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Determining Break Down of Hydrocarbons in Effluent Discharges from Petroleum Service Stations

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Abstract

The study sought to find out how hydrocarbons are broken down in effluent from petrol stations by the existing bacteria in the effluent. This was established by collecting samples from two petrol station interceptors and subjecting them to chromatography tests as well as bacterial enumeration experiments over a period of forty days. It was observed that various changes took place in the samples from the chromatography results, whereby the constituent hydrocarbon quantities reduced significantly and in some cases they got eliminated completely. It was also confirmed that there existed various strains of bacteria in the samples and their population increased for a period of time until it reached an optimum where the population stagnated. Both observations realized enhanced changes and faster growth of microorganisms where nitrogen and phosphorous had been added to the samples.

Keywords

Hydrocarbons (HC), Microorganisms, Effluent, Bioremediation, Degradation

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1. Introduction

1.1. Background

One of the major environmental problems we face today is hydrocarbon contamination resulting from the activities related to the petroleum industry; these activities keep on growing by the day as population increases. Petrol is the main source of energy for industry and daily life. Petrol consists of complex hydrocarbons which are released into the environment accidentally or due to human activities (9). Frequent accidental releases of petroleum products are of particular concern to the environment at large.

Hydrocarbons are organic compounds made of carbon atoms bound to each other forming a backbone with hydrogen atoms attached to the remaining sites on carbon. The carbon backbone can be straight or normal, branched, or cyclic (17).

Compounds containing only carbon and hydrogen are often referred to as parent compounds.

Compounds containing substitutions of other elements or smaller carbon and hydrogen groups onto the original carbon backbone in place of the hydrogen are called derivative compounds (15).

Accidental spills, leaks and accidents are a common occurrence during the exploration, production, refining, transport, and storage of petroleum and petroleum products in oil depots as well as petrol service stations.

With regulatory agencies like National Environmental Management Authority (NEMA) in Kenya becoming more concerned with the release of petroleum products into the

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environment, there is a growing need to develop more effective and less expensive technologies to remediate the petroleum-contaminated effluent from petroleum sites to acceptable standards. All firms or persons discharging effluent into the aquatic environment are required to submit quarterly discharge monitoring records to NEMA based on prescribed procedures of sampling and analysis.

The physical property classification of the various petroleum products is derived from the crude petroleum oil separation process. Most of the hydrocarbon compounds used by industry are derived from crude petroleum oil, coal, and natural gas. However, hydrocarbons are natural compounds produced by animals, plants, and bacteria (20). Therefore, it is not surprising that hydrocarbons can be biologically degraded by a variety of microorganisms.

Bioremediation is the most promising technology for the treatment of these contaminated sites since its technology is believed to be natural, less toxic and relatively cost-effective (1) and is cheaper than other remediation technologies. Bioremediation operates on biodegradation, which may refer to complete mineralization of organic contaminants into carbon dioxide, water, inorganic compounds, and cell protein. Complex organic contaminants are transformed in to other simpler organic compounds by biological agents like microorganisms through bioremediation (16)

The degrading microorganisms utilize hydrocarbons as an energy source and a carbon source.

Most hydrocarbons used as an energy source are degraded under aerobic conditions to carbon dioxide and water. This degradation process is a catabolic process and the degradation to inorganic compounds is called mineralization. However, some hydrocarbons are not mineralized but transformed into simpler compounds (7). The hydrocarbons used as a carbon source are degraded to smaller compounds and incorporated into the cell materials. This degradation process is a combination of catabolic and anabolic processes (3).

The specific degradation mechanisms are determined by the compound structure.

Linear alkanes degrade through β-oxidation in which the backbone is broken up two carbons at a time and the resulting acetyl-CoA is mineralized in the TCA cycle. Some cyclic alkanes degrade through cometabolism (12). Aromatic compounds are generally degraded via a dioxygenase enzyme, which converts the compound to a catechol followed by ring fission in the ortho or meta positions (19).The degradation mechanism as well as its rate and extent are determined by a number of factors. These factors are: the chemical structure of hydrocarbons, the presence and capabilities of microorganisms, the presence of nutrients. This Paper will be showing how effective existing microorganisms are in breaking down hydrocarbons in effluents from petroleum service stations.

