, is a significant environmental and health problem because many communities in the world depend upon groundwater as main source of drinking water (Farhadian *et al.,* 2008). This pollution may be related to leaks or releases of petroleum, gasoline, diesel and other petrochemical products from storage tanks and wastes from oil industries, or accidents during hydrocarbon transportation by trucks, ships or oil pipelines. In Costa Rica, there are reports of at least three sites for which subsoil or superficial water contamination has been demonstrated.

In the last few years, biological methodologies have proved to be versatile, economical and efficient for the remediation of petroleum by-products, pesticides and other pollutants of industrial origin. Bioremediation has been an effective tool in soils contaminated with low molecular weight polycyclic aromatic hydrocarbons (PAHs) (Banerjee *et al.,* 1995; Kästner and Mahro, 1996). PAHs are known as toxic, carcinogenic and mutagenic compounds. Moreover, because of their low water solubility PAHs are persistent in the environment and tend to adsorb onto organic matter and sediments. Since these pollutants are more recalcitrant than non-polycyclic fuel components, PAH biodegradation may be a good indicator that removal of those other compounds is also taking place in a polluted site.

The oil processing plant of RECOPE (Costa Rican Petroleum Refinery) located near the Caribbean coast in the area of Moín was established over the river deposits near the aquifer of the same name. In 1999 RECOPE began the construction of 11 wells for monitoring total hydrocarbon concentrations in groundwater within the plant area, which led to the detection of a hydrocarbon groundwater plume in the surrounding area of the well P-8. Research was held in order to determine the exact origin of the pollution and to give recommendations for the remediation (Guzmán, 2006).

The purpose of this study was to determine the ability of indigenous microbial strains from fuel-contaminated groundwater to degrade PAHs, in order

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to assess the feasibility to eventually apply a bioremediation process. Even though a major part of Costa Rica's international image is based in environmental protection, there has been only a reduced amount of research in bioremediation topics in this country; for that reason, this work is a pioneer approach to find potential microorganisms for removal of petroleum-derived pollutants.

MATERIALS & METHODS

Naphthalene (99%), phenanthrene (97%), fluorene (95%) and pyrene (96%) were obtained from Merck Schuchdart OHG (Hohenbrunn, Germany); dibenzothiofene (99+%) was purchased from Sigma-Aldrich Inc. (San Luis, MO); hexadecane (99.9%) was obtained from Fischer Scientific Company (Fair Lawn, NJ); ethyl ether (99.9%) was obtained from Mallinckrodt Baker Inc. (Phillipsburg, NJ); anthracene (practical grade) and hexane (practical grade) were kindly donated by the School of Chemistry of the Universidad de Costa Rica and the Water Analysis Laboratory of RECOPE, respectively.The study area is located within the oil refinery of RECOPE in Moín, Limón, Costa Rica (Fig. 1). According to Guzmán (2006) there is a shallow unconfined aquifer at the study site, composed by fluvial deposits and three geological layers (0-3 m clay, 3.5-12 m course to fine sand and 12-20 m clay). Specific sampling area included the surroundings of well P-8, where 16 sampling-piezometers were installed in a previous geological research (Guzmán, 2006). Each piezometer consisted of a galvanized pipe of 4.0 m length and 5.08 cm of internal diameter. Five piezometers were selected considering the direction of the contamination plume, reported by Guzmán (2006), from south-east to north-west.

Static levels and hydrocarbon layer levels were determined using an interface probe Solinst Interface Meter model 122 (Georgetown, Ontario, Canada). The width of the hydrocarbon layer was corrected for surface tension effect on light non-aqueous-phase liquids (LNAPL) (CONCAWE, 1979). Before sampling, four times the volume of the water/hydrocarbon mixture contained inside each piezometer was purged using a peristaltic pump (Cole Parmer, 12 V, 8.0 A, Vernon Hills, IL). 200 mL samples were used for water quality determinations. 40-60 mL samples were taken with a device constituted by a sterile syringe fixed to a PVC tube for microbiological analyses.

