PAHs Utilizing Bacterial Population and Physico-chemical Variability in Oil Contaminated Soils

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Abstract: Poly-aromatic hydrocarbons (PAHs), the known xenobiotic agents, persist as potentially hazardous soil contaminants. Various bioremediation strategies (indigenous microflora, introduced microorganisms, priming with bioremediated soil), applied for their removal from polluted soils, have rarely been successful. Biostimulation (nutrient amendment) and bioaugmentation (suitable microbial population) have shown accelerating effect on naturally occurring biodegradation of PAHs. However, a plausible bioremediation tool is still awaited. A study was conducted to examine PAH utilizing population and physico-chemical characteristics of the oil contaminated soil from different geographical locations. This could evolve an effective soil inoculum, with defined soil properties, for use as a bioremediation and bioaugmentation tool. Sixteen oil contaminated soil samples (AS-1 to AS-16) were collected from different sites under varying geographical locations in India. All the samples were analyzed for moisture content, pH, carbon (%), total organic content (TOC), inorganic phosphate, nitrate, poly-aromatic hydrocarbons (PAHs) and PAHs utilizing bacterial populations. All the samples showed different soil characteristics. AS-15 exhibited maximum pH (9.82) where as minimum pH (3.47) was observed in AS-8. The soil sample (AS-4), with higher content of PAH, Chloride, inorganic phosphate, TOC, % carbon, moisture and pH (7.45) was found to harbor comparatively higher PAH utilizing bacterial population. The results of this study highlight the possible higher degradation potential of PAHs utilizing bacteria under natural conditions. This study provides a baseline to understand the population dynamics, possible biodegradation potential of oil contaminated soils, at different locations, and may be used to prepare an appropriate inoculum with defined soil characteristics to evolve a plausible bioremediation technology.

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Key words: poly-aromatic hydrocarbons (PAHs), carbon, total organic content, oil contamination

1. Introduction

Poly-aromatic hydrocarbons (PAHs) have been identified as xenobiotic agents causing toxic, carcinogenic and tetragenic effects on life (Ruma et al., 2007). United States Environmental Protection Agency (USEPA) has designated sixteen PAHs (naphthalene, acenaphthylene, acenaphthene, fluorine, phenantherene, anthracene, fluoranthene, pyrene, benz-a-anthracene, chrysene, benzo-b-fluoranthene, benzo-k-fluoranthene, benzo-a-pyrene, dobenz-a-h-anthracene, benz-g-h-i-perylene and indeno-1,2,3-cd-pyrene), as priority pollutants. They originate from incomplete combustion of fossil fuels, oil spoilage near to oil reservoirs, forest fire and pyrolysis of organics (Kanaly et al., 1997). These are lipophilic in nature and are relatively insoluble in water, and persist as potentially hazardous soil contaminants (Johnsen et al., 2005, Hafez et al., 2008). Several studies such as the use of indigenous microflora, introduced microorganisms, priming with bioremediated soil and treatment with nitrogen, phosphorus and potassium have been carried out for the degradation or removal of PAHs from polluted soils (Juhasz et al., 2005, Johnsen et al., 2005, Johnsen et al., 2007, Hafez et al., 2008). These studies have rarely been promising. The physico-chemical factors control microbial metabolic and nutritional requirements, and the availability of pollutants to the microbial population (Lion, 1990, Amellal et al., 2001, Johnsen et al. 2005). Thus, de-aromatization of the PAHs could be influenced by the physico-chemical and biological characteristics of the soil. In present study, an effort has been made to determine the PAH utilizing population and the physical/chemical properties of different oil contaminated soils. This work provides a baseline to understand population dynamics and possible biodegradation potential of oil contaminated soils at different geographical locations. The findings would provide an appropriate soil inoculum with defined soil characteristics, and explore further possibilities to evolve a plausible bioremediation technology.

2. Materials and Methods

2.1 Chemicals and Reagents

All the chemicals used in this study, were of analytical grade. Anthracene was obtained from Sigma-Aldrich (Copenhagen, Denmark), and other chemicals and media were obtained from Himedia (India).

2.2 Collection of Soil Samples

Subsurface (1-10 cm below the surface) oil contaminated soil samples were collected in sterile plastic bottles from different sites. The samples were coded as AS-1 to AS-16 and were stored at low temperature (4^0C) for further investigations.