1.2. Effects of Addition Nitrogen and Phosphorus on Microorganisms

Nutrients are very important ingredients for successful biodegradation of hydrocarbon pollutants especially nitrogen, phosphorus, and in some cases iron (6). Some of these nutrients could become limiting factors thus affecting the biodegradation processes. In 1985 Atlas reported that when a major oil spill occurred in marine and freshwater environments (2), the supply of carbon was significantly increased and the availability of nitrogen and phosphorus generally became the limiting factor for oil degradation. In marine environments, it was found to be more pronounced due to low levels of nitrogen and phosphorous in seawater (8).

Therefore, additions of nutrients were necessary to enhance the biodegradation of oil pollutant (5). On the other hand, excessive nutrient concentrations can also inhibit the biodegradation activity (4)

For oil sludge containing large quantities of hydrocarbons, microorganisms must be able to use hydrocarbons as substrates (22). They must be able to synthesize enzymes that can catalyse the reaction in which these contaminants are degraded to simpler, lower molecular chains and less toxic compounds (CO_2 and H_2O), through obtaining the nutrients and energy necessary for their survival in the process (11). The initial step in this mechanism is the catabolism of oil sludge by bacteria and fungi, which involves the oxidation of the substrate by oxygenases, in which molecular oxygen is required. Aerobic conditions are necessary for this route of microbial oxidation of hydrocarbons to take place.

2. Methodology

2.1. Study Area and Sampling

2.1.1. Study Area

The study was carried out on samples collected from two Service Stations in Nairobi. The stations were TOTAL on Limuru road as well as Baba Dogo Oilibya Service Station on Outering Road in Baba Dogo area. The main reason why these particular stations were chosen was to have a contrast in the composition of the samples hence make the tests more interesting. The TOTAL sample was heavily contaminated with fuel and oil sediments since the interceptor had not been cleaned for some time while the Oilibya sample was clean, since the interceptor had been cleaned a day before the sample collection.

2.1.2. Sampling

One sample of 1.5 litre effluent polluted water and sediments from each of the interceptors of the two service stations were collected. The effluent was collected in clean two liter sterile bottles. Samples collected from the different stations were not bulked for provision of making many comparisons. The two different samples were transported to the laboratory and stored at 4°C to ensure that no reaction would take place before the actual experiment was started. Each Sample from each station was divided in two so that we had one sample being tested as a natural sample while the other sample had Nitrates & phosphates added to it (Bacteria growth enhancing nutrients)

2.2. Chromatographic Analysis

Residual total petroleum hydrocarbons (TPH) were extracted from the samples and quantified using GC-MS – Gas chromatography–mass spectrometry.

2.2.1. Extraction

10ml of oil was transferred into disposable centrifuge tube and extracted with 20ml Hexane. 6g anhydrous magnesium sulphate and 1.5g sodium chloride was added. The tubes were then hand-shaken vigorously for the components to mix. They were then centrifuged at 10000rpm for 5min.

2.2.2. Solid-Phase Dispersion (SPD) Clean-Up

The Hexane extract was cleaned up using two solid phase dispersive steps. The extract (10ml) was transferred to a second tube containing 330mg of C18 and 1.2g anhydrous magnesium sulfate. The tube was vortexed and centrifuge at 10000rpm for 5min. The extract (200µl) was transferred to a clean vial and further diluted with 2ml of hexane. 10µl of the solution was transferred to a smaller vial and 1µl of internal standard added.