Horiba Water Quality Checker U-10 (Horiba, Irvine, CA) was employed for the field determination of DO, temperature, salinity, pH, electric conductivity and

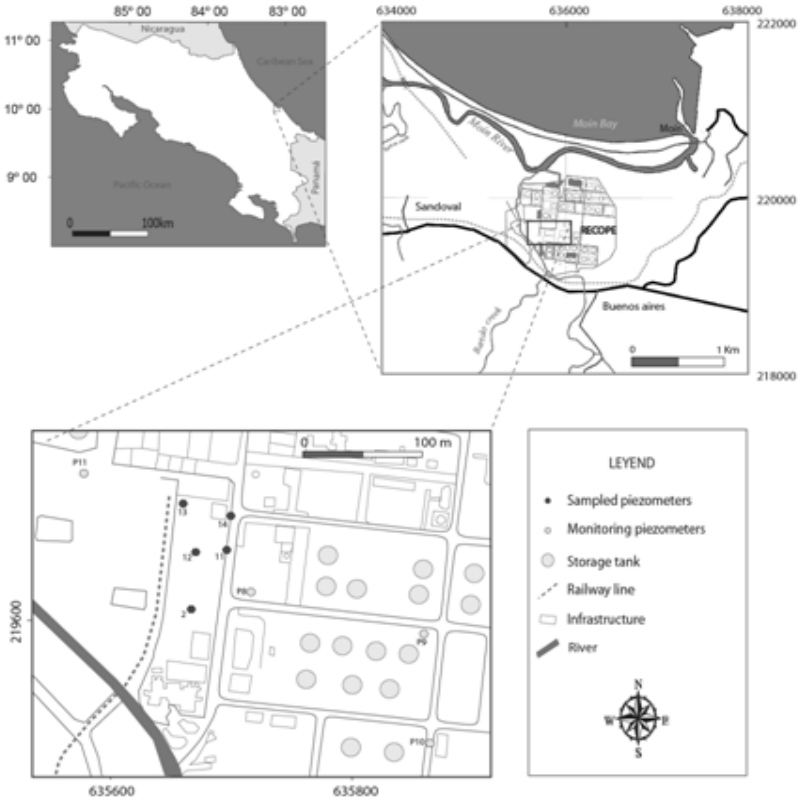

Fig. 1. Location of the contaminated site in the Costa Rican territory

turbidity in samples taken immediately after piezometer purging.

Aliphatic hydrocarbon (AH), polycyclic aromatic hydrocarbon (PAH) and total hydrocarbon (TH) degrading bacteria counts were performed. A most probable number (MPN) technique employing 96-well microplates was used, according to a modified methodology from Wrenn and Venosa (1996), using TTC (2,3,5-triphenyl tetrazolium chloride) as a metabolic activity indicator. The addition of TTC produces an insoluble reddish formazan precipitate in the wells with microbial growth. Positive patterns were analyzed with the Most Probable Number Calculator, version 4.04 (Klee, 1993) software and expressed as MPN/mL. Results were expressed as the percentage of degrading bacteria with respect to the total population, determined by the plate count technique in tripticase-soy agar (TSA) after a 72 hour incubation period.

A modified methodology by Kiyohara et al. (1982) was employed for isolation of bacterial strains capable of degrading selected PAHs (naphthalene, phenanthrene, pyrene and fluorene). Briefly, groundwater samples were inoculated in Bushnell-Hass agar (BHA) plates and immediately sprinkled with an ethereal solution of each PAH which formed an opaque layer as the ether volatilized. Plates were incubated at 25 °C for 7-14 d and analyzed for the presence of colonies with a surrounding translucent halo, produced by the presumptive degradation corresponding PAH. Each strain was purified to new plates of the same composition.

