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Dynamics of Polyaromatic Hydrocarbon Degradation Under Monitored Bioattenuation System

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Abstract: *Bioattenuation encompasses processes that lead to reduction of the mass, toxicity, mobility, or volume of contaminants without human intervention. This study was designed to determine the dynamics of polyaromatic hydrocarbon degradation under a monitored bioattenuation bioremediation system. Preliminary analysis was conducted to determine the extent of pollution by chemical analysis and to enumerate the microbiological population in the sample by standard microbiological methods. The identities of the hydrocarbon degrading bacteria population were ascertained using 16SrRNA molecular typing algorithm. The experimental duration was 35 days and the microcosm was monitored at weekly interval under a controlled system. A 4% spike in crude oil level was conducted to increase the concentration of PAH in the sample to 989.1 mg/kg. Total viable heterotrophic and hydrocarbon degrading bacteria populations were CFU X 108g and the dominant class of microbes was Grammaproteobacteria and genus Pseudomonas. The overall degradation rate was recorded at 52%. Anthracene was 100% degraded while benzo(b)fluoranthene, fluorine and pyrene had above 50% removal. All other PAHs present in the sample experienced some level of degradation except acenaphthylene, dibenz (a,h)anthracene and chrysene which increased slightly in concentration. The natural attenuation process was effective in degradation of the pollutant within the experimental period.*

Keywords: *Polycyclic aromatic hydrocarbon, bioattenuation, bioremediation, bacteria, degradation*

1. INTRODUCTION

The growth and activities of petroleum and petroleum associated industries in Nigeria and in other parts of the world have led to increased pollution. In Nigeria, the exploration for, and exploitation of, crude oil has been mainstay for over five decades [1, 2] accounting for more than 90% foreign exchange as well as 90% of environmental degradation and socio-economic degeneration [3]. The incidences of oil spills have however, negatively impacted the Niger Delta communities and environment in particular so much to be described as one among the worst impacted zones globally.

Petroleum contaminants include aliphatic, monoaromatic BTEX (benzene, toluene, ethylbenzene, xylene) and polyaromatic hydrocarbon (PAH) compounds which can be found in crude and refined oils in different ratios. Several researchers have found that crude oil contains PAHs which have toxic effects, such as immunotoxicity, embryonic abnormalities, and cardiotoxicity for humans and animals [4, 5, 6]. The Environmental Protection Agency (EPA) have identified 16 PAHs as priority pollutants, because they show greater toxicity than others. Some of these PAHs are toxic, mutagenic, and carcinogenic and they are all highly hydrophobic and thus raise considerable environmental concern [7].

PAHs released into different environmental compartments can be removed through different bioremediation processes such as biostimulation, bioaugmentation and bioattenuation. Bioremediation is the application of the metabolic capacity of biological systems (plants and microbes) to degrade hazardous substances into less toxic or innocuous ones in the environment and has gained popularity in the global conservation and environmental sustainability strategies. It is a natural process that takes advantage of nature's recycling and self-purification capabilities and as such is accepted by the public for cleanup of polluted ecosystems [8].

Biaottenuation or monitored natural attenuation (MNA) is an in-situ bioremediation technology that relies on the activities of naturally occurring hydrocarbon degrading microbes without human intervention under favourable environmental conditions reduces the mass and concentration of the contaminants in the environment [9, 10]. Bioattenuation has been

recorded to be increasing in use as a low cost means of remediating contaminated soil compared to other bioremediation approaches. The prospect of more efficient, economically advantageous management strategies has driven the development of contaminated land management concepts particularly for large and complex contaminated sites during the last years. Consequently, the conscious and controlled use of naturally occurring degradation and retardation processes of pollutants in soil (monitored natural attenuation—MNA) has gained increasing attention [11]. Bioattenuation makes use of processes such as biodegradation; dispersion; dilution; sorption; volatilization; and chemical or biological stabilization, transformation, or destruction of contaminants to clean up contaminated soils thereby reducing levels of risk to human health and the environment. This approach has gained wide acceptance for restoring the hydrocarbon contaminated soils because they are cost-effective.

One of the most important components of natural attenuation is biodegradation and this biological treatment of an oil-contaminated soil can be affected by hydrocarbons structure and the characteristics of the soil. Picariello et al. [12] investigated indigenous microbial community involvement in soil PAH degradation. The obtained findings show a capability of indigenous native microbial community to degrade almost completely PAHs after one year, with phenanthrene and pyrene degrading faster than benzo[a]pyrene, according to their molecular weight.