2.2.3. Determination of Composition of the Petroleum Hydrocarbons

GC-MS analysis of the samples was performed with Agilent 7595 Instrument. Chromatographic separation was achieved using a fused silica capillary column (Hewlett Packard ,50M x 0.32mm ID) coated with carbowax 20M (0.3µM film thickness) with Helium as a carrier gas. All the GCMS analysis was made in the split less mode with Helium as carrier gas. The Oven temperature was programmed from 60°C for 7 minutes increasing to 120°C at a increase rate of 5°C per min, then to 180°C at a rate of 10°C per min then finally to 220°C at a rate of 10°C per minute where it was maintained for 10 min. Constituent of the analytes were identified by analysis of their mass spectra, direct comparison of these with those in the Wiley National Bureau of Standards (NBS) and National Institute of Standards and Technology (NIST) databases.

2.3. Enumeration of Total Heterotrophic Bacteria

Total heterotrophic bacterial (THB) counts were determined using the spread plate method on a plate count agar (PCA) using nutrient Agar**.** Each sample of 1 ml was homogenized in 9 ml of 0.85% sterilized distilled water.

2.3.1. Isolation of Hydrocarbon Utilizing Bacteria

Isolation of oil degrading bacteria was carried out under aerobic condition with kerosene as sole source of carbon. 500µl of 10^{-3} serial diluted sample was inoculated in liquid mineral salt media (MSM) and 100 µl of the same was inoculated on solid MSM. The MSM was made up of the following components (g/L): 0.2 $MgSO_4$, 0.02 $CaCl_2$, 1.0 $KH2PO_4$, 1.0 $K2HPO_4$, 1.0 NH_4NO_3, and 0.05 $FeCl_3$, and the pH was adjusted to pH 7.4 containing 1% kerosene oil by spread method (13) who used crude oil as a source of carbon. Culture plates were incubated for 10 days at temperature 28°C. After incubation period the growth of microbial colonies was observed on the petri dishes. In the morphological characterization study, Grams positive bacterial cells retained purple stain and the bacterial cells that retained pink safranin color were gram negative. It is due to the dark purple crystal violet stain retained by the thick layer of peptidoglycan which forms the outer layer of the gram positive cell. In gram negative bacteria, the thin peptidoglycan layer in the periplasm does not retain the dark stain, and the pink safranin counter stain stains the peptidoglycan layer. Both the gram positive and gram negative bacteria were rod shaped Bacillus cells.

2.3.2. Purification and Characterization of Heterotrophic Bacteria

Discreet colonies of different Hydrocarbon utilizing Bacteria were randomly picked using a sterile inoculating wire loop and sub cultured for purification by streaking on nutrient agar plates and incubated for 28°C for 24 h. To monitor cell numbers and biodegradation, 1 ml of effluent was removed from each container at the set times and re suspended in 10 ml of sterile saline in sterile centrifuge tubes. 0.1 ml of mixture was sampled for CFU (Colony Forming Units) counts.

The study was conducted at temperature of 28°C and monitoring was performed on Days 0, 10, 20, 30, and 40. The bacterial species indigenous to the effluent samples were isolated by spread plate technique using 0.1 ml aliquots of appropriate dilution onto nutrient agar plates. Individual cultures were identified morphologically using the taxonomic

scheme of Bergey's Manual of Determinative Bacteriology (10). Just like in the other experiments, this was repeated after adding nitrogen and phosphorous to compare the counts.

3. Results Analysis and Discussions

3.1. Introduction

All experiments undertaken were directed towards establishing the effectiveness of microorganisms in breaking down hydrocarbons. This was established by observing;

- Looking at the hydrocarbon characteristics over time using a chromatographic machine
- Lastly, monitoring growth of microorganisms in all the samples.

It was expected that the samples would definitely show physiochemical changes reflecting the activities by the micro-organisms. This was mainly to be reflected through reduction in the hydrocarbon quantities and an increase in the number of bacteria. It was also expected that the changes in the hydrocarbon composition as well as increase in micro-organisms number will be faster where the bacterial growth nutrients of Nitrogen and phosphorous had been added.

3.2. Chromatographic Tests

When the Oilibya samples were run, the GC-MS machine could not detect any substantial hydrocarbons to record peaks. This was because they had minimal quantities of hydrocarbons. The study concentrated on TOTAL samples only, the tables 1, 2, 3, 4 and 5 show the reduction in the specific hydrocarbons

Table 1. Showing Composition of Initial TOTAL sample.

