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Examining the Soils Adjacent to the Historical Pitch Lake for Levels of Polycyclic Aromatic Hydrocarbons (Pahs) and Naturally Occurring Pah-Degrading Bacteria

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Abstract: The soils adjacent to the La Brea Pitch Lake in Trinidadare expected to have high levels of Polycyclic Aromatic Hydrocarbons (PAHs) and PAH-degrading bacteria. This is due to the historical influx of hydrocarbons (bitumen) since the Cretaceous period. The PAHs are toxic, carcinogenic and genotoxic compounds which are natural components found in bitumen and natural oil seepages. This study investigates the occurrence of five selected PAHs (*viz*. naphthalene, acenaphthene, anthracene, phenanthrene and pyrene) andthe presence of bacteria that can utilize these selected PAH in soils neighbouring the PitchLake. To do this, soil samples from three distance zones to the perimeter of the Pitch Lake (zone 1: within 200 m; zone 2: within 400m and zone 3: >500m) were analyzed for levels of five selected and screened for their degrading bacteria using selective enrichment procedures. The results showed that the levels of these individual PAHs were generally above the standard acceptable limit of 1mg/Kg among all 3 zones except for anthracene in Zone 2. The levels of the total PAHs (∑5PAHs) showed a general trend of decreasing concentrations with high variability as thedistance from the Pitch Lake increased.The results of the soils screened for potential PAH-degrading bacteria showed that isolates in zone 1 and 2 (HIS) produced more PAH degraders for the more recalcitrant PAHs (*viz.* phenanthrene, anthracene,and pyrene) compared to naphthalene and acenaphthene, which bothappeared independent of zones.

Keywords: Polycyclic aromatic hydrocarbons (PAHs), naphthalene, acenaphthene, anthracene, phenanthrene, pyrene, PAH-degrading bacteria, La Brea Pitch Lake

INTRODUCTION

Polycyclic Aromatic Hydrocarbons (PAHs) exhibit carcinogenic and/or mutagenic properties and these are listed by the United States Environmental Protection Agency (US EPA) and European Commission (EC) as priority pollutants (Amellal et al., 2001). The European standards for PAHs in soils, sediments, and groundwater has been well documented and evaluated in recent years (Lijzen et al., 2001) and will be used as the baseline for purposes of this research. Utilizing the revised clean up standards for an average soil for the Netherlands (10% organic matter and 25% clay) should range between 1- 40 mg(PAH)/Kg dw, if it is greater than 40 mg(PAH)/Kg dw, then remediation should be undertaken(Harmsen, 2004). Generally in the natural terrestrial environment from biological processes, soils are estimated to have levels of 1–100 μg/Kg PAHs (Wilcke, 2000), whereas concentrations of PAHs in highly polluted soils can range anywhere from 10 mg/Kg to 10 g/Kg dry weight (Stieber et al., 1994).

In Trinidad, the health of the environment is of grave concern due to the continuous influx of contaminants through oil spills or by-products of factory processes. Although legal requirements for monitoring and controlling these contaminants are stipulated within the Environmental Management Agency Act (2002), the Act only requires a reduction of Total Petroleum Hydrocarbons (TPH) to be less than 10 mgL-1. However, studies have shown that during remediation processes the structurally simple compounds are degraded rapidly whereas complex compounds such as the PAHs tends to be recalcitrant to degradation (Mueller et al., 1996). Thus, although the TPH levels maybe within the stipulated levels, PAHs and other recalcitrant compounds can be relatively high(Cerniglia, 1992; Lehmann, 1998).

The La Brea Pitch Lake is one of the three largest natural petroleum seepages in the world(Mohammed & Agard, 2004). It is located on the southwestern coast of the island of Trinidad and measures approximately 41 hectares (100 acres) in an area with a depth of 76 m (250 ft) in the centre. The Lake is estimated to contain 10 million tons of natural asphalt, referred to locally as pitch, which contains a plethora of hydrocarbons (LATT, 2004). Considering that the Lake has been in existence since the Cretaceous period(Kugler, 1965), there is limited information on possible influences of the Pitch Lake on its surrounding terrestrial environment.

To our knowledge, this is the first study done on PAH levels in the soils of Trinidad. The aim of this study is to establish baseline levels of selected PAHs in the soils adjacent to the La Brea Pitch Lake and explore their potential, as sources of selected PAH-degrading bacteria.

