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HYDROCARBON-DEGRADING BACTERIA IN PETROLEUM-CONTAMINATED SOIL AT SUAME MAGAZINE

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ABSTRACT
Accidental oil spill has been a global problem especially the oil producing developing countries. Bioremediation remains the best and safest option for eradicating these toxic substances from the environment. In this study, petroleum hydrocarbon-degrading bacteria associated with crude oil contaminated soils were investigated. Samples were taken from 3 different garages at 3 different soil depths (5, 10 and 15 cm) in Suame, a suburb of Kumasi and analyzed microbiologically. Colonies were isolated and cultured on nutrient agar covered with used engine oil to check for their hydrocarbon-degrading ability. The enumeration of the microbes for sites A, B and C were 1.695×10^7, 1.817×10^6 and 1.511×10^5 CFU/g respectively. *Pseudomonas spp., Acinetobacterium spp., Bacillus spp., Aeromonas spp. and Micrococcus spp.* were found to possess hydrocarbon-degrading ability. Thus, in this study, bacteria with hydrocarbon-degrading ability were found to inhabit engine oil-contaminated soils at Suame magazine in Kumasi, Ghana.

KEYWORDS: Petroleum hydrocarbons, *Pseudomonas spp., Acinetobacterium spp., Bacillus spp., Micrococcus spp.*

1. INTRODUCTION
Disposal of waste on land or water bodies is a common practice in Ghana and this can be found in almost every part of the country. However, certain wastes are associated with particular sites. Garages are well noted for oil disposal on soil. Unlike solid wastes, further procedures are required to remove these pollutants from the soil. Various crude oil fractions are used in these garages and end up being dumped on soil. Different chemical contaminants are introduced into the soil and mostly require diverse bioremediative processes to curate the pollution. Contaminants usually found at the garages include brake fluid, engine/dirty oil, petrol, kerosene and bitumen [1]. 218

It is estimated that between 1.7 and 8.8 million metric tons of oil end up in water bodies and on soil annually of which deliberate and non-deliberate human activities account for more than 90% [2]. The emergence of industrialization in Africa has led to tremendous increase in the use of oil fractions as fuel for private and com-

mercial purposes. This generate large amount of waste which mostly end up disposed in the soil [3]. Human activities such as disposal of used lubricating oil, coal tar, and oil filters have led to consistent contamination of soil.

Polycyclic aromatic hydrocarbons (PAHs) contamination of soil is a common practice in Ghana. These contaminations take their source from industries and homes. However, greater proportion of these contaminants is known to be from industries such as mining industries and automobile industries [4].

Research has shown that though guidelines for oil waste disposal is outlined by Environmental Protection Agency of Ghana and made known to oil companies, pollution still exists at these companies. Enforcement of guidelines is hindered due to various reasons and oil contamination of soil continues to persist at a higher level [4]. Those who act contrary to these guidelines are left unpunished and these are often seen in most developing countries. Safety of the environment is a priority in every country and therefore proper measures must be put in place [5].

Suame is a suburb of Kumasi and an area noted in the colonial days for army depot. This led to the coining of the name "Magazine" till date. The area has since been taken over by craftsmen establishing workshops for various duties [6].

The transition from army depot to cluster of workshops started in the 1950s and 1960s. Suame magazine is 1.8 km long and 0.3 km wide. It is estimated to house 40 000 workers in the early 1980s and 100 000 in 2005 [7]. It is the biggest and most efficient in automobile workshop in the country. Vehicles plying the road in Kumasi either to the northern or southern region of the country mostly access services from the magazine [8-9].

Servicing of vehicles leads to oil spill and oil deposits which in most cases are washed off into water channels present at the site. Consistent oil spill gives the soil a greasy feel which cake at the soil surface. Pollution at the magazine is at a high peak making this place suitable for this research. The aim of this study is to identify hydrocarbon-degrading bacteria in petroleum-contaminated soil at Suame magazine.