2.3 Moisture Content

10 g soil of each sample was weighed (w_1) and dried at 100ºC in a hot air oven (Narang Scientific Works, New Delhi.) for 24 hours. The final weight (w_2) of each sample was recorded using an electronic weighing balance (Sansui, Japan), and the moisture content (w_1-w_2) was determined as mg of moisture per gram soil.

2.4 pH

10% (w/v) suspension of air dried soil of each sample was prepared in de-ionized milli-Q water. This suspension was allowed to settle for one hour and filtered through Whatman filter paper no.42. pH for all the soil filtrates was checked using a calibrated pH meter.

2.5 Total Organic Content (TOC)

250 mg of air dried soil of each sample was taken in 250 ml conical flasks, and 5 ml of 1N potassium dichromate solution was added. After that, 10 ml concentrated sulfuric acid was added gradually and the contents were incubated for 30 minutes at room temperature. Then, 100 ml de-ionized water, 5 ml of concentrated Phosphoric acid, 0.1 g of dry sodium fluoride and 0.5 ml of diphenylamine indicator were added sequentially. The contents of the flask were titrated against 0.5 N ferrous ammonium sulfate. The end point was noticed as dull green through turbid blue to brilliant green. Distilled water blank was run simultaneously, and the TOC was calculated as described by Hooda and Kaur, 1999.

TOC (mg/g soil) = 6.791/W (1-T_1/T_2) X 10

Where, T_1= Volume of titrant used against samples (ml)

T_2= Volume of titrant used against distilled water blank (ml)

2.6 Poly-aromatic Hydrocarbons (PAHs)

100 ml solvent (n-hexane & dichloromethane, v: v = 1:1) was added to 5 g of soil sample in a soxhlet. After 14 h, contents of the flask were evaporated and concentrated exactly to 3 ml. The PAH content in each soil sample was measured by observing absorbance at 220 nm. (Tsai et al. 2002).

2.7 Inorganic Phosphate Content (PO_4^{-3})

Stock solution was prepared by dissolving 136 mg of KH_2PO_4 in 100 ml distilled water, and diluted ten times. Different volumes viz. 0.2, 0.4, 0.6, 0.8 and 1.0 ml of this solution were taken and the final volume was made to 1.0 ml with distilled water. Simultaneously, the test sample was also prepared by adding 0.1 ml. soil suspension (10% w/v) to 1.0 ml. distilled water. Then, added 1 ml 5 N H_2SO4, 1 ml of $(NH_4)6Mo_7O_{24}.4H_2O$ and 0.1 ml ANSA to each test tube containing stock and test sample, and waited for 10 minutes. Final volume in all the test tubes was made to 10 ml and the absorbance (O.D) was measured at 690 nm. (Hooda and Kaur, 1999).

2.8 Nitrate Content (NO_3^-)

10% (w/v) soil suspension was prepared in de-ionized water and was filtered through Whatman filter paper no 42. The filtrate was treated with 0.4 ml concentrated aluminium hydroxide suspension to remove color and avoid the organic interference from the filtrate. The mixture was swirled; the suspension was allowed to settle for 5 minutes, and filtered through Whatman filter paper no 42. Then, 0.1 ml of 1 N HCL was added to 5 ml clear filtrate. A nitrate calibration curve was plotted using a standard curve in range of 0-350 µg of nitrate. The absorbance was recorded at 220 nm and 275 nm (Hooda and Kaur, 1999).

2.9 Chloride Content (Cl^-)

100 ml (10%) soil suspension was taken and the pH was adjusted to 7.0. Then, added 9.0 ml of 5% K_2CrO_4, stirred well and titrated with 0.0282 N $AgNO_3$ to a permanent reddish tinge. The chloride content was calculated as outlined by Hooda and Kaur, 1999.

Chloride (mg/g) = [Volume of $AgNO_3$ used against samples (ml) - Volume of $AgNO_3$ used against blank (ml)] X [Normality of $AgNO_3$] X 35.46 X 1000/ mg of soil sample taken.

2.10 Total Colony Forming Unit (CFU)

10% (w/v) soil suspension in sterile normal saline was serially diluted under aseptic conditions. The dilution was inoculated on nutrient agar media using spread plate method, and incubated. The results were recorded as total colony forming units (CFU) per gram of soil sample (Aneja, 2002).

