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Aerobic Biodegradation of Petroleum Hydrocarbons in Laboratory Contaminated Groundwater

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Authors' contributions

This work was carried out in collaboration between both authors. Author OA designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript and managed literature searches. Authors OA and OAG managed the analyses of the study and literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

Two treatment options A and B were set up in quadruple using Erlenmeyer flasks containing spiked groundwater samples from a hand dug well to study the effects of nutrient amendment and natural attenuation on hydrocarbon removal under laboratory aerobic conditions. Treatment A received 0.5g of N.P.K (15:15:15) fertilizer as nutrient amendment while treatment B received no treatment in order to measure the rate of hydrocarbon removal by natural attenuation. The bioremediation process was monitored for 56 days by periodic (0, 14, 28, 42, 56) measurements of total hydrocarbon content, pH, nitrate, phosphate, sulphate and dissolved oxygen to establish their effects on hydrocarbon removal under laboratory conditions. Enumeration of total heterotrophic bacteria and total hydrocarbon utilizing bacteria was also periodically carried out. The total hydrocarbon content removal in nutrient amendment and natural attenuation at day 56 showed percentage removal of 89 and 74, respectively. Total heterotrophic and total hydrocarbon utilizing bacterial count increased progressively in all the treatment options. The nutrient amendment

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(treatment A) showed greater removal of the hydrocarbon pollutants. The physicochemical analyses at day 56 were within the World Health Organization standard for drinking water. Statistical analysis revealed that there was significant difference in the data obtained from treatment A and B for total hydrocarbon content, total heterotrophic bacteria, hydrocarbon utilizing bacteria, conductivity and nitrate at $p<0.05$. Bacterial strains isolated include *Bacillus* sp., *Arthrobacter* sp., *Micrococcus* sp., *Pseudomonas* sp., *Alcaligenes* sp. and *Flavobacterium* sp. Biodegradation of petroleum hydrocarbons in laboratory contaminated groundwater under nutrient amendment conditions was found to be higher than the untreated, unamended condition.

Keywords: Aerobic biodegradation; petroleum hydrocarbon; groundwater; nutrient amendment; natural attenuation.

1. INTRODUCTION

Groundwater is an important source of water for agricultural and domestic use especially in developing countries like Nigeria, due to long retention time and natural filtration capacity of aquifers [1]. It is less contaminated as compared to surface water [2]. However petroleum hydrocarbons can be introduced into groundwater via oil spills, leaking or unplugged oil wells, the disposal ponds of waste petroleum products, abandoned oil refinery sites, pipe line ruptures, incomplete combustion of fossil fuels and accidental discharge during transport in tanks and ships failures [3].

Oil spill contaminating underground water are considered to have deleterious effects on the overall survival of man, not only because man depends on water for various domestic and industrial uses, but also because underground water can also be used for irrigation of land for agricultural practices [4].

It is worthy to note that groundwater is one of the many media by which human beings, plants and animals come into contact with petroleum hydrocarbon pollution. In the Niger delta area of Nigeria, extensive farm land has been lost due to contamination with crude oil [5]. Also sources of drinking water and traditional occupation such as fishing and water transportation are greatly affected by crude oil contamination.

Remediation of petroleum contaminated sites could be achieved by either physicochemical or biological methods. Due to negative consequences of the physicochemical approach, more attention is now given to the exploitation of biological alternatives [6].

Besides, bioremediation technology is believed to be non-invasive and relatively cost effective [7]. In some cases it may not require more than the addition of some degradation enhancers to the polluted system. It could end up being the most reliable and probably least expensive option for solving some chemical pollution problems [8].

Microbial degradation of pollutants has intensified in recent years as humanity strives to find sustainable ways to clean up contaminated environment [9]. Biodegradation of oil is one of the most important processes involved in weathering and the eventual removal of petroleum from the environment, particularly for the non-volatile components of petroleum [10]. Bioremediation technology holds a lot of promise not only for developed but also for the developing nations such as Nigeria because of its cost effectiveness and environmental friendliness benefit [11,12].