MATERIALS AND METHODS

Sample Site

Three zones along a distance gradient adjacent to the southern perimeter of the La Brea Pitch were taken sampled (see Figure 1). The study zones were situated within 200m (zone 1), within 200 - 400 m (zone 2) and more than 500 m (zone 3 and the reference site) from the southern perimeter of the La Brea Pitch Lake in the island of Trinidad.Triplicate core samples of soil at a depth of 8-10 cm (approximately 100 g each) were taken per quadrant. Samples were transported on ice-cooled containers, stored at 0^{o}C until analysis could be performed.

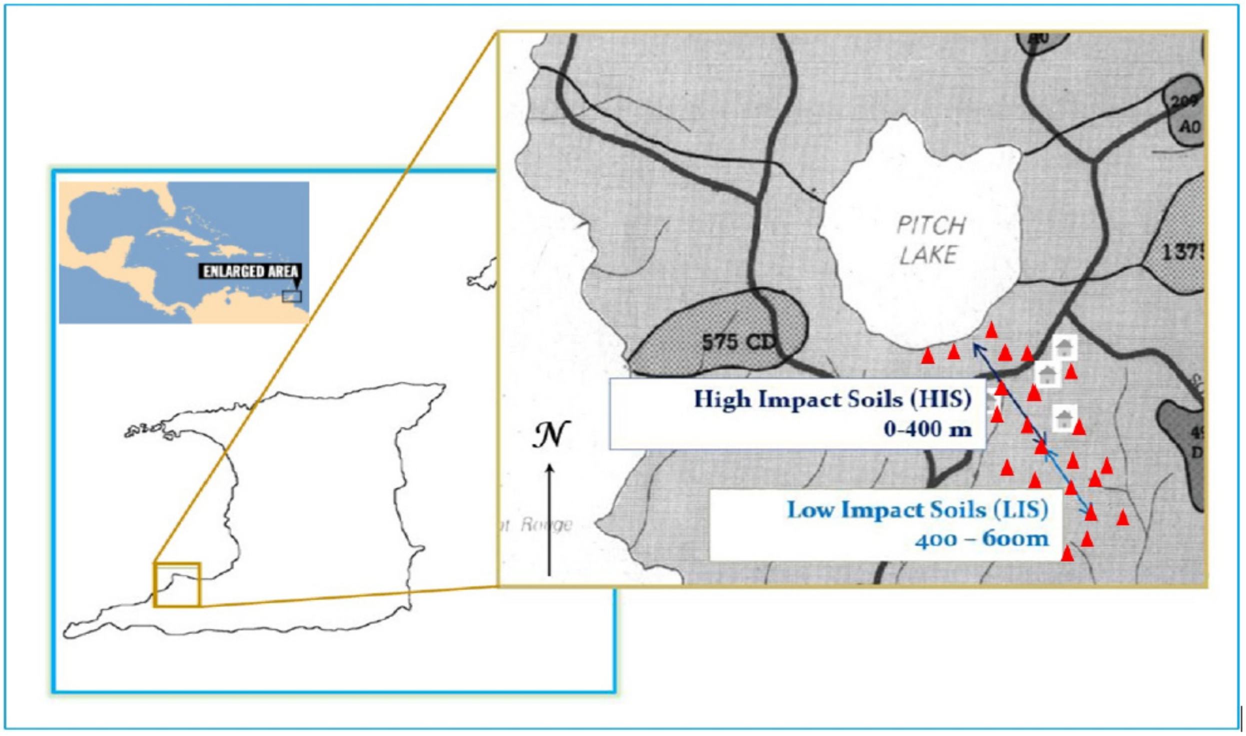


Fig1. *Location of sample site, impact zones and distribution of sampling points*

PAH Extraction, Purification and Analysis

Soxhlet extractions of PAHs were conducted using hexane as the solvent(ECRC, 2003). This involved weighing approximately 5 g of each soil into a porous cellulose (94 mm external length by 33 mm internal diameter) Whatman thimble and then extracting the PAHs in the soil using a Soxhlet apparatus consisting of a 500 mL round-bottomed flask, a condenser and extractor tube, seated in a temperature-controlled heating mantle. Extractions were done at 55-60°C for 8 hours with 120 mL hexane solvent.