2.11 PAHs Utilizing Bacterial Population

10% (w/v) soil suspension in sterile normal saline was serially diluted under aseptic conditions. The dilution was inoculated on a synthetic basal salt mineral medium (BSM medium: K_2HPO_4 - 0.38g/l, $MgSO_4.7H_2O$- 0.2 g/l, NH_4Cl - 1.0 g/l, $FeCl_3$ - 0.05 g/l; pH- 7.0) using spread plate method. 0.05% (w/v) anthracene in diethyl ether (DEE) was sprayed on the plates as a carbon source. The plates were incubated at 37^0C. The results were recorded as PAH utilizing bacterial population (CFU) per gram of soil sample (Aneja, 2002).

3. Results and Discussion

Table.1 describes all the sixteen soil sampling sites. Among all the samples analyzed for pH, four samples (AS-1, AS-12, AS-15, AS-16) were found to show pH above 8.0. The samples, AS-7 to AS-9 were acidic in nature. However, nine samples were found to have pH between 7 to 8 (Table 2). AS-8 showed minimum pH (3.47). The maximum pH (9.82) was measured for AS-15 (Table 2a , 2b).

Table 1. Description of Soil Samples

Sample code	Site & expected time of oil contamination	Locations(latitude/longitude/ elevation)	Collection Site
AS-1	Workshop & 21 years	30º17'41.52"N/78º03' 25.99"E/2078ft	Dehradun, Uttrakhand, India
AS-2	Workshop & 20 years	30º17'39.20"N/ 78º 03' 49.08"E/2109ft	Dharampur, Dehradun, Uttrakhand, India
AS-3	Workshop & 21 years	30º17'27.55''N/78º03'42.90'' E/2095ft	Dharampur, Dehradun, Uttrakhand, India
AS-4	Railway Engine repairing site & 100 years	29º56'53.82''N/78º09'20.1"E /964ft	Haridwar Uttrakhand, India
AS-5	Workshop & 15 years	29º22'51.30''N/78º88'22.82" E/771ft	Bijnor, Uttar predesh, India
AS-6	Workshop & 14 years	29º22'50.05''N/78º08'21.64'' E/775ft	Bijnor, Uttar predesh, India
AS-7	Workshop & 16 years	29º22'25.05''N/78º48'29.04'' E/774ft	Bijnor, Uttar predesh, India
AS-8	Service centre & 16 years	29º22'34.21''N/78º08'45.45'' E/777ft	Bijnor, Uttar predesh, India
AS-9	Service centre & 15 years	29º18'52.69''N/79º30'16.98'' E/769ft	Dhampur, Uttar predesh, India
AS-10	Workshop & 15 years	29º19'23.08''N/78º29'53.78'' E/773ft	Dhampur, Uttar predesh, India
AS-11	Service centre & 12 years	29º19'17.00''N/78º29'51.45'' E/772ft	Dhampur, Uttar predesh, India
AS-12	Service centre & 9 years	23º02'00.11''N/72º33'58.32'' E/176ft	Ahemdabad, Gujrat, India
AS-13	Petrol pump & 8 years	23º02'60.41''N/72º13'51.12'' E/172ft	Ahemdabad, Gujrat, India
AS-14	Petrol pump & 10 years	25º12'04.03''N/85º31'03.70'' E/197ft	Purnia, Bihar, India
AS-15	Petrol pump & 20 years	25º12'10.20''N/85º31'13.08'' E/201ft	Purnia, Bihar, India
AS-16	Sulphur water & 100 years	30º23'09.41''N/78º07'51.21'' E/2822ft	Shashtradhara, Dehradun, Uttrakhand, India

Table 2a. Physico-Chemical analysis of soil samples

Sample Codes	pH of the Samples	Moisture Content (mg/gm soil)	Carbon %	Total organic matter (mg/gm soil)
AS-1	8.09	116±0.48	6.93	119.5±0.015
AS-2	7.58	88±0.35	9.60	165.6±0.024
AS-3	7.40	111±0.38	12.12	209.13±0.042
AS-4	7.45	190±0.33	12.44	214.56±0.350
AS-5	7.59	114±0.39	5.04	86.91±0.078
AS-6	7.99	13±0.14	8.03	138.51±0.022
AS-7	3.55	58±0.22	5.67	97.77±0.042
AS-8	3.47	93±0.42	3.94	67.90±0.056
AS-9	6.27	101±0.28	3.30	57.03±0.072
AS-10	7.55	85±0.28	5.35	92.34±0.003
AS-11	7.51	86±0.54	3.78	65.18±0.054
AS-12	8.36	113±0.27	4.25	73.33±0.011
AS-13	7.48	23±0.32	9.13	157.52±0.028
AS-14	7.52	65±0.38	4.88	84.19±0.088
AS-15	9.82	44±0.26	6.30	119.50±0.008
AS-16	8.37	96±0.26	4.23	82.22±0.102