Degradability of hydrocarbon, organisms involved and the physicochemical characteristics of the laboratory contaminated groundwater under natural attenuation and biostimulation conditions were determined.

2. MATERIALS AND METHODS

2.1 Source of Samples

The groundwater samples were collected from a hand dug water well at Ejama Ebubu community in Eleme Local Government Area of Rivers State in Nigeria. The groundwater sample was collected using a sterile string of about 20m which was tied to a sterile plastic container and lowered inside the well. Groundwater collected in the sterile plastic container was transferred into a 25- litre plastic container and transported to the laboratory for analysis within 24 hours.

2.2 Experimental Set-up

Eight Erlenmeyer flasks (250 mL) were used to set up microcosms of the groundwater sample to simulate various treatment options. The sample was spiked with 0.25 mL of fresh crude oil into the various flasks set for the experiment. Since the site of collection of the sample was polluted over 40 years ago, abiotic weathering processes would have reduced appreciably. In order to monitor these processes alongside biodegradation, the sample was spiked, thoroughly mixed, and allowed to settle.

The conditions simulated were as follows: Treatment A (day 14), A(day 28), A(day 42), A(day 56), contained 250 mL of groundwater sample plus 0.5 g of NPK fertilizer. This simulated biostimulation under aerobic condition (Nutrient Treatment). Treatment B (day 14), B(day 28), B(day 42), B(day 56), contained 250 mL of groundwater sample kept under aerobic condition. This stimulated natural attenuation.

2.3 Experimental Monitoring Parameters

The behavior of the crude oil in the given flasks was monitored for a period of 56 days by recording changes or otherwise in total heterotrophic bacteria (THB), hydrocarbon utilizing bacteria (HUB), total petroleum hydrocarbon (TPH), total hydrocarbon content (THC), pH, DO, conductivity as described in the standard methods for examination of water and waste water. To further determine the rate of disappearance or otherwise of the crude oil and distinguish biodegradation from other weathering processes, gas chromatographic (GC) tracing of the experiments was done.

2.4 Physico-chemical Analysis of Groundwater

The groundwater samples were analyzed to access its physico-chemical properties. Nitrate, phosphates (total phosphorus), dissolved oxygen, pH, conductivity and total hydrocarbons were determined using APHA method.

2.5 Characterization and Identification of Isolates

Stock cultures of the isolates with different cultural characteristics were made on nutrient agar slants. Gram staining was used to check for morphology and biochemical tests were performed to aid in identification. Various tests performed and used in probable identification of isolates included the oxidase test, motility test, catalase test, urease test, coagulase test, indole test, methyl red test, Voges-Proskauer test and citrate utilization test.

2.6 Gas Chromatographic (GC) Analysis

Residual total petroleum hydrocarbon (TPH) was extracted from the water samples and quantified using Gas Chromatograph- Flame Ionization Detector (GC-FID). The analysis was carried out using a Varian 1440 GC-FID. A DB-I column was used with the following dimensions: 30 m × 0.2 mm; 0.25 µm film thickness; 0.32 i.d. Helium was the carrier gas at a flow rate of 1 ml/min. Analyses were carried out in split injection mode using a split ratio 5:1. The injection port was set at 250ºC. The oven temperature was programmed from 40ºC for 10 min, the 20ºC per min to 330ºC, holding this temperature for 10 min.

All data generated was presented in tables and subjected to statistical analysis (the P2-test, with the level of significance set at $p < 0.05$ using statistical package for social sciences (SPSS) to determine any significant relationship between aerobic degradation under natural attenuation and biostimulation condition, physicochemical changes.