Sample extracts were reduced to approximately 1mL and added to an assembled chromatography column (1cm diameter with 25 mL total volume). The column consisted of a wad of glass wool (~1 cm thick), approximately 1 cm of sodium sulphate, 5 g KOH-modified silica gel and another 1 cm of sodium sulphate were added to the column in that order. The column was then immediately saturated with approximately 25 mL of hexane. A 125 mL collection flask was placed beneath each column which contained approximately 2 g of copper filings activated with concentrated nitric acid to remove organic acids and residual sulphur.The entire 1 mL sample extract was added to the column and the residue from the sample vial was rinsed sequentially three times with 1-2 mL of 5% dichloromethane in hexane solvent which was also added to the column. The PAHs were then eluted with 40 mL of 5% dichloromethane in hexane and collected in the 125 mL glass beakers. The solvent containing the PAHs were then reduced to 0.5 mL by evaporating with nitrogen gas. Tubes were stored under refrigeration conditions (0 - 4°C) until GC-MS analysis.The 0.5 mL extracts were shot into a PE Claris Gold Gas Chromatograph coupled with a 500 Turbomass mass selective detector (GC-MS). The column used for GC was PE 5 MS capillary column with a length of 25 m, 0.2 mm i.d., 0.33 mm film thickness (Perkin Elmer, Waltham, Massachusetts, USA). The conditions for analysis are seen in Table 1. Five PAHs (viz. naphthalene, acenaphthene, anthracene, phenanthrene,and pyrene) were selected based on their relatively low tetratoxicity (Mueller *et al*., 1997). The GC-MS chromatographs were used to quantify the levels of naphthalene, acenaphthene, anthracene, phenanthrene,and pyrene. A standard solution of the five priority PAHs were prepared for analysis. PAH concentrations were calculated on a dry weight basis. The limits of detection were 0.01 mg/Kg for the PAHs tested.

Table1. *GC-MS program used in the analysis of five selected PAHs for the Pitch Lake impacted soils of La Brea*

GC/MS: Perkin Elmer Clarus 500			
Column: PE Elite 5			
Carrier Gas: Helium			
Splitless:1:10			
Oven Temperature Program (28:33min)			
	Temperature	Delay	
Initial Temperature	75°	Hold for 3:00 min	
Ramp 1	12°/min to 300°	Hold for 5:00 min	

Culture Conditions

A basal salt medium (BSM) was prepared for enrichment and isolation of PAH-degrading bacteria based on a modification of the method described by Juhasz *et al.* (1997). The BSM contained (L^{-1}): 0.4 g K_2HPO_4; 0.4 g KH_2PO4; 0.4 g $(NH_4)_2SO4$; 0.3 g NaCI; 5 mL trace element solution (L^{-1}: 400 mg $FeSO_4.7H_2O$, 400 mg $MnSO_{4.}4H_2O$, 200 mg $ZnSO_4.7H_2O$, 40 mg $CuSO_4.5H_2O$, 300 mg KI, 50 mg $Na_2MoO_4.2H_2O$;); 5 mL of Difco yeast extract solution (5 g L^{-1}) and 5 mL of magnesium and calcium solution (0.4 g $MgSO_4.7H_2O$ and 0.4 g $CaCl_2.2H_2O$ in 1 L). Trace elements, yeast extract,and magnesium/calcium solutions were sterilised by filtration (0.22 µm, Millipore) and added to BSM after autoclaving at 121^{o}C and 15 psi for 15 minutes. Stock solutions of each PAH were prepared in acetone at the following concentrations: 10 mg mL^{-1}, naphthalene (NAP), acenaphthene (ACE), phenanthrene (PHE) and pyrene (PYR); and 5 mg mL^{-1} for anthracene (ANT). BSM was supplemented with individual PAHs to achieve a final concentration of 100 mg L^{-1}. When used in plates BSM media was solidified with 1.5% bacto agar. Naphthalene, acenaphthene, anthracene, phenanthrene,and pyrene were purchased from Sigma Chemicals Co. (St. Louis, MO). Bacterial media components and reagents were purchased from Voigt Global Distribution Co. (Kansas City, MO) and Oxoid (Hamshire, UK). All solvents and chemicals used were high purity grade reagents.