Hydrocarbons interact with the environment and environmental microorganisms determining the fate of the contaminants relative to their chemical nature and microbial degradative capabilities, respectively. Chikere et al. [13] reported that bioremediation method can be very successful in the detoxification of polluted sites provided the polluted environment has requisite values for environmental factors that influence microbial activities and there are no inhibitors. It is worthy of note that petroleum hydrocarbon-metabolizing microorganisms are widely distributed in nature. These microorganisms could include bacteria, fungi, cyanobacteria and microalgae and are actively involved in the process of mineralization of hydrocarbon pollutants. The role of several indigenous hydrocarbon microbial groups in PAH natural attenuation could be a very effective method of cleaning up spill site even without human intervention.

From literature, PAHs released into the environmental compartments were removed through different bioremediation processes however, the dynamics of the individual PAH with regards to Nigeria oil pollution have not been critically investigated and analysed. The objective of this study is to analyse the dynamics of individual PAH degradation by indigenous microbial communities during a bioattenuation study.

2. METHODOLOGY

2.1 Sample Collection and Characteristics

Soil sample was obtained from an aged crude oil spill site in Komkom community, Rivers State, Nigeria. The soil was spiked with 4% crude oil to increase the concentration of hydrocarbons in the soil. The soil was characterized and baseline properties obtained to reveal the extent of contamination and to determine the physicochemical properties of the soil.

2. 2 Microbiological and Molecular Analysis

Microbial enumeration is used as a screening tool to determine the population of micro-organisms in a sample. Microbiological analysis was conducted to reveal the microbial community population of both heterotrophic and hydrocarbon degraders. Ten-fold serial dilution was performed using 1g of the soil sample and 0.1 ml of 10^{-4}, 10^{-5} and 10^{-6} dilutions were plated out in triplicates on plate count Agar. After 24hrs at 37^{o}C, viable counts were determined and expressed as colony forming units per gram (CFU/g).

Also, distinct morphologies of colonies were selected on the basis of their abundance, and were cultured on Nutrient agar medium until their purity was determined. Purified bacteria were identified by 16S rDNA gene sequencing after the amplification of the gene by polymerase Chain Reaction (PCR) using a MinicyclerTM (MJ Research). An almost full-length bacterial 16S rDNA sequence (1500 bp) was amplified using primers 27F and 1492R, and purified using the an ABI 3130 XL genetic analyzer incorporating the ABI Big Dye Terminator Kit (Amersham Biosciences) for automated DNA sequencing. 1Amplified DNA was examined by electrophoresis in 1.5% agarose gel with 2 µl aliquots of PCR products in 1x Tris-Acetate-EDTA buffer and visualized on UV-trans illuminator. Then, 16S rDNA sequences showing the same profile were grouped and considered a unique ribotype. Only one representative of each ribotype was sequenced. The 16S rDNA sequences were obtained using an ABI PrismTM 3100 Genetic Analyzer (Perking Elmer) and were compared with the GenBank sequences; this was done using the USA National Center for Biotechnology Information NCBI´s Basic Local Alignment Search Tool (BLAST).

2.3 Chemical Analysis of Soil Sample

Residual PAH was extracted from the samples and quantified using gas chromatograph-flame ionization detector (GC-FID). Residual PAHs were extracted with 20 ml of n-pentane from 10 g of each soil sample. The samples were shaken for 15min by the ultrasonic apparatus and allowed to settle for 60min at room temperature. Organic extract (10 ml) was transferred into a vial and analyzed by GC-FID. GC-FID analysis of the PAH was done on a Hewlett Packard 5890 Series II-Plus gas chromatograph equipped with an HP 7673 autosampler and FID detector coupled with a 30´0.32 mm DB-5 (5% phenyl, 95% methyl-polysiloxane) fused silica capillary column. The oven temperature was programmed from 40°C (3 min.) to 300oC at 15°C/min. Samples were injected in splitless mode, with the relay open at 20S. Injector and detector temperatures were 250 and 320°C, respectively. Helium was used as the carrier gas at a linear velocity of 38 cm sec-1 (15 psig). Data

handling was done with Agilent Chemstation chromatography software (version 10). For quantification purposes, PAH area of each peak was calculated using the baseline-baseline mode and external response factor quantization [14].