3. RESULTS AND DISCUSSION

3.1 Baseline Properties of the Groundwater Sample (Day 0)

The total hydrocarbon (oil and grease) in the groundwater sample was 23 mg/L but after the spiking was 1500 mg/L. The concentration of the spiked exceeds the limits recommended by World Health Organization (WHO) for potable water. The total heterotrophic bacterial count (THB) was 1.84×10^5 cfu/mL while the hydrocarbon utilizing bacterial count (HUB) was 7.6×10^4 cfu/mL. The level of hydrocarbon degraders present in the sample at day 0, i.e. (41.3%) of the total heterotrophic bacterial count is a reflection of the degree and age of contamination of the groundwater sample. The DO value of 8.0 mg/L depicts the water as oxygenated.

Microbiological analysis of Total and Faecal coliform count, physicochemical parameters and heavy metals of the baseline studies are given in Table 1.

3.2 Percentage (%) of the Total Hydrocarbon Content on Treatment Options

Total hydrocarbon content (oil and grease) remaining and the percentage in the groundwater as the experiment progressed was determined every 14 days for the period of the experiment, this is shown in Table 2. In the course of the research it was also observed that the groundwater sample in Treatment A (Nutrient amendment) showed a higher percentage removal of the total hydrocarbon content (89%) compared to Treatment B (natural attenuation) which showed 74% removal at the end of the fifty six days. The contributions of natural attenuation to the bioremediation of impacted media such as water have been reported at other times too [13].

3.3 Microbial Counts in Treatment Options

The total heterotrophic bacterial count in treatment A ranges between 1.84 x 10^5 – 4.2 x 10^5 cfu/mL while treatment B ranged between 1.8 x 10^5 – 3.0 x 10^5 cfu/mL. Total hydrocarbon utilizing bacterial count in treatment A ranges between 7.6 x 10^4 – 4.0 x 10^5 cfu/mL in treatment B it ranged between 7.6 x 10^4 -2.8 x 10^5 cfu/mL. Statistical analysis shows there was significant difference in the total heterotrophic bacterial count and hydrocarbon utilizing bacteria since p-values (0.0189) < 0.05 (Table 6) Bioremediation can be effective only where environmental conditions permit microbial growth and activity. In some cases, the environment can be modified to support or accelerate microbial growth, for example, by fertilizer application [14].

The effect of NPK fertilizer treatment on the groundwater sample was monitored for fifty six days by measurement of hydrocarbon degrading activity and gravimetric loss of the oil with time.

The results indicate a steady increase in bacterial counts throughout the period of study. The increase in counts of the heterotrophic population is in agreement with results obtained by other researchers that hydrocarbon pollution

Table 1. Baseline (Day 0) properties of Ejama Ebubu groundwater

Parameter	Measurement
pH	6.5
Temperature °C	26
Phosphate (mg/L)	8.3
Sulphate (mg/L)	18.6
Nitrate (mg/L)	14.0
Dissolved oxygen (mg/L)	8.0
Conductivity (µs/cm)	63
Total organic carbon	0.800
Iron (Fe^{2+}) (mg/L)	6.230
Copper (Cu) (mg/L)	0.014
Zinc (Zn) (mg/L)	0.435
Lead (Pb) (mg/L)	0.023
Chromium (Cr^{6+}) (mg/L)	0.043
Total heterotrophic bacteria (cfu/mL)	1.84×10^5
Total hydrocarbon utilizing bacteria (cfu/mL)	7.6×10^4
Total hydrocarbon content (mg/L)	23 (before spiking), 1500 (after spiking)

Total and faecal coliform count	MPN (presumptive)	EMB (*E. coli*)	BGLB (Total coliform)	EC medium (*E. coli*)
Groundwater	0	Absent	Absent	Absent

Table 2. Percentage (%) of total hydrocarbon content removed in the laboratory contaminated groundwater

Days	% of total hydrocarbon content removed in treatment A	% of total hydrocarbon content removed in treatment B
14	32	4
28	51	15
42	68	43
56	89	74

does not enrich only hydrocarbon utilizers but also other populations that utilize breakdown products of hydrocarbons [15]. The growth in the heterotrophic count and hydrocarbon utilizing bacteria were also observed to be more in Treatment A (Artificial Treatment) than Treatment B as the experiment progressed. Other researchers [16,11,13] have also demonstrated the use of inorganic nutrients in bioremediation of hydrocarbon impacted media with overall positive results.