Peak number	Retention time(min)	Compound name	Conc(ng/µl)
1.	11.8	Decane	0.82
2.	13.82	Dodecane	2.46
3.	21.5	Naphthalene	1.32
4.	22.5	2-Methyl naphthalene	0.30
5.	30.93	Eicosane	2.86
6.	32.5	1-Chloro-octadecane	4.63
7.	33.5	Fluoranthene	12.68
8.	34.0	pyrene	2.51
9.	34.5	Tetracosene	2.62
10.	36.3	Hexacosane	4.82
11.	38.3	Triacontane	0.28

Table 2. Showing TOTAL Sample without NP after 20 Days.

Peak number	Retention time(min)	Compound name	Conc(ng/µl)
1.	11.8	Decane	0.82
2.	13.82	Dodecane	2.46
3.	21.5	Naphthalene	1.32
4.	22.5	2-Methyl naphthalene	0.30
5.	30.93	Eicosane	2.86
6.	32.5	1-Chloro-octadecane	17.53
7.	33.5	Fluoranthene	760.1
8.	34.0	pyrene	12.62
9.	34.5	Tetracosene	12.51
10.	36.3	Hexacosane	17.43
11.	38.3	Triacontane	0.28

Table 3. Showing TOTAL Sample with NP after 20 Days.

Peak number	Retention time(min)	Compound name	Conc(ng/µl)
1.	11.8	Decane	0.74
2.	13.82	Dodecane	2.40
3.	21.5	Naphthalene	1.29
4.	22.5	2-Methyl naphthalene	0.30
5.	30.93	Eicosane	2.72
6.	32.5	1-Chloro-octadecane	17.53
7.	33.5	Fluoranthene	760.2
8.	34.0	Pyrene	12.62
9.	34.5	Tetracosene	12.49
10.	36.3	Hexacosane	17.41
11.	38.3	Triacontane	0.25

Table 4. Showing TOTAL Sample without NP after 40 Days.

Peak number	Retention time(min)	Compound name	Conc(ng/µl)
1.	13.1	Decane	0.41
2.	14.8	Dodecane	1.82
3.	24.8	Naphthalene	0.83
4.	31.2	2-Methyl naphthalene	0.32
5.	32.93	Eicosane	1.68
6.	33.1	1-Chloro-octadecane	0.81
7.	33.8	Fluoranthene	0.42

Table 5. Showing TOTAL Sample with NP after 40 Days.

Peak number	Retention time(min)	Compound name	Conc(ng/µl)
1.	14.8	Dodecane	0.38
2.	21.5	Naphthalene	0.29
3.	22.5	2-Methyl naphthalene	0.31
4.	40.0	Eicosane	1.32
5.	35.5	1-Chloro-octadecane	0.26

3.3. Isolation, Enumeration and Characterization of Bacteria

3.3.1. Purification and Characterization of Heterotrophic Bacteria

In the morphological characterization study, Grams staining bacterial cells retained purple stain and they were referred to as gram positive and some bacterial cells retained pink safranin colour and they were referred to gram negative. It is due to the dark purple crystal violet stain retained by the thick layer of peptidoglycan which forms the outer layer of

the gram positive cell. In gram negative bacteria, the thin peptidoglycan layer in the periplasm does not retain the dark stain, and the pink safranin counter stain stains the peptidoglycan layer. Both the gram positive and gram negative bacteria were rode shaped Bacillus cells. Of all the heterotrophic bacteria that were established to exist there was none whose elevation was raised. They were all flat and circular, none was wavy.

Table 6. The results were as tabulated below.