2.4 Microcosm Experimental Setup

In order to determine the degradation potentials of the naturally occurring hydrocarbon degraders in thse soil, the crude oil polluted soil sample was tested under laboratory conditions. Crude oil polluted soil (3kg) was dispensed into a well aerated container with 50% water holding capacity under a controlled greenhouse system. The setup was frequently tilled with a sterilized hand trowel to maintain aerobic conditions. At weekly intervals from day 0 to day 35, samples were collected for chemical analysis using GC-FID.

2.5 Statistical Analyses

One-way analysis of variance (ANOVA) and multiple comparison tests (posthoc) were used to determine level of significance of the data generated in this study. The analysis was carried out using Statistical Package for Social Science (SPSS) Version 20.0. Descriptive statistics such as mean, standard deviation, and standard error of means were carried out.

3. RESULTS AND DISCUSSION

3.1 PAH concentrations in soil sample

The result for PAH analysis revealed the presence of sixteen (16) Polycyclic hydrocarbons ranging from low molecular to high molecular weight hydrocarbons all of which make up the sixteen high priority pollutants as designated by the Environmental Protection Agency [15]. These PAHs are of environmental concern because of their potential toxicity in humans and other organisms as well as their prevalence and persistence in the environment. In this study, The highest PAH concentration was recorded in Benzo(a)anthracene (115.83669 mg/kg; 23%) while anthracene and naphthalene had the least concentrations (13.00487 m/kg (1%)) (Figure 1). A research conducted by Adekunle et al. [16] on biodegradation of petroleum polluted soils, revealed the presence of five PAHs also detected in this study and benzo(g,h,i)perylene topped the chart. The total PAH concentration in the soil sample as revealed by GC-FID analysis was 989.1 mg/kg indicating a high level of PAH contamination which required remediation or detoxification. The 4% soil spiking with crude oil successfully increased PAH concentration to support and validate the reason for bioremediation of the polluted sample using the bioattenuation approach. The soil spiking also allowed for effective monitoring of the individual PAH degradation dynamics from day 0 to 35. In Nigeria, the intervention limit for PAH contamination as set out by Environmental Guidelines and Standards for the Petroleum Industry in Nigeria (EGASPIN) is 40mg/kg [17] This information reveals that the soil sample after spiking was ideal for this study.

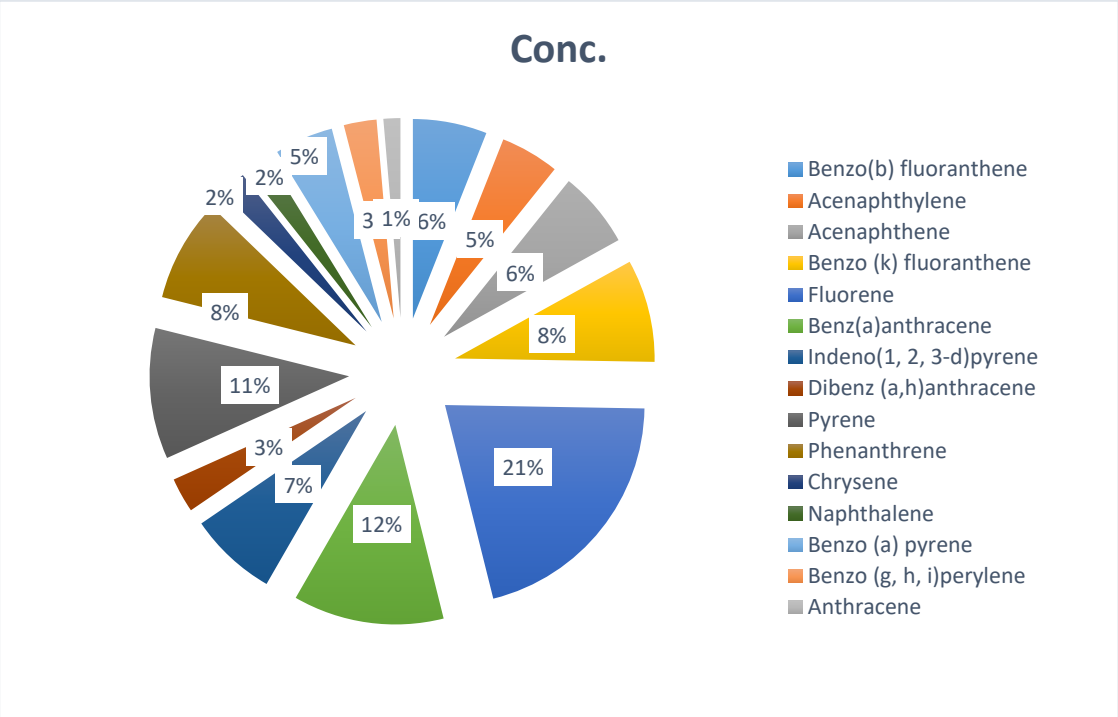

Figure 1. Percentage composition of PAH compounds relative to the total PAH found in the petroleum-impacted soil