3.4 Gas Chromatographic Profile of Groundwater

At the end of the 56 days experimental period, it was observed through the GC tracing that some of the target analytes were either not detected or reduced in A than B. when compared to the original sample, treatment A witnessed appreciable reduction in peak height and activity than treatment B which was moderate. The gas chromatogram of the treated Ejama Ebubu groundwater revealed biodegradation of biomarkers (Pristane C_{17} & Phytane C_{18}). In treatment A (nutrient treatment) there was a near and or complete disappearance of the pristane C_{17} and phytane C_{18} from the total petroleum hydrocarbon concentration. The total petroleum hydrocarbon reduced from 15.33955 mg/L to 3.90361 mg/L in treatment A at the end of day 56 (Table 3), treatment B (nutrient attenuation) also showed decrease in pristane C_{17} and phytane C_{18}. The total petroleum hydrocarbon in treatment B reduced from 15.33955 mg/L to 9.49634 mg/L at the end of day 56. (Table 3). Gas chromatograph of the groundwater sample at the initial stage was compared with those obtained after fifty six days for Treatment A and B. The chromatograms showed most noticeable difference between Treatment A and B at the end of the experimental period compared to the initial stage. Generally, it is believed that microbes preferably degrade or metabolize C_8-C_{15} n-alkanes followed by C_{16}-C_{36} n- alkanes due to simplicity of these hydrocarbons [17]. Most of the sharp peaks observed in the original sample were either reduced or not found in Treatment A after fifty six days. GC results indicate presence of volatile biodegradable substances in the groundwater sample. The commonly used biomarkers (pristane and phytane) for the evaluation biodegradation of crude oils were found to be degraded in Treatment A containing the nutrient amendment at day 56. Treatment B (natural attenuation) also showed a decreased amount in pristane and phytane at day 56. This suggests that biodegradation occurred in both treatment options. While the isoprenoids pristane and phytane (C_{17}, C_{18}), are somewhat more resistant to biodegradation than the n-alkane with similar boiling points they should only be used to monitor the earliest stages of a biodegradation treatment program, as they are known to be biodegradable under natural conditions [18].

3.5 Physicochemical Parameters (pH, Dissolved Oxygen, Conductivity)

Treatment B maintained a pH of 6.5 from day 0 to day 42 but increased to 7.28 at day 56. Treatment A pH value reduced from 6.5 to 4.85 on day 14 and gradually increased to 6.5 and 7.57 at day 42 and 56 respectively. The pH of the groundwater in the study period ranged between 4.8 –7.5 in treatment A and 6.5 -7.2 in treatment B (Table 4). Groundwater with pH below 6.5 is generally considered acidic. This is applicable day fourteen and day twenty-eight in treatment A, the slight decrease in pH may be attributed to the increase in the rate of microbial metabolism [19] and effect of fertilizer application. The observed pH values throughout the study are of special consideration since microbial populations are highly dependent on this parameter (20). The results are in agreement with observation made by other workers that a pH range of 6-8 provides better conditions for mineralization of hydrocarbons since most bacteria capable of metabolizing hydrocarbons develop best at pH conditions close to neutral [20].

The initial dissolved oxygen was determined to be 8.0 mg/L. The dissolved oxygen gradually decreased in both treatments. In treatment B dissolved oxygen ranged from 5.0 mg/L to 8.0 mg/l while in treatment A, it ranged from 3.20 mg/L to 8.0 mg/L (Table 4).The dissolved oxygen concentrations measured in the flasks in the course of the study were lower than the initial concentration of 8.0mg/l. Oxygen is a microbial electron acceptor and a redox indicator. High oxygen (>2mg/L) shows aerobic conditions and oxygen will be the preferred electron acceptor until depleted. Nutrient amendment enhanced oxygen uptake.