In order to select the strains with the highest ability to degrade each PAH, a second test was carried out using a modified technique from Hanson et al. (1993). A 0.1 mL volume of a 5% m/v solution (naphthalene, phenanthrene or fluorene, in ether) or 0.167 mL of pyrene 3% m/v in ether was placed into sterile tubes. After ether volatilization, 9 mL Bushnell-Hass medium (BHM, Bushnell and Hass, 1941) were added and tubes were agitated overnight at 200 rpm in the dark, before adding 0.75 mL TTC (3 g/L) and 0.15 mL of bacterial inoculum. Inocula were prepared dissolving colonies from a 48 h TSA culture to 30% T at 600 nm in sterile saline solution (0.85%). Tubes along with negative controls were incubated in the dark at 25 °C for 21 d, and changes in color were monitored by daily readings. Relative degrading potential was estimated based on the time necessary to produce a visible change in TTC or the production of macroscopically evident metabolites derived from PAH degradation. Purified strains were grown in new TSA plates and identification was performed using GN2 Microplate® miniaturized biochemical-test galleries (Biolog Inc, Hayward, California), according to manufacturer's recommendations.

An aerobic slurry system was designed to simulate a groundwater system in order to determine the extent of microbial transformation of PAHs. Young cultures of the degrading strains NAPH 2, PHEN 1, FLU 1 and PYR 1 were employed to prepare individual suspensions in BHM (21.5% T at 600 nm, approx. 1.2 x 10^9 bacteria/mL). Cells were then washed and pelleted by centrifugation (4000 × *g*, 10 min, twice) using BHM as diluent and finally suspended in 18 mL to give a final concentration of approx. 1.0 x 10^{10} bacteria/mL; the latter suspension was used to inoculate PAH-spiked soil slurries. Soil (N: 0.59 %, organic matter: 9.1%, pH: 5.4, Ca: 2.44 cmol/L, Mg: 0.44 cmol/L, Na: 0.02 cmol/L, K: 0.30 cmol/L, cation exchange capacity: 3.81 cmol/L, P: 3 mg/L, Zn: 0.5 mg/L, Cu: 5 mg/L, Fe: 115 mg/L, Mn: 6 mg/L) was sieved (mesh: <0.5 mm), autoclaved and finally dried for 24 h (100-105 °C). Soil bioslurries were prepared by adding 2.6 g of soil in chemically clean 40 mL teflon-sealed screw capped containers (I-CHEM Brand, Thermo Fisher Scientific, Rockwood, TN) and spiked with 100 µL of a PAH ethereal solution (naphthalene, phenanthrene, fluorene and pyrene, 5000 mg/L each). The solvent was allowed to evaporate prior to addition of 3 mL of BHM and 1 mL of each bacterial suspension. The final reaction mixture in the slurries contained approx. 50 mg/L each PAH and 4.0 x 10^9 total bacteria/mL. The remaining headspace in the containers helped to support the aerobic conditions. Containers were incubated at 30 °C on an orbital shaker at 90 rpm and the amount of PAHs was analyzed over time. At each time point triplicate sample containers were completely sacrificed as well as duplicate sterile controls (inoculums were substituted by BHM) for hydrocarbon analyses. The material contained in each slurry was subjected to successive extractions with dichloromethane and sonicating for 10 min. Extracts were cleaned with silica-gel, eluted with hexane and hexane-dichloromethane and finally adjusted to 2 mL with acetonitrile after concentration. Analyses were performed in a HPLC 1200 (Agilent, Santa Clara, CA) using a Waters PAH C18 column (5µm, 4.6 × 250 mm) and equipped with a diode array detector. Chromatographic separation was done by supplying acetonitrile (ACN)-water at 1 mL/min (gradient: 0-3 min 60% ACN; 3-20 min 100% ACN; 35-40 min 60% ACN).