Table 2b. Physico-Chemical analysis of soil samples

Sample Codes	Inorganic PO_4^{-3} (mg/gm soil)	NO_3^- (mg/gm soil)	Cl^- content (mg/gm soil)	PAHs Content (ppm)	PAHs utilizing Bacterial population
AS-1	12.40±0.004	2.56±0.002	123.60±0.35	27.817±0.004	$102X10^3$
AS-2	17.60±0.014	0.15±0.006	476.98±0.31	56.759±0.008	$150X10^3$
AS-3	10.20±0.026	0.001±0.000	527.09±0.45	85.701±0.009	$43X10^3$
AS-4	18.60±0.048	0.002±0.000	544.44±0.50	131.567±0.002	$163X10^3$
AS-5	01.60±0.002	2.5±0.004	12.06±0.64	31.471±0.001	$19X10^3$
AS-6	15.20±0.066	2.4±0.005	56.24±0.66	56.759±0.009	$17X10^3$
AS-7	10.40±0.089	3.5±0.004	102.04±0.24	120.22±0.007	$63X10^3$
AS-8	07.40±0.033	3.54±0.004	66.82±0.54	62.432±0.008	$13X10^3$
AS-9	14.60±0.018	1.0±0.007	65.67±0.33	120.22±0.004	$6X10^3$
AS-10	04.20±0.018	2.52±0.003	114.0±0.31	120.22±0.002	$4X10^3$
AS-11	01.40±0.049	0.32±0.006	86.87±0.19	20.028±0.007	$57X10^3$
AS-12	02.80±0.076	1.42±0.008	96.0±0.19	29.836±0.008	$42X10^3$
AS-13	02.00±0.008	3.42±0.006	50.36±0.34	46.663±0.004	$24X10^3$
AS-14	00.08±0.002	2.51±0.004	147±0.64	30.798±0.002	$54X10^3$
AS-15	00.06±0.002	4.53±0.003	42.66±0.44	104.548±0.006	$8X10^3$
AS-16	02.55±0.004	2.33±0.003	42±0.26	39.451±0.004	$11X10^3$

The moisture content measurement showed maximum moisture (190 mg/g) for AS-4. The moisture content in AS-6, recorded as the minimum, was about 1/15th of the highest value (Table 2). The carbon content ranged between 3.30 (AS-9) to 12.44% (AS-4) for different soil samples. Among all the soil samples, minimum total organic content was 57.03 mg/g soil (AS-9), and the maximum was found to be 214.56 mg/g soil for AS-4. The nitrate content of AS-3 and AS-4 was negligible; AS-15 showed highest (4.53 mg/g soil) value. The inorganic phosphate content for different soil samples varied from 0.06 mg/g soil (AS-15) to 18.60 (AS-4). The chloride content was estimated to be maximum (544.44 mg/g) in AS-4.

The poly-aromatic hydrocarbon (PAH) content was 131.567 ppm in AS-4. The lowest PAH content was

observed in AS-11. Similarly, the PAH population was highest (163X10^3/g soil) in AS-4. Only 4X10^3/g, PAH utilizing bacteria were found in AS-10. It was further noticed that there was significant variation of PAH utilizing population with respect to chloride/PAHs (Figure. 1), total organic matter/nitrate (Figure. 2) and inorganic phosphate/moisture (Figure. 3). This variation can be attributed to different soil properties and geographical location.