3.2 Microbial Community Profile in the Soil Sample

The microbiological assay revealed significant microbial availability and activity in the soil sample. The total culturable heterotrophic and hydrocarbon utilizing bacteria counts are presented in Table 1. The results indicates that the indigenous

hydrocarbon degraders are available in appreciable quantity which is typical of an aged spill site and also supports the natural attenuation process whereby the microbes are involved in the mineralization of the crude oil into CO_2 and water.

No significant difference ($P \leqslant 0.05$) was observed between total hetrotrophic and total hydrocarbon degrading populations. This observation is in line with Teknikio et al. [18] and Chikere et al. [19]. In this study Eight (8) known hydrocarbon degrading bcateria were isolated and identified by 16S rRNA typing and Pseudomonas was recorded as the most dominant genera while Gammaproteobacteria the most dominant class. Microorganisms with the capacity to degrade hydrocarbons are among the best-studied microbial groups in applied and environmental microbiology. Indeed, more than 200 bacterial, algal, and fungal genera, encompassing over 500 species, have been recognized as capable of hydrocarbon degradation [20, 21]. There is no single bacterium capable of degrading all the oil components. We observed that the eight HUBs isolated belonged to five genera with expected different substrate specificity based on the genetic capabilities of the organism [22].

Oil degrading bacteria or hydrocarbon degrading bacteria are ubiquitous in areas when after an oil spill has occurred. The influx of oil causes the immediate increase in the number of hydrocarbons degrading populations. The degradation of both crude and refined oils seems to involve a consortium of microorganisms as evidenced in this study. The most common genera known to be responsible for oil degradation comprise mainly *Nocardia, Pseudomonas, Acinetobacter, Flavobacterium, Micrococcus, Staphylococcus, Enterocabter, Arthrobacter, Corynebacterium, Achromobacter, Rhodococcus, Alcaligenes, Mycobacterium, Bacillus* amongst others [23, 24]

Table 1: THB and THUB counts from soil sample

Microbiological Parameters	**Counts**
Total heterotrophic bacteria	$3.11x10^8$
Total culturable hydrocarbon utilizing bacteria	$2.43x10^8$

3.3 Degradation Dynamics of Individual PAH Undergoing Natural Attenuation

Soil contaminated by polycyclic aromatic hydrocarbons (PAHs) can be candidates for remediation via an approach like monitored natural attenuation. Monitored natural attenuation involves the involvement of indigenous oil degrading microbes in the breakdown of hydrocarbon related compounds. Although the natural attenuation process is markedly slower than another bioremediation approaches, it can be enhanced to facilitate the process. In this study, the microbiological analysis revealed that active known hydrocarbon degraders were harboured in the soil sample in appreciable quantity which is the most important requirement for a successful natural attenuation process to occur. The experiment for a 35-day period and samples were collected weekly for chemical analysis. At the end of day 35, results show variations in the degradation pattern of the individual PAH components which could be attributed to the different molecular weight, structural angularity and number of rings of the different PAHs [25] The results in Figure 2. Shows the degradation profile of the various PAHs at different sampling days for a duration of 35 days. Benzo(a)fluoranthene, benzo(k)fluoranthene, indeno(1,2,3, d)pyrene, pyrene, Naphthalene showed a similar trend of PAH reduction from day 0 to 35 while all other PAH components showed fluctuations and inconsistencies in the in the degradation process.

However, it was observed that Anthracene had 100% removal at day 35 while Benzo(b) fluoranthene, fluorine and pyrene had above 50% removal (Figure 3). Acenaphthylene, Dibenz (a,h)anthracene and chrysene increased in concentration from the initial value which could be as a result of the breakdown product from other hydrocarbons increasing the concentration of other lower PAHs. Based on statistical analysis, significant difference ($P \leqslant 0.05$) was observed in the PAH concentration between day 0 and day 35. Kosnar et al. [26] conducted a study on the bioremediation of PAHs using different biostimulation approaches and natural attenuation for 120 days. In the experiment the initial total PAH content significantly decreased ($P < .05$) in all treatments including natural attenuation after 120 days. However, compared to other treatments NA had the least degradation rate. The removal of PAH in the other treatments was enhanced by addition of stimulants, nutrient and other growth factors that facilitate the activity of indigenous microorganisms or the introduction of PAH degrading microbes to augment the existing population in the sample [27]. On the other hand, the removal of PAHs from natural attenuated soils is mainly caused by autochthonous microorganisms and abiotic losses of LMW PAHs [28].