Researches made over the last decade have led to the emergence of new and advanced technologies that use biological agents to remediate contaminated soils [10]. These technologies are carried out at a low cost and are more efficient than the physical methods. However, bioremediation processes are used on few occasions despite the benefits. It is gradually gaining public recognition and will with time be the ultimate choice for remediating hydrocarbons from soils. Bioremediation techniques involve the use of plant or microbes or both. This tends to offer many options in carrying out this process due to diverse nature of plants and most especially microorganism [11]. Cleaning of pollutants can be done via phytoremediation, bioventing, bioleaching, landfarming, rhizoremediation, biostimulation and bioaugmentation.

2. MATERIALS AND METHODS

The methods used in this study to identify petroleum hydrocarbon-degarding bacteria is a modification to that of Hamza *et al*., (2010) and Latha and Kalaivani, (2011).

2.1. Soil Sampling Method and Processing

Soil samples were aseptically obtained from 3 oil-contaminated soil sites at 3 different soil depth (5, 10 and 15 cm) using hammer, chisel, scraper, builder's square and were transferred into sterile zip-lock bags (Figure 1). Soil samples from sites A and B were contaminated with used diesel whilst samples from site C contained used gasoline. The 9 oil-contaminated soil samples were stored at 4^{o}C and transported to the Biotechnology Laboratory for microbial analysis.

Figure 1 : Soil samples from site C in zip-lock bag.

2.2. Media and diluent preparation

Nutrient agar was used to culture and isolate the various microbes present in the samples since it supports growth of every microorganism. The agar was prepared by dissolving 1.0 g Lab-Lemco Powder, 2.0 g of yeast

extract, 5.0 g peptone, 5.0 g sodium chloride and 15g agar in one liter distilled water and autoclaved for 15 minutes.

Triple Sugar Ion agar was prepared by dissolving 64.6g of the powdered agar in 1 liter distilled water and autoclaved for 15minutes.

For sulfide-indole-motility agar, 30.0g of the agar was dissolved in 1 liter distilled water and autoclaved for 15 minutes.

Tween 80 solution was used as the diluent for the serial dilution method and this was to uniformly dissolve the oil content (diesel and petrol) present in the soil samples. The preparation of Tween 80 solution was done by dissolving 1.0g peptone, 10.0g tween 80 stock solution and 30.0g of NaCl in 1000ml distilled water. The mixture was autoclaved at 121^{o}C for 15 minutes.

2.3. Microbial Growth and Selection

Nutrient agar was used for microbial culturing and was poured into each Petri dish to solidify and kept in an incubator for 24 hours to check media contamination. Soil samples were each serially diluted from 10^{-1} to 10^{-6}. The samples were first sieved to obtain finer particles and 1g of the finer particles of each sample was measured with electric balance. It was dissolved in 9 ml of tween 80 solution in test tube, shaken and allowed to settle. Using a pipette, 9ml of blank tween 80 solution was transferred into 5 test tubes labelled from 10^{-2} to 10^{-6}. Spread plating was done by transferring 0.1 ml of a dilution onto nutrient agar using a micropipette. Spreading was done uniformly using sterile glass rod which was sterilized with alcohol and flamed to avoid contamination. Plating of each dilution was done in triplicate for accuracy of result. This plating technique was repeated for all dilutions and for all samples. Incubation was done at 37^{o}C for 24 hours after inoculation.

Enumeration of microbial colony was done manually after 24 hours and plates were again incubated for 72 hours to ensure adequate growth and distinction of bacteria colony. Isolation of bacteria colony was done by considering the different colony present in all the plates. Based on morphology and growth on agar, different bacteria colony were selected from the plates of all samples and sub-cultured to obtain pure culture of each selected isolate.

Sub-culturing was carried out by inoculating the colony on nutrient agar. Sterile inoculation loop were used to pick each bacteria colony and streaked on fresh agar to obtain pure colony on each plate for further analysis.