It was observed that conductivity of the contaminated groundwater sample with initial value of 63 µs/cm decreased progressively in all the treatments indicating uptake and exchange of ions during the period of study. Statistically there was a significant difference since p-value (0.0248) < 0.05 (Table 6) The reduction in conductivity in treatment A and treatment B from

initial of 63 µs/cm to 43 µs/cm and 63 µs/cm to 26 µs/cm (Table 4) after fifty six days, respectively suggests that there was uptake and exchange of ions in the samples in course of the study confirming that biodegradation of hydrocarbon was achieved. The maximum permitted conductivity by Nigerian Standard for drinking water quality is 1000 µs/cm [21].

3.6 Time Series Analysis of Nitrate, Phosphate and Sulphate

The increase in nitrate and phosphate concentrations in treatment A in the first 14 days of the study period can be attributed to biostimulation with NPK fertilizer. This agreed with the results of Odokuma and Akponah. (2010) who observed that the addition of nitrogen and phosphorous sources to contaminated environmental media increased the proliferation of biodegrading bacteria, resulting in an increased in degradation rates. The reduction of these nutrients as the experimental period progressed suggests utilization by microorganisms. Sulphate concentrations over the study period revealed an appreciable and steady reduction from initial concentration of 18.6 to final concentration of 5.6 mg/L in treatment A and 18.6 to final concentration of 4.3 mg/L in treatment B (Table 4) suggesting uptake by microorganisms, although sulphate is not a preferred terminal electron acceptor when oxygen and nitrate are present in growth medium. Statistically, there was no significant difference in phosphate and sulphate, as the p-values were all greater than 0.05. For nitrate, there was significant difference since the p-values (0.0386) is < 0.05 (Table 6) Nutrient utilization measurement revealed that there was general uptake of nutrients in the various treatment options indicating that these nutrients were critical to the metabolism of hydrocarbon degraders in the water sample. Application of inorganic fertilizers (NPK 15:15:15) led to the increase in the concentrations of nitrate and phosphate utilized in flask A. This explained why the concentration of phosphate at day fifty six in treatment A (8.9 mg/L) was greater than the baseline concentration of 8.3 mg/L. The addition of nutrient proved to be beneficial in terms of hydrocarbon removal in flask A compared to treatment B. The benefit of nutrient amendment was also observed by Abu and Ogiji (1996), who noted that the response of the indigenous hydrocarbon degrading microorganisms to the bioremediation treatment was positive and differed according to the type and concentration of the nutrients added. Sulphate in the groundwater sample was also utilized in the course of the study. The concentration of nitrate, phosphate and sulphate in the groundwater sample and treatment options at the end of the study period were less than the allowable limits set by the World Health Organization [22].

3.7 Biochemical Characteristics and Identification of HUB Isolates

Hydrocarbon utilizers were identified to genus level on the basis of colonial morphology, biophysical, physiological and biochemical characteristics (Table 5). The genera include; Bacillus, Arthrobacter, Micrococcus, Pseudomonas, Alcaligenes and Flavobacterium. Microorganisms isolated and identified in the course of the study include; *Bacillus* sp, *Arthrobacter* sp, *Micrococcus* sp, *Pseudomonas* sp, *Flavobacterium* sp, *Alcaligenes* sp. The microorganisms capable of utilizing oil and oil products as a sole source of carbon and energy occur practically everywhere in the air, water and soil [23]. Some of the common genera of bacteria involved in bioremediation of hydrocarbon contaminated site include Nocardia, Pseudomonas, Mycobacterium, Vibrio, Serratia, Achromobacter, Acinetobacter, Flavobacterium, Brevibacterium, Micrococcus, Arthrobacter and Corynebacterium, Alcaligenes, Bacillus and Actinomyces [11]. These results clearly show that a large diverse aerobic bacterial population capable of utilizing organic carbon is present in groundwater.