Bacterial inoculum

Two sets of bacterial inoculum were used in this study. They were obtained by grouping soil samples taken from the soil surrounding the La Brea Pitch Lake. The first setincluded homogenized soil samples from zones 1 and 2 and wasreferred to as the High Impact Soils (HIS) since they were the closest to the PitchLake. The second setincluded homogenized soil samples from zone 3 and werereferred to as the Low Impact Soils (LIS) since they were the most distant from the PitchLake. The bacterial inoculum for each sample was prepared from 20g soil (wet weight) which was shaken for 24 hours in 100 mL of Ringer's solution at 30°C and 175 rev min^{-l} (modified after Juhasz *et al.,* 1997).

Determination of total viable counts for PAH degraders

For the soil sets (HIS and LIS), the total viable counts of bacteria capable of degrading the five PAH compounds (NAP, ACE, ANT, PHE and PYR) were determined by inoculating BSM plates amended with individual PAHs and observing for the appearance of "clear zones"(Kiyohara *et al.*, 1982). The BSM agar plates were overlayed with 20 µL of a single PAH (100 mg/mL acetone) followed by spreading 0.1 mL bacterial (soil) suspension on the surface of plates. The plates were then incubated at 30^{o}C and the numbers of "clear zones" appearing on plates were recorded every 2 days for 22 days. Tests were performed in triplicate.

Data Analysis

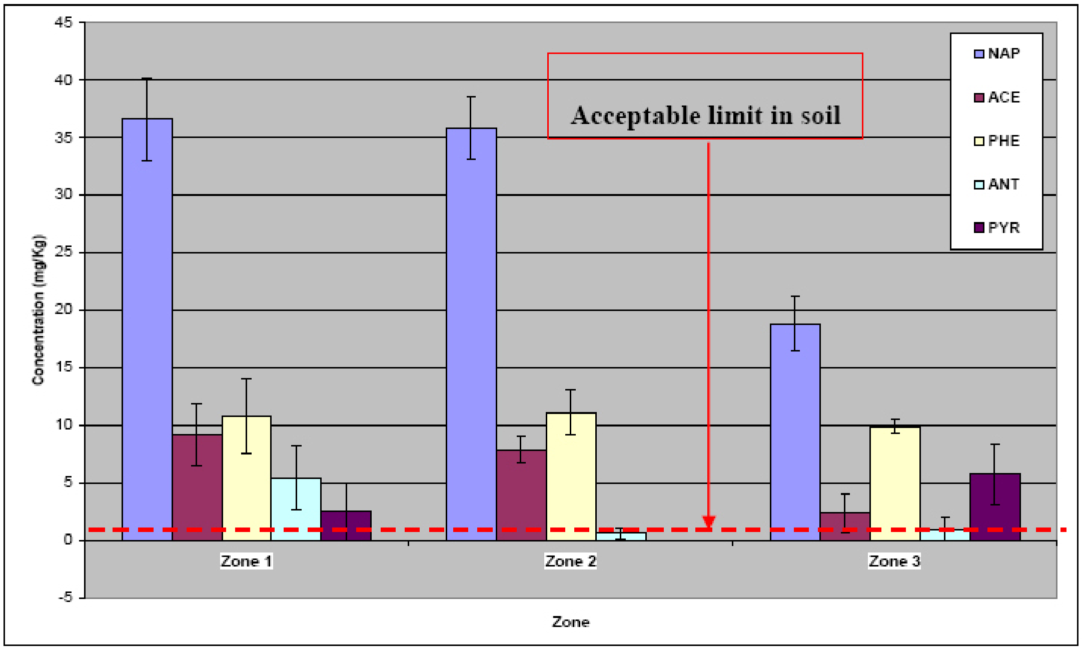
ANOVAs and correlations analysis was performed on Minitab 14 to determine differences of physicochemical parameters among Zones and the level of association between the various parameters.