2.4. Characterisation and Identification of Selected Isolates

Gram staining and microscopy, and biochemical analysis were used to characterize and identify isolates. Bacteria colonies were identified based on morphology, reaction with Gram stains and ability to degrade biomolecules to produced characteristic colours.

2.5. Gram Staining and Microscopy

A drop of sterile physiological saline water was placed on a clean slide and a loopful of bacteria colony (isolate) from pure culture was emulsified in it using sterile inoculation loop. It was air-dried and heat-fixed by passing the slide over Bunsen flame. The slide was flooded with crystal violet solution for about 2 minutes, gently washed off with water and drained with filter paper. The slide was further flooded with Gram's iodine solution (mordant) for about 2 minutes to fix the stain in the bacteria cell wall. Excess dye was washed off using 95% ethanol and drained. Safranin was used to counterstain for 3 minutes and gently washed off with water. The slides were dried and viewed under oil immersion objective lens [12].

2.6. Biochemical tests

The isolates were carried through series of biochemical tests to analyse their ability to degrade some biomolecules. These tests included catalase, citrate, triple sugar ion (TSI) agar test and sulfide-indole-motility (SIM) test.

In the catalase test, a drop of 30% hydrogen peroxide (H_2O_2) was placed on clean slide. A loopful of each isolate was transferred into each drop on a slide and observed for bubble formation.

Sterilized citrate agar was poured into test tubes and slanted in a rack. It was allowed to solidify and incubated for 18 hours to check contamination. Inoculation was done by streaking isolate on agar slant using sterile inoculation loop. Incubation was done at 37^{o}C for 24 hours. Observations were recorded based on colour changes.

In TSI agar test, the sterilized agar was poured into tubes and slanted in a rack to solidify. The media was stabbed with the isolates in each tube using sterile inoculation pin. Incubation was done at 37^{o}C for 24 hours and colour changes observed.

Sulfur-indole-motility agar slants were prepared and incubated for 18 hours to check media contamination. The isolates were picked from sub-culture and stabbed into the agar slant to the bottom using sterile inoculation pin. Incubation was done at 37^{o}C for 24 hours.

2.7. Hydrocarbon-degrading ability of isolates

Hydrocarbon-degrading ability of isolates were determined. Nutrient agar was prepared without glucose (hydrocarbon source). Five millilitres of used diesel oil was spread on the agar in different plates and 3ml of each isolate was inoculated (Figure 2).

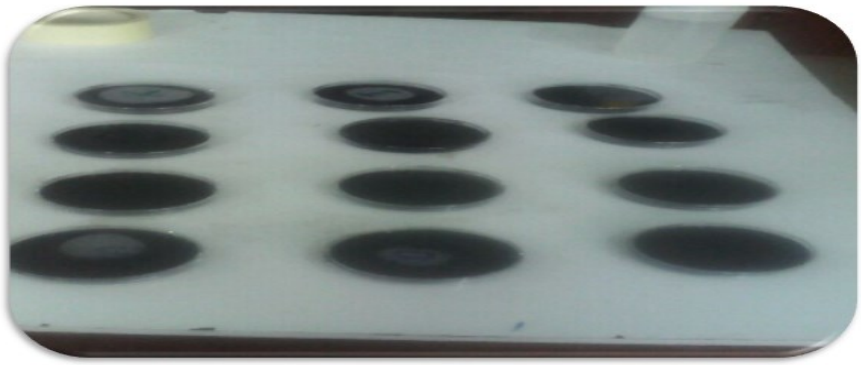

Figure 2. : Media covered with diesel oil before incubation

2.8. Statistical Analysis

Data analysis was carried out using GraphPad Prism 5 software of which standard error of mean was calculated to show the extent of deviation of mean value.