Table 3. Summary of initial and final TPH concentrations in the groundwater

Days	TPH conc. in treatment A (mg/L)	TPH conc. in treatment B (mg/L)
Initial conc. at day 0	15.33955	15.33955
Final conc. at day 56	3.90361	9.49634

Table 4. Physicochemical properties of the laboratory contaminated groundwater

Parameters	Treatment A					Treatment B				
	Day 0	14	28	42	56	Day 0	14	28	42	56
THC (mg/L)	1500	1020	740	480	160	1500	1440	1280	860	390
pH	6.5	4.8	5.3	6.5	7.5	6.5	6.5	6.5	6.5	7.2
DO (mg/L)	8.0	6.67	5.40	4.50	3.20	8.0	5.60	5.60	5.20	5.00
CONDUCTIVITY (µs/cm)	63	50	43	31	26	63	60	53	47	43
NITRATE (mg/L)	14	27.2	21.4	16.8	7.5	14	9.4	7.0	4.6	2.6
PHOSPHATE (mg/L)	8.3	18.2	12.4	10.6	8.9	8.3	9.1	8.7	7.7	6.5
SULPHATE (mg/L)	18.6	13.4	11.2	9.6	5.6	18.6	12.3	7.7	5.9	4.3

Table 5. Biochemical characteristics and identification of HUB isolates of remediation treated Ejama Ebubu groundwater

Iso. no.	GS	Shape	Ind.	Cat.	Ox.	MR.	VP.	CIT.	Spore	Starch hydro	Urea	O_2	Sugar fer SLG	Identification
A	+	Rod	-	+	-	+	+	-	+	+	-	Ae	-**	*Bacillus Sp.*
B	+	Rod	-	+	-	-	-	-	-	-	-	Ae	**,*,**	*Arthrobacter Sp.*
C	+	Coccus	-	+	+	-	-	-	-	+	-	Ae	* * -	*Micrococcus Sp.*
D	-	Rod	-	+	+	-	-	-	+	-	+	Ae	* **	*Pseudomonas Sp.*
E	-	Rod	+	+	+	-	+	-	-	-	-	Ae	- * -	*Flavobacterium Sp.*
F	-	Rod	-	+	+	-	+	+	-	+	-	Ae	****	*Alcaligenes Sp.*

KEY: *ISO No. = Isolate Number; GS = Gram's stain, Ind. = Indole test; Cat.= catalase test; Ox.= Oxidase test; MR. = Methyl Red; VP= Voges Proskauer; Urea= urease test; O2 Req= Oxygen requirement; S= Sucrose fermentation; G = Glucose fermentation; L= lactose fermentation; Ae= Aerobic; *= Acid; **= Acid &Gas; += positive; - = negative. Id = identification*

Table 6. Statistical analysis of data that are significantly different

	Mean	Variance	t-cal	t.cr	p-value
HBC					
Treatment A	6.56×10^{5}	4.4×10^{11}	3.8068	2.7764	0.0189
Treatment B	5.74×10^{5}	5.3×10^{11}			
HUB					
Treatment A	2.51×10^{5}	1.51×10^{10}	3.8139	2.7764	0.0189
Treatment B	1.71×10^{5}	7.11×10^{9}			
Conductivity					
Treatment A	42.60	220.30	3.502	2.7764	0.0248
Treatment B	53.20	1.472			
Nitrate					
Treatment A	17.38	55.492	3.0359	2.7764	0.0386
Treatment B	7.5	19.632			

HBC: heterotrophic bacterial count. HUB: hydrocarbon utilizing bacterial

4. CONCLUSION

The results of this study revealed utilization of hydrocarbon by microorganisms in hydrocarbon polluted groundwater, the results also revealed that enhanced natural attenuation proved to be effective. The method is cheap as microorganisms responsible for biodegradation are present in-situ.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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