RESULTS & DISCUSSION

Hydrocarbon layer width is a trustworthy indicator regarding the contaminant's distribution in the geological strata (Guzmán, 2006). Values obtained for the hydrocarbon layer width (corrected for the effect

of surface tension) were the following: 17.8; 16.5; 3.3; 2.2; and 3.5 cm for piezometers P2, P11, P12, P13 and P14 respectively. The maximum value is similar to that obtained in a previous study conducted by Guzmán (2006). This indicates that the contamination persists despite natural processes in the groundwater and efforts to reduce it, which have mainly involved pumping and disposing adequately of the extracted contaminant. Table 1 presents water quality parameters obtained from the groundwater samples. Dissolved oxygen values in all samples (5.70-6.35 mg/L) may provide adequate levels to maintain aerobic and facultative aerobic bacterial populations. Static water levels measured in the piezometers ranged from 0.63 to 2.08 m. According to Atlas (1981) shallow aquifers are ideal for the hydrocarbon biodegradation since aerobic conditions are frequently found in these environments. pH values obtained were near neutrality and are in accordance with those found in a previous study (Guzmán, 2006); values between 6.5 and 8.0 units are in general in the optimal range for normal biological activity (Testa and Winegardner, 2000).

Total heterotrophic bacteria and hydrocarbon-degrading bacteria population counts are shown in Table 2. Only small variations were observed between the total heterotrophic counts for each of the piezometers, always in the range of 10^2 and 10^3 CFU/mL, which are one to four orders of magnitude lower than those found in other studies of contaminated groundwater (Cavalca *et al.*, 2004, Ferguson *et al.*, 2007). If compounds with higher toxicity are found making up the contaminant mixture in this study, these may have a more severe effect towards autochthonous bacterial populations, limiting their numbers. The percentage of the total bacterial population constituted by hydrocarbon-degrading bacteria in soil or water is low in pristine environments; Atlas (1981) states a percentage lower than 0.1%; other authors set the percentage below 1% or even 10% (Mulkins-Phillips and Stewart; 1974; Prince, 2005). Groundwater from all the sampled piezometers showed total hydrocarbon degrading bacteria percentages greater than 1%, and two of them (P11 and P14) showed values greater than 10%. The influx of hydrocarbon contaminants in the groundwater system throughout time could contribute to the increased percentages of total hydrocarbon degrading bacterial populations. Reports of total hydrocarbon-degrading bacteria percentages in contaminated groundwater settings could not be found in the literature reviewed. However, high percentages were found compared with studies in which samples from other hydrocarbon-polluted freshwater and marine environments were analyzed. Maximum values of hydrocarbon degrading bacteria populations ranging from 0.3% to 2.0% have been reported from contaminated rivers and coastal waters (Suborna *et al.*, 2002; Adebusoye *et al.*, 2006), far less than the 28% obtained in the present study. Total hydrocarbon degrading bacterial counts were performed using diesel as the sole carbon source, as opposed to aliphatic hydrocarbon degrading bacteria counts and PAH degrading bacteria counts, where only one compound (n-hexadecane) and a mixture of four different PAH compounds were used as a sole carbon source respectively. Given the variety of compounds found in diesel it could be expected for total hydrocarbon degrading-bacteria percentages to be the highest of all. However, in general, percentages of total hydrocarbon degrading bacteria were lower than those for aliphatic-hydrocarbon and polyaromatic-hydrocarbon degrading bacteria. This could be due to the presence of compounds with toxic growth-inhibiting properties as part of the diesel or its additives (Yemashova *et al.*, 2007).