RESULTS AND DISCUSSION

Although not significant, there was a general decrease in the levels of total PAH as the distance from the PitchLake increased. The reference zone (i.e. Zone 3), was expected to have levels of PAHs below the acceptable limit (i.e. 1 ppm for individual and 40 ppm for total PAHs), however as the results showed, Zone 3 located more than 500 m SW from the perimeter of the Pitch Lake possessed levels above the acceptable limits (Table **2**). With respect to individual PAHs, there were significantly lower levels of NAP in Zone 3 compared to Zones 1 and 2 and a decreasing trend (not significant) for ACE, ANT,and PHE. These results suggest that the Pitch Lake had an effect on the soil PAH levels and conform with the findings of (Mohammed, 2000)who reported higher levels of PAHs in coastal sediments closer to the pitch lake as compared to more distant areas. The lack of significant differences among Zones is considered as a result the nature of the Pitch Lake, which according to(Chaitan & V.R.Graterol, 1992), possesses a network of pitch dykes or "veins" that can emerge at any random point outside the basin of Pitch Lake.

Table2. *Mean and data range of selected PAHs (mg/kg dw) in the soil samples from three different distance zones from the La BreaPitchLake.*

	Zone 1			Zone 2			Zone 3	
PAH (mg/kg)	**Mean**	**Range**		**Mean**	**Range**		**Mean**	**Range**
NAP	36.6±3.57	16.0 - 54.0		35.9±2.67	23.8 - 51.0		18.8±2.34	13.2 - 28.3
ACE	9.2±2.70	ND - 26.0		7.9±1.13	ND - 14.4		2.4±1.74	0.0 - 10.5
PHE	10.8±3.29	ND - 29.0		11.1±1.94	ND - 25.4		9.9±0.63	8.0 - 12.1
ANT	5.5±2.76	ND - 20.2		0.6±0.43	ND - 5.0		1.0±1.00	0.0 - 6.0
PYR	2.5±2.52	ND - 22.7		ND	ND		5.8±2.64	0.0 - 14.0
Σ5PAHs	60.4±11.49	16.0 - 127.4		52.8±3.59	30.7 - 74.4		7.9±3.19	27.2 - 49.3



Red dashed line indicates acceptable limits in soil (1 ppm).

Keys: NAP: naphthalene, ACE: acenaphthene, PHE: phenanthrene, ANT: anthracene, PYR: pyrene, (*n*=30)

Fig2. *Distribution of five of the selected PAHs (mg/Kg dw soil) along a distance gradient from the La Brea Pitch Lake*

There is limited published data on PAH levels in natural soils in Tropical regions. Findings from one study by (Wilcke *et al.*, 2003)reported total levels of the same five PAHs (NAP, ACE, ANT, PHE & PYR) to be 0.145 mg/Kg in soils of the Amazon Basin, Brazil (Table 3). This is relatively low compared to soils surrounding the La Brea Pitch Lake which had total levels up to 127 mg/kg for the equivalent PAHs (NAP, ACE, ANT, PHE & PYR). This supports the hypothesis that the petrolic compounds of the pitch lake influence the relatively high levels of PAHs in the soils neighbouring the La Brea pitch lake.

Analysis of the results showed that NAP (58% of Σ5PAHs) was the most dominant among the five PAH followed by PHE, ACE, ANT,and PYR (20%, 13%, 4.0% and 3.8%, respectively). (Wilcke *et al.*, 1999)observed a similar abundance pattern for the natural soils of the tropical Brazilian forest in the Amazon basin with the order of abundance being: NAP>PHE>ACE>PYR≈ANT. Interestingly the dominant PAHs in the Amazon forest were also NAP (~56% of total PAHs) and PHE (~21% of total PAHs) which showed similar ratios to that found in the pitch lake soils. In temperate regions, PYR tended to be the most dominant of the five selected PAHs (Bucheli *et al.*, 2004; Kulakow & Erickson, 2000)of the five selected PAHs chosen in this study, regardless of land use. A possible explanation purported by (Wilcke *et al.*, 1999)is that the tropics have biological processes that contribute to the relatively high NAP and PHE concentrations in the soils.

Table3. *Soil polycyclic aromatic hydrocarbon (PAH) concentrations compiled from literature data as compared to those determined in this study.*