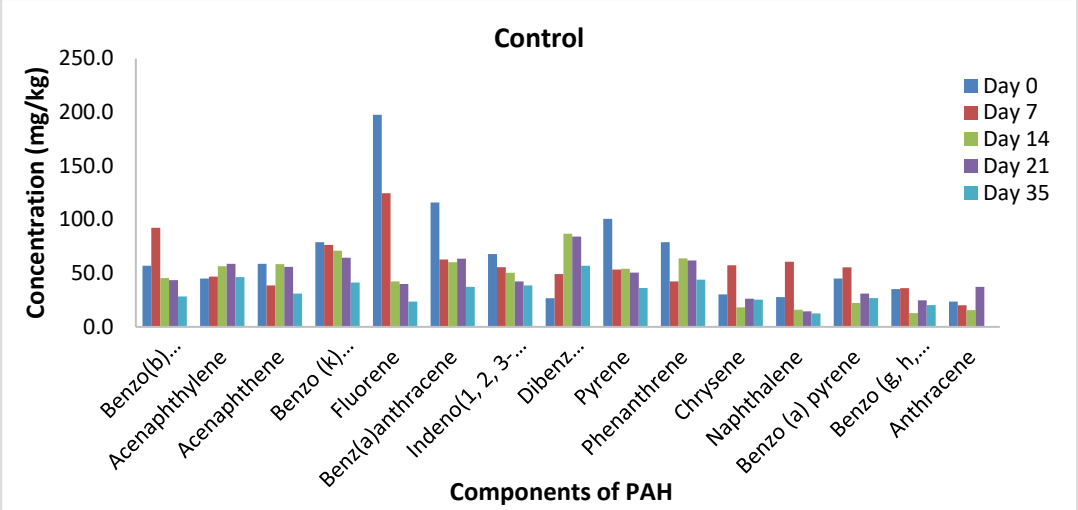

Figure 2. Components of PAH degradation profile at different sampling days (35 days)

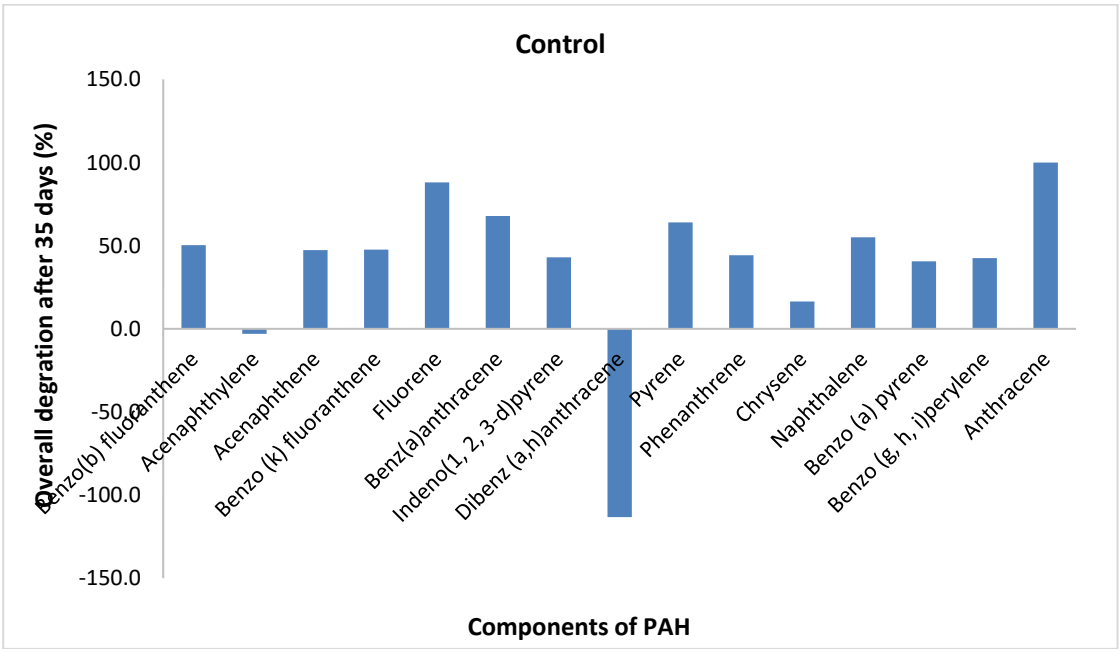

Figure 3. Percentage degradation of individual PAH components

The higher removal of LMW PAHs than MMW and HMW PAHs in the same treatment of soil phytoremediation is in agreement with previous studies of Feng et al. [29]. Stogiannidis and Lanne [30] described LMW PAHs as more volatile, water soluble and less lipophilic which make them more easily biodegradable than HMW PAHs which tends to be more recalcitrant to microbial attack and hydrophobic.