3.0 RESULTS AND DISCUSSION

3.1 Microbial load determination for soil samples

A gradual decrease of microbial load from 10^{-1} to 10^{-6} dilutions was observed. The first two dilutions had too numerous microbial loads for most samples while 10^{-6} had no growth for majority of the plates. The average colony forming unit for each soil showed a decline with increasing soil depth for site B and C whereas 10 cm depth had the highest load for site A as shown in Table 1. Different bacteria colonies characterized by shape were found to grow on the agar. There were distinct features of the growth pattern with gradual decrease in total viable count (TVC) from 10^{-1} to 10^{-6} dilution factor. Also some bacteria were found to be dominant in 10^{-4} dilutions while 10^{-1} to 10^{-3} dilution showed mixed colony growth. This is because the microbial load on plate decreases with increasing dilution which provides a wide area for fast growing colonies to occupy more space. Also, colonies of some bacteria were wide in size accounting for the dominance. From the result in Table 1, the highest TVC was observed for samples taken at 5cm depth while 15cm depth samples had the least TVC. This is in line with assertion made by Fierer *et al.*, (2003), that microbial load decreases with increasing soil depth due to the reduction in soil nutrient, moisture and air.

Sample	Average Colony Forming Unit (CFU/g)
A_5	$1.707 \times 10^7 \pm 18.99$
A_{10}	$1.860 \times 10^7 \pm 6.429$
A_{15}	$1.517 \times 10^7 \pm 13.84$
B_5	$2.383 \times 10^6 \pm 31.69$
B_{10}	$1.790 \times 10^6 \pm 12.42$
B_{15}	$1.277 \times 10^6 \pm 6.333$
C_5	$2.467 \times 10^5 \pm 21.46$
C_{10}	$1.570 \times 10^5 \pm 10.69$
C_{15}	$4.967 \times 10^4 \pm 3.756$

Table 1: Microbial enumeration (CFU/g) for each soil depth

3.2 Microbial load and morphology for each soil site

According to Hamza *et al*., (2010), microbial load of a diesel-contaminated site is higher than gasoline-contaminated site. This assertion is line with result obtained for each site in Table 2. The diesel oil contaminated sites (A and B) had higher total viable count or average coliform unit/gram (CFU /g) than the gasoline-contaminated site (C) . The highest microbial count, 1.695×10^7, was recorded for site A while site C had the least count, 1.511×10^5. The colonies observed from each site showed a distinctive colony growth. Site C had cream and white coliforms (bacillus) with wide size dominating most of the plate, while that of site A and B had different colonies but dominated by brown, green and yellow colonies (coccobacillus, rods and cocci) with small spread size on (Figure 3). This may have been influenced by the type of oil contaminant at the site as observed also by Latha and Kalaivani, (2011).

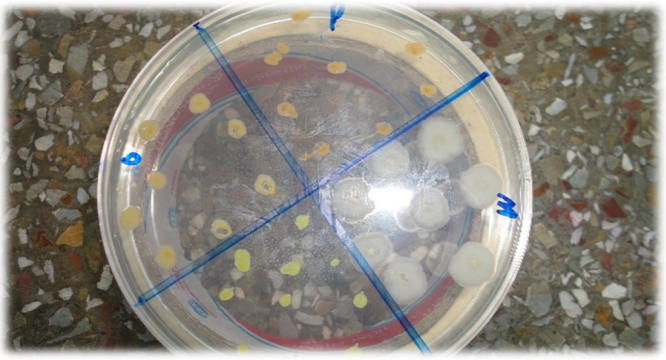

Figure 3: Representative plate showing pure culture of bacteria

Table 2: Average Bacterial loads from the 3 sites

SITE	Average Colony forming unit (CFU/g)
A	$1.695 \times 10^{7} \pm 0.09921$
B	$1.817 \times 10^{6} \pm 0.3196$
C	$1.511 \times 10^{5} \pm 0.5695$