The present studies highlight that all the samples, collected from different locations, vary in their soil parameters. The distribution of organic matter, humus substances, xenobiotics and microorganisms have been found to be affected by soil structure (Jocteur-Monrozier et al, 1991; Christensen, 1992; Kukkonen and Landrum, 1996). It is evident from the findings that the samples collected from site AS-4 (pH 7.45) were rich in soil properties viz. PAH content, chloride, inorganic phosphate, total organic content, carbon (%) and moisture content, as compared to all concentration and the number of PAH degrading bacteria has earlier been reported (Amellal et al., 2001). The highest values of PAH and PAH utilizing bacteria in AS-4 are also suggestive of intermediate aggregate size soil, as evidenced by Amellal et al., 2001. The results indicate possible higher PAH degradation potential of AS-4. Johnsen and Karlson, 2005 found that the PAH degradation potential of PAH polluted soil was also linked to the level of PAH pollution. Our results corroborate with earlier findings that the indigenous PAH degraders respond favourably, when the growth conditions improve (Hestbjerg et al, 2003, Juhasz et al., 2005). The improved levels of soil parameters might be a favorable set of growth conditions for the PAH utilizing bacteria.

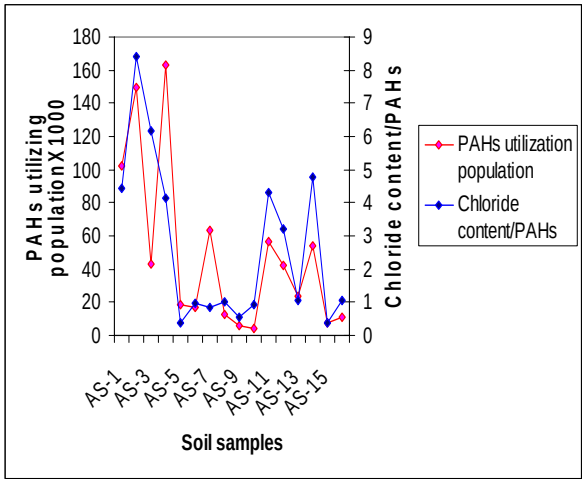

Figure 1: PAHs utilizing population kinetics with Chloride content/PAHs.

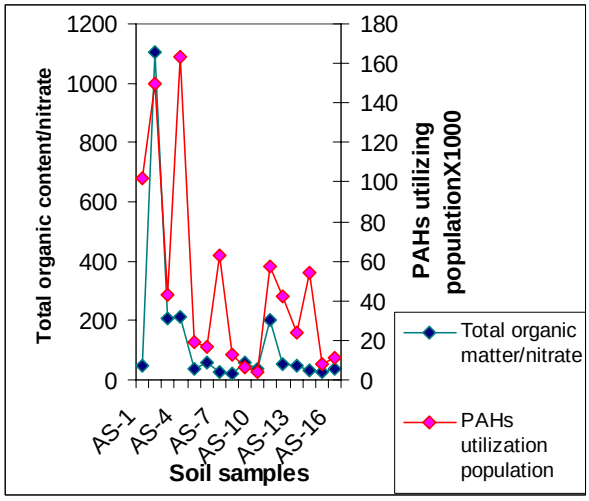

Figure 2: PAHs utilizing population kinetics with Total organic content/nitrate.

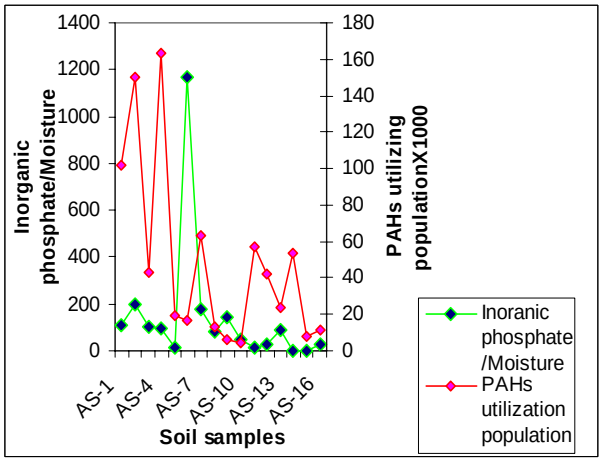

Figure 3: PAHs utilizing population kinetics with Inorganic phosphate/Moisture.

4. Conclusion

Results of this study emphasize on the importance of soil characteristics on PAH utilizing population. This can contribute to develop an effective soil inoculum for use in bioaugmentation and bioremediation of oil contaminated soils. However, in depth studies would be required to establish the bioavailability of the polluting PAHs and their effective biodegradation, to design new protocols and strategies, which could curb the adverse effects of hazardous soil pollutants.

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