Likewise, previous reports of aliphatic hydrocarbon and PAH-degrading bacteria populations in groundwater are scarce. However, similar absolute MPN values have been reported for hydrocarbon-contaminated coastal waters (Bonner *et al.*, 2002, Yun *et al.*, 2003), with maximum populations in the order of 10^3 MPN/mL, slightly below the maximum values found in this study (10^4 MPN/mL). Absolute and relative counts for PAH-degrading bacteria were the highest compared to the other hydrocarbons analyzed in this study. If the composition of the oil mixture in the contaminant is PAH-rich, then it is possible that an adaptation process has taken place in the bacterial

Table 1. Water quality parameters for each of the piezometers sampled in the RECOPE oil refinery in Moín

Piezometer	Dissolved Oxygen (mg/L)	Temperature (°C)	Salinity (%)	pH	Electric conductivity (mS/cm)	Turbidity (NTU)
P2	6.00	29	0.00	6.4	0.92	2
P11	5.70	31	0.10	6.6	1.20	2
P12	6.31	28	0.05	6.6	1.19	56
P13	6.35	28	0.02	6.2	0.57	11
P14	6.00	30	0.01	7.4	0.38	304

Table 2. Bacterial populations present in the hydrocarbon-contaminated groundwater in the RECOPE oil refinery in Moín

Piezometer	Heterotrophic bacteria (CFU/mL)	Aliphatic hydrocarbon-degrading bacteria		Aromatic hydrocarbon-degrading bacteria		Total hydrocarbon-degrading bacteria	
		(MPN/mL)	(%)	(MPN/mL)	(%)	(MPN/mL)	(%)
P2	2100	1.2×10^2	6	8.3×10^2	39	8.3×10^1	4
P11	3400	1.2×10^3	36	1.2×10^3	36	4.7×10^2	14
P12	940	1.9×10^2	20	2.7×10^2	28	2.8×10^1	3
P13	5200	1.4×10^2	3	1.0×10^4	---	1.4×10^2	3
P14	670	8.3×10^1	12	3.8×10^2	57	1.9×10^2	28

community, in which bacteria with the ability to degrade this compounds have a selective advantage over other populations, which contributed to their increased numbers.

A total of 16 PAH-degrading strains were isolated from the PAH spray-covered BHA plates inoculated with groundwater samples; 5, 3, 6 and 2 isolates from naphthalene, phenanthrene, fluorene, and pyrene-covered plates respectively. The fact that the number of isolates for each hydrocarbon was not high could have been a consequence of the relatively low heterotrophic bacterial counts found in the groundwater samples. Ferguson et al. (2003) pointed out that in areas in which the levels of hydrocarbon pollutants are high and prone to have a toxic effect over bacterial populations, PAH degradation may be dominated by a reduced number of particular types of bacteria. A similar process could have occurred at the study site, thus yielding a low number of strains resistant to these high contamination levels, a useful characteristic for bioremediation purposes.

The patterns obtained in Biolog's Microlog galleries provided an adequate identification for more than half of the isolated PAH-degrading strains, as shown in Table 3. All of the isolated strains corresponded to gram-negative bacteria. All of the identified isolates are considered strict aerobes, and the majority corresponded to *Pseudomonas* species.

It has been established that *Pseudomonas* species play an important role in the degradation of toxic chemicals of anthropogenic and natural origin. They are well-known degraders of compounds such as hydrocarbons, aromatic compounds, polycyclic compounds and their derivatives (Palleroni, 2005), and have comprised the most frequent degrading-fuel genus in contaminated aquifers (Stapleton *et al.,* 2000). *Sphingomonas* species were also isolated. Members from this genus have a wide distribution in the environment and a diverse metabolic capacity, being able to degrade many persistent compounds of environmental importance, including aromatic and polyaromatic hydrocarbons of low and high molecular weight, using them as a sole source of carbon and energy or through co-metabolism (Shuttleworth *et al.,* 2000; Pinyakong *et al.,* 2003). Similarly, strains from the genus *Comamonas* are capable of degrading a wide variety of complex aromatic hydrocarbons, steroids and many organic xenobiotics (Goyal and Zylstra, 1996).

Organisms from the species *Stenotrophomonas maltophilia*, although not as nutritionally versatile as *Pseudomonas* or *Sphingomonas* species, have been isolated from enrichments using kerosene and petroleum (Iizuka and Komagata, 1964) and have been shown to degrade pyrene as a sole source of carbon and energy, as well as benzo(α)pyrene through co-metabolism (Boonchan *et al.,* 2000).