Table 3: Gram staining and Microscopic Identification of isolates

ISOLATES	COLOUR ON MEDIA	GRAM STAINING	MICROSCOPY
A	Green	-	Rods
B	Orange	+	Clustered cocci
C	Green	-	Rods
D	Green	-	Rods
E	Cream/White	+	Rods
F	Cream/White	+	Rods
G	Cream/White	+	Rods
H	Pink	-	Coccobacillus
I	Yellow	+	Clustered cocci
J	Cream/White	+	Rods
K	Cream	+	Rods
L	Brown	-	Coccobacillus
M	Cream	+	Rods
N	Green	-	Rods

3.3 Gram staining and microscopy for isolates

The ability of the cells of the isolates to retain Gram stains gave variable results. Six isolates (A, C, D, H, L and N) were Gram negative as shown in Table 3. Gram positive isolates were mainly long rods (bacillus) with white/cream colour on nutrient media and clustered cocci with yellow colours. Gram negative isolates had a mix of short rods and coccobacillus when observed under microscope. These isolates had different colours on nutrient media including green, orange, pink, white/cream and brown. Gram positive *Bacilli* were the predominant bacteria among the isolates from site C. According to Latha and Kalaivani, (2011), Gram positive *Bacillus* species are good gasoline-degrading bacteria. The presence of a peptidoglycan cell wall makes them tolerant to high concentration of gasoline. *Bacillus* (rods) species are known to be the best petroleum hydrocarbon-degrading bacteria [14, 15]. Gram negative isolates (rods, coccobacillus and spheres) were the dominant isolates for diesel-contaminated sites. Unlike Gram positive bacteria, their cell wall contains

porins which makes it impermeable to these hydrocarbons.
Gram negative rods (*Pseudomonas spp. and Aeromonas spp.)* contain additional efflux pumps in their cell membrane which ensure excretion or removal of metabolites from the cell to prevent feedback inhibition or saturation of end products. This account for the reason *Pseudomonas spp* are known to be the best petroleum hydrocarbon-degraders.
The shapes of the isolate were identified by microscopy under oil immersion objective lens. From Table 3, 6 isolates (E, F, G, J, K and M) were Gram positive rods belonging to the *Bacillus* species. Two isolates (B and I) were Gram negative cluster of spherical shaped bacteria and therefore belong to the *Micrococcus* group. Isolate H and L showed both Gram negative spheres and Gram positive rods. This is associated with *Acinetobacter* genus. The remaining isolates (A, C, D and N) were Gram negative short rods, characteristic of *Pseudomonas spp.* and *Aeromonas spp.*
+ = Purple coloured bacteria cells (Gram positive)
- = Pink coloured bacteria cells (Gram negative)

3.4 Biochemical assay
Petroleum hydrocarbons (PHC's) are converted to less toxic substances either by biotransformation, biodegradation or mineralization. Bacteria species have different capacity in degrading PHC's and therefore different end products are produced which sometimes become substrates for other bacteria species. PHC's is metabolised by microbes in the soil via oxidation. Due to the hydrophobic nature of PHC's, micelles are formed by bacteria to enable oxygen transport. The oxidative degradation of PHC's by microbial consortium produces different end product as well as reactive oxidizing species (ROS) which are harmful to microbes that lack those enzymes. The result from Tables 4 and 5 showed the ability of isolates to degrade these end products and reactive oxidizing species.
Petroleum hydrocarbon-degradation is an oxidative process carried out by cytochrome P450 mono- or dioxygenase present in most facultative and obligate aerobes. Toxic forms of oxygen such as H_2O_2 are produced by bacteria that partially reduce electron acceptors (oxygen) [17]. Catalase test was used to identify bacteria that can convert H_2O_2 to oxygen. From Table 4, all the isolates produced bubbles when dropped in the peroxide solution, showing the presence of catalase enzyme in all the isolates.