Colony Characteristics	General Observations	
Gram reaction	+ve	-ve
Colony configuration	Circular	Circular
Colony margin	Entire	Wavy
Colony elevation	Flat	Flat
Colony surface	Smooth	Smooth
Colony texture	Moist	Slimy
Cell shape	Rod	Rod

3.3.2. Microbial Monitoring and Enumeration of Heterotrophic Bacteria

The naturally occurring bacteria in the effluent numbered $237x10^6$ cfu/ml for the samples collected from Oilibya petrol station and $217x\ 10^6$ cfu/ml for the samples collected from TOTAL petrol station respectively. This enumeration was done on nutrient agar.

The addition of nitrogen and phosphorus suspensions into the effluents resulted in the growth of bacteria population to $513x10^7$ and $486x10^7$ cfu/ml for the samples collected from Oilibya and TOTAL petrol station respectively after 10 days of this study. This was a significant increase in population growth considering that for the samples where nitrogen and phosphorus were not added the population stood at $824x10^6$ and $698x10^6$ for Oilibya and TOTAL petrol stations respectively.

The bacterial count in the enriched effluent samples increased to$776x10^8$ and $668x10^8$ CFU/ml for the Oilibya and TOTAL petrol station respectively by the 20th day into the study but there was a decrease in the population as on the 30th day of the study. The populations stood at $135x10^8$ and $348x10^8$ CFU/ml for the Oilibya and TOTAL petrol stations respectively.

Many microbial strains are capable of degrading only specific components of oil sludge. However, oil sludge is a complex mixture of different petroleum hydrocarbon (14). Single bacterial species have limited capacities to degrade all the fractions of hydrocarbons presents (21). Hence, a mixture of different bacterial species that can degrade a broad range of the hydrocarbon constituents such as present in oil sludge would show more potential.

The results were as tabulated as below in table 7 and table 8;

Table 7. Bacterial count for the samples without nitrogen and phosphorus suspension added in 10^6.

Number of days	Oilibya	TOTAL
	No. of bacteria (10^6)	No. of bacteria (10^6)
0	273	217
10	824	698
20	1596	1268
30	1038	1834
40	958	1628

Table 8. Bacterial count for the samples with nitrogen and phosphorus suspension added in 10^6.

Number of days	Oilibya	TOTAL
	No. of bacteria (10^6)	No. of bacteria (10^6)
0	273	217
10	5,130	4,860
20	77,600	66,800
30	13,500	12,400
40	10,900	10,600

4. Summary of Findings and Conclusions

4.1. Chromatographic Analysis Conclusions

When quantifying the residual total petroleum hydrocarbons (TPH) extracted from the samples and quantified using GC-MS – Gas chromatography–mass spectrometry. There was significant reduction in most constituent hydrocarbons and in some cases complete elimination in the sample after 40 days especially in the aromatic hydrocarbons. This clearly showed that they must have been broken down.

4.2. Conclusions Arising from Enumeration, Isolation and Characterization of Bacteria

The experiments done established the presence of more than ten different varieties of bacteria. Whose population steadily grew up to the 30th day where the populations stagnated meaning the population had reached its optimum and could not grow further due to limited food and nutrients.

The addition of nitrogen and phosphorous increased the proliferation of biodegrading bacteria, resulting in an increase in degradation rates. We see faster reactions and increased populations of the bacteria in samples where nitrates and phosphates were added. Nitrates were consumed fast and exhausted clearly indicating higher requirement of the same but phosphates were not exhausted and by the 40th day we still had huge amounts of phosphorous within the samples, although there was increased activity in the samples with phosphorous. This was an indication that phosphorous is required to enhance the biodegradation process but in limited quantities.

4.3. Summary Conclusions

Looking at the outcome of various tests, the experiments conclusively demonstrated that micro-organisms can effectively be used for reduction and breakdown of hydrocarbons.

Recommendations

So as to realize the full benefit of this study it is important to do further studies of identifying the hydrocarbon utilizing bacterial species responsible for the breakdown then culture the same to grow the numbers. These can be used in the interceptors of petrol stations to see how well they decompose hydrocarbon contaminated effluents in situ.

This will require DNA isolation and extraction, Polymerase Chain Reactions, sequencing and eventually blasting using a sequencer. Biochemical reactions will also be applied to fully conclude the study.

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