At day 0 of the experiment PAH concentration was 989.1 mg/kg which decreased steadily to 468.4 mg/kg at day 35 and corresponded to a degradation rate of 52.6 % (Figure 4). The continuous decline of PAH concentration reveals the active contribution and role of the indigenous hydrocarbon degrading microbial population onsite. Guarino and Sciarrillo [31] evaluated through a pot experiment three bioremediation strategies: a) Natural Attenuation (NA), Landfarming (L), and Bioaugmentation-assisted Landfarming (LB) for the treatment of a contaminated soil with petroleum hydrocarbons. After a 90-days trial, LB approach produced the best results and the greatest evident effect was shown with the most polluted samples reaching a reduction of about 86% of total petroleum hydrocarbons (TPH), followed by L (70%), and NA (57%). Although NA had the least degradation rate but was still very effective and remains a promising low-cost alternative to other conventional techniques. The process can be enhanced by optimizing prevailing conditions indigenous hydrocarbon degraders require to thrive and function within the framework of an ENA approach and this could be a useful means to enhance the natural degradation process [32].

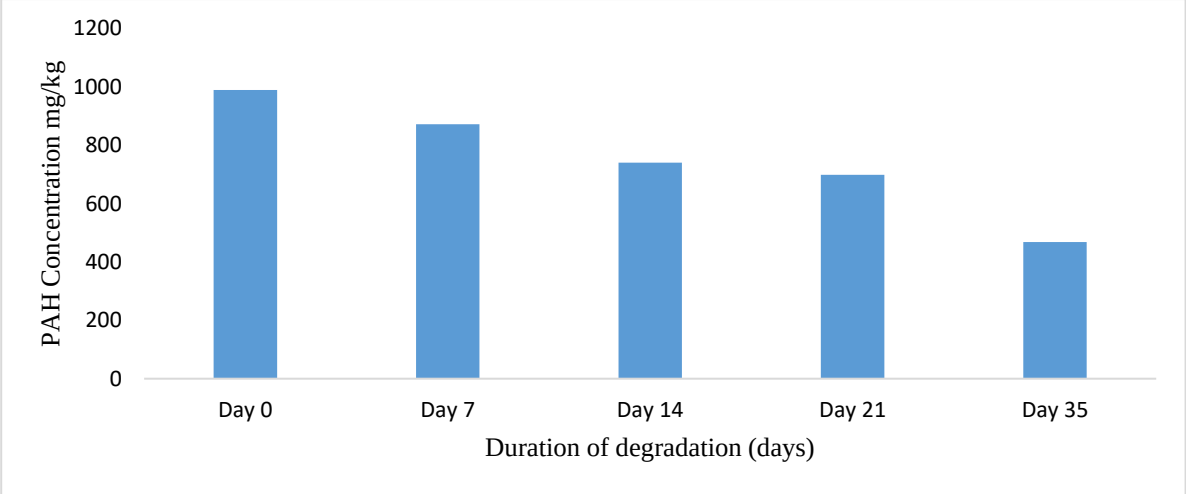

Figure 4. PAH degradation profile within a 35-day duration of natural attenuation

4. CONCLUSION

The sample location which is an aged-spill site was confirmed to harbor an appreciable number of both heterotrophic and hydrocarbon utilizing bacteria population which supported and contributed to the bioattenuation process. PAH concentration in the polluted sample was reduced by natural attenuation to 52% after a 35-day period. Anthracene was 100% degraded while benzo(b)fluoranthene, fluorine and pyrene had above 50% removal. All other PAHs present in the sample experienced some level of degradation except acenaphthylene, dibenz (a,h) anthracene and chrysene which increased slightly in concentration. The dynamics of the individual PAH degradation within the experimental period shows that monitored natural attenuation is an effective approach although slower than other bioremediation protocols. This process can be enhanced by optimizing the conditions required for hydrocarbon degrading microbes to break down hydrocarbons at a faster rate.

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