Citrate test was used to identify bacteria capable of using citrate as a sole carbon source. Isolates C, D, I, K, L, M and N were citrate positive as shown in Table 4 and Figure 4. Blue colour was observed after incubation. Citrate is an intermediate in Kreb's cycle which can only be accomplished by bacteria that show complete mineralization of PHC's. Three Gram negative rods (C, D and N) were positive which confirm their ability to metabolise citrate as the sole source of carbon. Coccobacillus and cocci had positive isolate each, L and I respectively. Citrate positive isolates were J, K and M.

TSI test was used to identify isolates with the ability to ferment sugars, produce CO_2 gas and also hydrogen sulphide. The agar used contained glucose, sucrose and lactose as the sugar constituent, peptone and phenol red (pH indicator). Colour changes were due to the production of acid from sugar catabolism. Gram negative rods isolates (A, D and N) showed no colour change and hence no sugar catabolism under anaerobic condition. It implies that these isolates are obligate aerobic bacteria and therefore require oxygen for cellular metabolism. Isolates with colour change in either butt or slant such as E, F and M catabolized only glucose under anaerobic condition while those with colour change in both butt and slant such as B, C, J and K metabolized all the sugars present. All isolates with colour changes are facultative anaerobes and can therefore thrive well with or without oxygen. Colour change to yellow depict acid (A) production while red or orange shows alkaline (K) production as shown in Table 4 and Figure 5a.

From Table 5 and Figure 5b, motility of colony growth along the stabbed path, H_2S production and indole production were identified in SIM test. Slight motility was observed for 7 isolates. No H_2S production was recorded while isolates B and K produced a red ring when Kovac's reagent was added. This means that isolates B and K contained tryptophanase enzyme to produce indole from aromatic amino acid tryptophan which is a component of the agar. It also implies that these isolates can use amino acids as a carbon source to survive.
All isolates showed degradation ability at different rates. However, Gram negative rods showed fastest degrading ability followed by the clustered cocci. Gram positive bacillus showed the lowest degrading ability after 24 hours of incubation. This is because *Bacillus* degrade diesel oil at a slower rate [18-19] .

Comparing results obtained from Gram staining and microscopy, and biochemical assay with that obtained in other research work [19-22], *Pseudomonas spp*, *Aeromonas spp*, *Micrococcus spp*, *Acinetobacter spp* and *Bacillus spp* were present and had degradative ability.

Pseudomonads are ubiquitous Gram negative rods that possess flagella for motility. These are non-spores and have green colony on nutrient media. These are obligate aerobes, citrate positive, catalase positive, and TSI negative and are widely known for their ability to degrade petroleum hydrocarbons. Strains with such ability

ISOLATES	CATALASE	TSI	CITRATE
A	+	K/K	-
B	+	A/A	-
C	+	A/A	+
D	+	K/K	+
E	+	K/A	-
F	+	A/K	-
G	+	A/K	--
H	+	A/A	-
I	+	A/K	+
J	+	A/A	+
K	+	A/A	+
L	+	K/A	+
M	+	K/A	+
N	+	K/K	+

Table 4: Biochemical Identification of isolates

include *Pseudomonas aeruginosa* and *Pseudomonas putiba.* New genus identified with PHC's degradation include *Stenotrophomonas*. From result obtained, it can be deduced that isolates D and N were *Pseudomonas spp. Aeromonas spp* are also Gram negative rods with the same characteristics as *Pseudomonas spp*. However, unlike *Pseudomonas spp*, these are citrate negative and show variable result for TSI due to their facultative anaerobic nature. *Aeromonas* also show variability in motility due to the absence of flagella in some species. The well-known specie with PHC's degrading ability is *Aeromonas hydrophila*. Isolates A was therefore identified as an *Aeromonas spp*.