Even though there is increasing evidence of the diverse metabolic capacity of *Delftia* species in the environment, particularly referred to the degradation of xenobiotics, it has been reported very few times as being able to degrade PAHs (Vacca *et al.,* 2005).

Table 3 also shows the performance of the strains in the PAH-degrading screening test. Several of the screened strains did not produce a color change during the scheduled time of the test (21 days). Based on the results, strains with a presumptive higher naphthalene and phenanthrene degrading potential were NAPH2 (*Pseudomonas maculicola*) and PHEN1 respectively. In the case of fluorene, two strains: FLU1, (*Sphingomonas paucimobilis*) and FLU10, (*Stenotrophomonas maltophilia*) showed a similarly high degrading capacity for this hydrocarbon. The two presumptively pyrene-degrading strains failed to show a color change during the test. Susceptibility of PAHs to microbial attack decreases with an increase in molecular weight and octanol:water partition coefficient (Cerniglia,

Table 3. Bacterial strains isolated from the hydrocarbon-contaminated groundwater in the RECOPE oil refinery in Moín. Results on the PAH-degrading capacity screening test are included

Hydrocarbon	Strain code	Identification	Time for color change (hours)
Naphthalene	NAPH 2	***Pseudomonas maculicola* (93%)**[a]	12
	NAPH 3	*Pseudomonas aeruginosa* (99%)	24
	NAPH 4	No ID (*Sphingomonas paucimobilis* group B)[b]	No change
	NAPH 5	No ID (*Phyllobacterium myrsinacearum*)	72
	NAPH 6	No ID (*Sinorhizobium meliloti*)	72
Phenanthrene	PHEN 1	**No ID (*Sphingomonas paucimobilis*) group B**	24
	PHEN 9	No ID[c]	72
	PHEN 10	*Pseudomonas fluorescens* biotipe G (86%)	No change
Fluorene	FLU 1	***Sphingomonas paucimobilis* group B (99%)**	24
	FLU 8	*Comamonas testosteroni* (91%)	264
	FLU 9	No ID (*Acidovorans delafieldii*)	No change
	FLU 10	***Stenotrophomonas maltophilia* (99%)**	24
	FLU 11	No ID (*Rhizobium rhizogenes*)	288
	FLU 12	*Delftia acidovorans* (99%)	No change
Pyrene	PYR 1	*Pseudomonas sp.*	No change
	PYR 2	*Pseudomonas aeruginosa* (99%)	No change

[a] Strains with the best performance in the PAH-degrading screening test for each compound are marked in bold

[b] For strains in which an adequate identification was not obtained, the closest strain in the Biolog's Microlog database is indicated

[c] This strain produced a color change in all of the gallery's micro-wells, invalidating the identification procedure

1992). Taking into consideration the PAHs used in this study, pyrene shows the highest values for both of these properties, which would explain its persistence. Also, it has been shown that even using strains in which pyrene degradation has been demonstrated, if the initial inoculum is not large enough, there may be no degradation of the compound at a detectable level (Daane *et al.,* 2001). Bioreactors can be used to control factors that have an influence over the level and rate of microbial growth and transformation of hydrocarbons in order to obtain an optimal degradation performance. Bioslurry reactors are useful for determining the plausibility and potential of a bioremediation strategy in the final restoration of a contaminated site (Fava *et al.,* 2000) as well as for the remediation of soils contaminated with recalcitrant compounds (Robles-González *et al.,* 2008) such as PAHs.