Country	Land use	ΣPAH concentration (mg/kg dry wt.)	Number of PAHs	PAH abundance	Reference
Brazil	Rural	0.10	20	Nap > Phe > Per > Pyr ≈ Fla	Wilcke *et al.* (2003)
UK	Rural	0.19	12	ND	Wild and Jones (1995)
Germany	Rural	1.90	6	ND	Tebaay et al. (1993)
India	Rural	6.70	11	Chr > BbF > Fla	Masih and Taneja (2006)
Switzerland	Urban	0.40 - 0.62	16	Fla > Pyr > Chr > Phe	Bucheli *et al*. (2004)
Germany	Industrial	16.00	6		Tebaay *et al.* (1993)
UK	Industrial	4.50	12	Fla > BaA + (C + T)	Wild and Jones (1995)
Iran	Industrial	100-10000	5-20	ND	Arbabi *et al.* (2004)
Austria	Industrial	0.28 – 79.00	18	ND	Weiss *et al*. (1994)
USA	Industrial	34 – 781	16	Flo ≈ Pyr > Phe > Chr	Kulakow and Erickson (2000)
South Africa	Industrial	>30,000	9	Phe > Fla > Pyr > Nap > Chr	Atagana, 2004
India	Industrial	13.70 - 28.50	11	Chr > Fla > BbF	Masih and Taneja (2006)
Trinidad	Rural	16.04 - 127.37	5	Nap > Phe > Ace > Pyr ≈ Ant	This study

Ace: acenaphthene, Ant: anthracene, BaA: Benzo[a]anthracene, BbF: Benzo[b]fluoranthene, Chr: chrysene, Fla: fluoranthene, Flo: fluorine, Nap: naphthalene, Per: perylene, Phe: phenanthrene, Pyr: pyrene, ND: no dataModified from (Masih & Taneja, 2006)