ISOLATES	MOTILITY	H_2S	INDOLE
A	+	-	-
B	+	-	+
C	+	-	-
D	+	-	-
E	-	-	-
F	+	-	-
G	-	-	-
H	+	-	-
I	-	-	-
J	-	-	-
K	-	-	+
L	-	-	-
M	-	-	-
N	+	-	-

Table 5: Identification of Isolates using SIM test

Figure 4: Representative tubes showing colour changes for citrate test

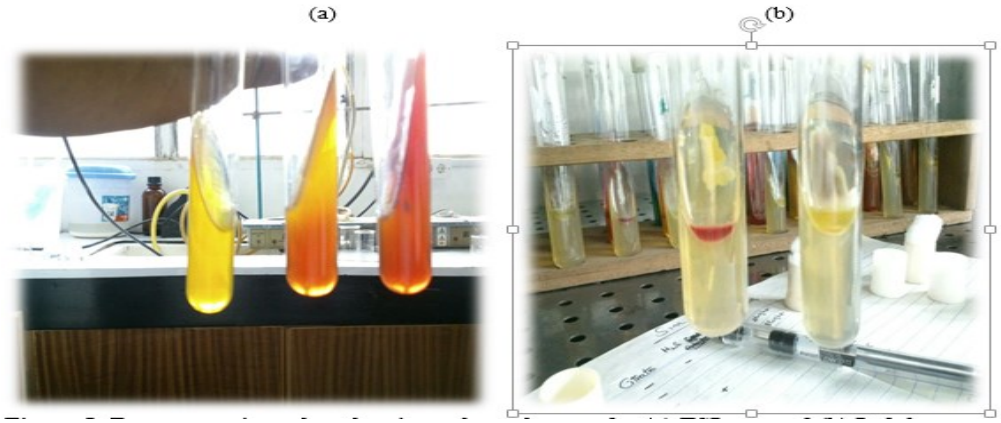

Figure 5: Representative tubes showing colour changes for (a) TSI test and (b) Indole test

Acinetobacter is a group of bacteria characterized by their Gram negative coccobacillus nature. These are known to be non-motile, catalase positive and aerobic. They are known to be highly resistant to different classes of antibiotic. *A. lwoffi* is the specie associated with PHC's degradation. Isolates H and L belonged to this group.
Micrococcus is a Gram positive clustered spheres that are catalase positive, citrate negative and indole positive. The cell wall constitutes 50% of cell size. This aid in their degrading ability of PHCs and known species include *Micrococcus luteus*. Isolates B and K were found to belong to this group.
Bacillus spp. are ubiquitous Gram positive rods, obligate aerobe or facultative anaerobe, catalase positive and endospore formation bacteria. Many species have been identified over the years. However, those associated with PHC's degrade include *B. subtilis*. Isolates F, G and J were found to be examples of *Bacillus spp.*

Key: Catalase (+) = Effervescence formation, A = Acid formation, K = Alkaline formation
Citrate (+) = Blue colour formation

Key: Motility (+) = Slight motility (-) = No motility
Indole (+) = Red ring formation (-) = No ring formation
H_2S (-) = No black precipitate formed

3.5 Hydrocarbon-degrading ability of isolates
All isolates showed degrading ability. However, Gram negative rods and Gram positive clustered spheres showed the fastest degrading potential after incubating for 24 hours.

4. CONCLUSION
The depth of soil has influence on the microbial consortium present. The 5 cm samples obtained from each site had the highest microbial load together with diverse bacteria colonies. This is basically dominated by aerobic bacteria species while that of the 15 cm depth had the least growth with anaerobes as the dominant colony. The various microbes identified in present study were Gram negative bacteria comprising rods and coccobacillus while Gram positive were clustered cocci and rods. The various biochemical test and microscopy tests revealed

the presence of *Pseudomonas spp*, *Micrococcus spp*, *Aeromonas spp, Bacillus spp* and *Acinetobacter species*.

Authors' Contribution

Mak-Mensah Ephraim drafted the research concept and design. Collection of sample and laboratory work was done by Agyeman-Duah Eric. Statistical analysis was done by Felix Charles Mill-Robertson and Agyeman-Duah Eric. All authors contributed to writing and revision of manuscript. Agyeman-Duah gave the final approval of manuscript.

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