PAH removal data is presented in Fig. 2, the percentage of PAHs remaining was calculated by comparing the concentrations with abiotic controls. The PAH with the most rapid and complete removal was naphthalene, 99.9% after the three-week period. The removal of fluorene, though not as complete as the one for naphthalene, reached to 41.2% by the end of the process. The two compounds with the highest molecular weight and octanol-water partition coefficients, phenanthrene and pyrene showed a slower and less complete removal (19.9% and 18.5%, respectively), as expected. Even though removal of PAHs was monitored during a relatively long three week period, complete removal of three of the four compounds could not be achieved. Aside from low water solubility, the production of toxic metabolites may have hindered degradation. Studies in which a faster and more complete removal occurred under similar conditions can be found in the literature (Shi *et al.,* 2001). This difference may have been caused by a higher PAH degradation ability of the strains employed in other studies or by the presence of more readily degradable substances in the soil used to prepare the slurries for this experiment. Also, even though precautions were taken to assure the presence oxygen in the slurries, faster consumption vs. renewal of this compound throughout the assay may have affected degradation.

Remediation of the contaminated site should start with the direct physical recovery of the hydrocarbon mixture; however, an important amount of diverse compounds, including PAHs, would remain adsorbed to the sediments, making a complete removal impossible through this method. Then, additional options such as bioremediation should be taken into consideration to be used once the floating hydrocarbon layer has been extracted.

According to our data, even though the groundwater contains high percentages of bacteria able to degrade different kinds of hydrocarbons, including persistent compounds such as PAHs, it was determined that their absolute population numbers are low. Therefore, strategies such as enhanced biorestoration

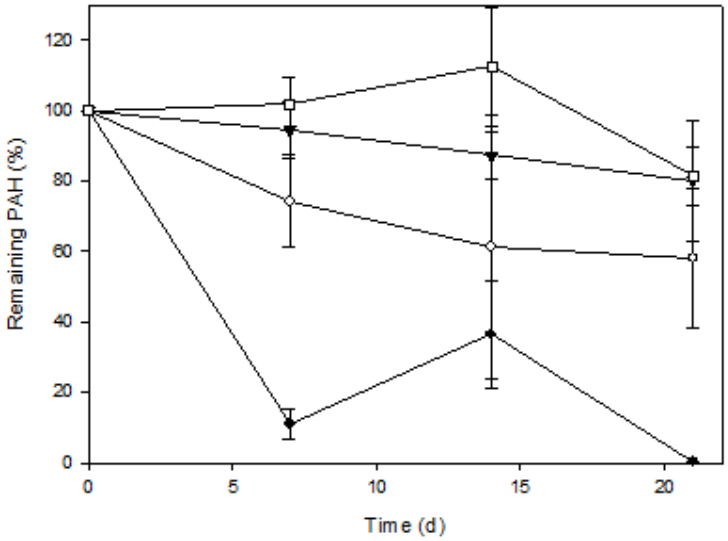

Fig. 2. Removal of naphthalene (● %), fluorene (○%), phenanthrene (Δ%) and pyrene (□%) in a spiked (50 mg/L each) soil slurry inoculated with a mixture of NAPH2 (*Pseudomonas maculicola*), PHEN1, FLU1 (*Sphingomonas paucimobilis*) and PYR1 (*Pseudomonas sp.*). Values plotted are means ± SD for triplicate slurries

at the site should be explored in order to allow hydrocarbon degrading bacterial populations already present at the site to increase their numbers and to degrade the contaminants more effectively. Temperature and pH conditions at the contaminated site are optimal for microbial growth and metabolism. Increasing the availability of electron acceptors, in this case oxygen, is a possibility that should be considered.

CONCLUSION

The present investigation provided information regarding the PAH-degrading ability of autochthonous bacterial strains from a fuel contaminated aquifer in a tropical region. A mixture of the presumptively most capable isolates was able to remove various PAHs in soil-bioslurry systems at different rates. Since the degrading-strains isolated were aerobic bacteria and the groundwater presents an aerobic environment, the possibility of applying a bioremediation process might be feasible in this groundwater-system. However, more studies related to the characterization of the contaminated site are required.

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