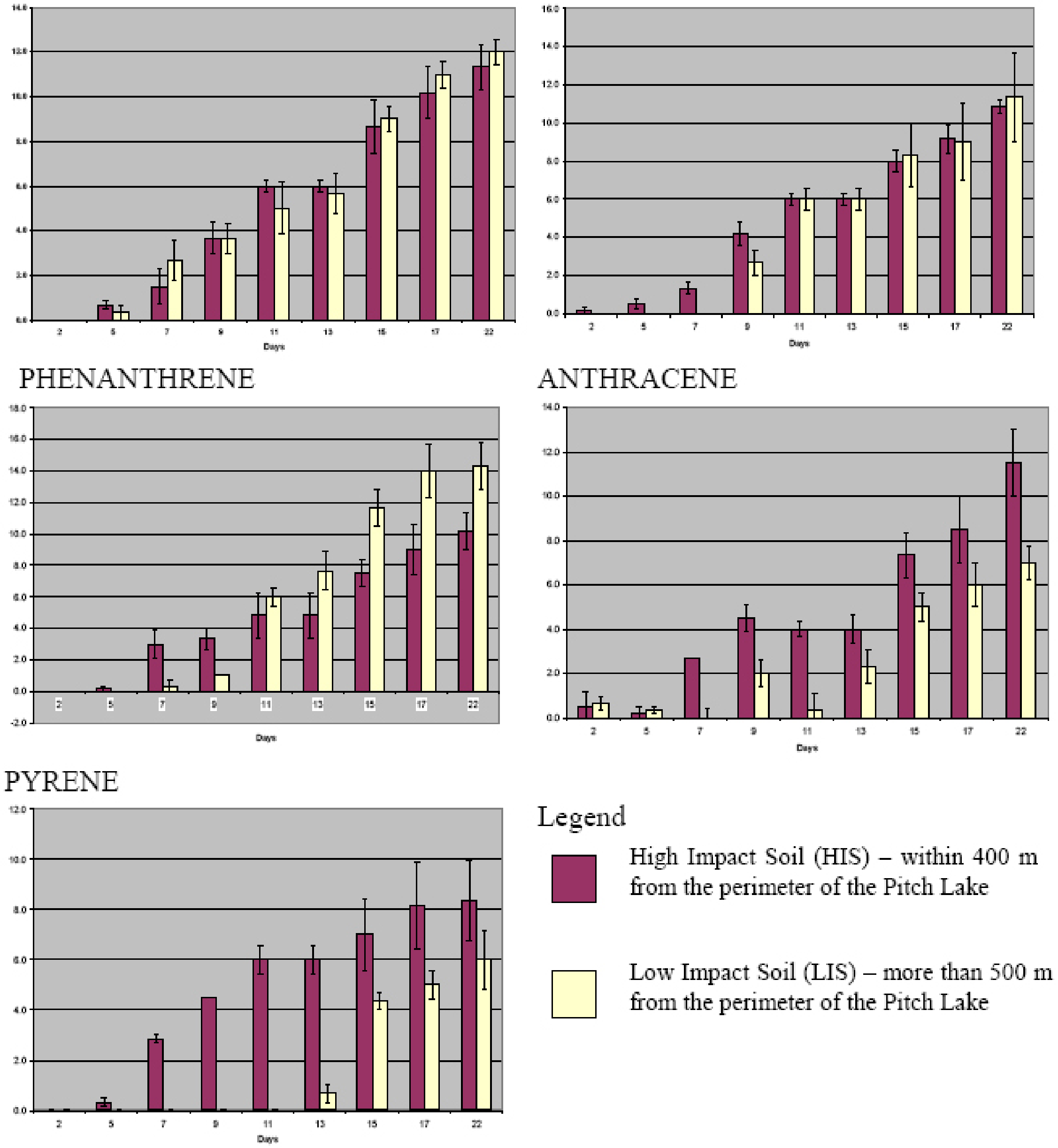


Fig3. *Changes in the mean number of bacterial clear zones formed for the HIS and LIS in each PAH enriched medium over a 22-day period*

The ANOVA of results showed that the level of $\sum$5PAHs was significantly ($p<0.05$) lower in Zone 3 compared to Zones 1 and 2. Such that the soil samples from these two zones were grouped, and collectively referred to as the High Impact Soils (HIS) and the soil samples from Zone 3 was separately referred to as the Low Impact Soils (LIS).

For the PAH-degrading bacteria, the lower molecular weight PAHs (NAP and ACE), showed no notable differences in the number of clear zones formed (an indicator of PAH utilization) in either the High Impact Soils (HIS – within 400m of the Pitch Lake) and the Low Impact Soils (LIS – more than 500m away from the

Pitch Lake) by bacterial colonies over a 22-day period (Figure 2). However, as the aqueous solubility and recalcitrance to biodegradation increasedof the PAHs, so does the number of bacterial clear zones formed in the HIS compared to the LIS (PHE, ANT and PYR). This supports the thought that PAH degraders exist in most soils as a normal part of the soils flora. However, for the higher molecular weight PAHs like (PHE, ANT and PYR) we note that the higher number of clear zones were seen in the HIS, suggesting that for the isolation of bacteria capable of degrading more complex PAHs, tends to be associated with a higher PAH impacted soil.

It was noted that the levels of the total PAHs (∑5PAHs) were not significantly different from each other in Zones 1 and 2 (Table **2**), so that soil samples from these two zones were grouped, and collectively referred to as the High Impact Soils (HIS). The ANOVA of results showed that the level of ∑5PAHs was significantly ($p<0.05$) lower in zone 3 compared to zones 1 and 2. Thus, the soil samples from zone 3 were separately referred to as the Low Impact Soils (LIS).

For the lower molecular weight PAHs (NAP and ACE), there were no notable differences in the number of clear zones formed (an indicator of PAH utilization) in either the High Impact Soils (HIS – within 400m of the Pitch Lake) and the Low Impact Soils (LIS – more than 500m away from the Pitch Lake) by bacterial colonies over a 22-day period. However, as the aqueous solubility and recalcitrance to biodegradation increases for the PAHs so does the number of bacterial clear zones formed in the HIS compared to the LIS (PHE, ANT,and PYR). This supports the thought that PAH degraders exist in most soils as a normal part of the soils flora. However, for the higher molecular weight PAHs like (PHE, ANT,and PYR) we note that more clear zones are seen in the HIS, suggesting that for the isolation of bacteria capable of degrading more complex PAHs, tends to be associated with a PAH impacted soil.

Concluding Remarks

Our results showed that the levels for most of the individual and total PAHs were generally above the acceptable limits, even in the reference zone (> 500m away from the pitch lake). The La Brea Pitch Lake also seemed to influence the levels of NAP in its surrounding soils as is observed by a progressive decrease in concentration, as distance increased from the homogenous source of PAHs (La Brea Pitch Lake). This is further supported by trends in the general decrease in the levels of PHE, ACE,and ANT as proximity increased from the La Brea Pitch Lake. Also, that the lack of significant difference among zones may be due to the high standard error associated with the sample means for the majority of parameters measured and this may be attributed to the presence of pitch dykes (“veins”), which may have skewed the levels of the physicochemical parameters tested.

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