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Crude Oil Degradation Potentials of Bacterial Communities from Humic Freshwater Ecosystem of Eniong River Sediment Samples

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ABSTRACT
Crude oil degradation potentials of bacterial communities from humic freshwater sediment of Eniong River were determined using diverse bacteriological and analytical procedures. The humic sediment harbours remarkable bacteria load such as heterotrophic bacteria with a mean count of 5.94±0.41 $\log_{10}$cfu/g, sulphate reducing bacteria with a mean count of 5.51±0.62 $\log_{10}$cfu/g and hydrocarbon utilizing bacteria with an average count of 4.48±0.95 $\log_{10}$cfu/g. The characterization studies of the isolates from the humic sediment ecosystem revealed 19 genera and 25 species of bacteria and 1 genus and 1 species of Actinomycetes. The screening tests revealed 14 out of 19 species of bacteria encountered in the black water ecosystem possess the ability to utilize hydrocarbons. Among the isolates, *Bacillus subtilis*, *Micrococcus* sp., *Bacillus cereus* and *Pseudomonas aeruginosa* showed very strong crude-oil degrading potentials. The results of the effect of environmental factors showed that the best growth was obtained by *Pseudomonas aeruginosa* ($2.60x10^2$cfu/g) at 30°C while the best growth was exhibited by *B. cereus* ($1.20x10^2$cfu/g) at pH of 6. *Pseudomonas aeruginosa* ($\log_{10}$6.93cfu/ml) exhibited the highest growth at day 10 and 180 revolution per minute (rpm). The profiles showing the relationship between cell numbers and pH indicate a steady increase in cell numbers and decrease in pH of the medium as incubation periods increase from day 1 to day 10. The result showed the rich assemblage of different bacteria with the ability to degrade crude oil and therefore should be harnessed for application in remediation of the crude oil polluted ecosystems.

Keywords:
Degradation potential,
Bioremediation,
Detoxification,
Contaminants,
Assemblage,
Humic freshwater ecosystem.

INTRODUCTION

Microorganisms play very important and basic roles in cycling of elements and soil structure formations (Bastida *et al.*, 2007). Current evidence suggests that in aquatic sediment ecosystem, microorganisms are the chief agents for biodegradation of substances of environmental concern. Therefore, the microbiological techniques are seen as the most important and promising for application in degradation of various organic contaminants in the environment. The employment of diverse microbiological techniques in the detoxification of contaminants typically is less expensive than the physical and chemical methods (Balba*et al.*, 1998) and is also environmental friendly. It is important to note that organic matters from the soil are composed of complex mixtures which affect some of the soil characteristics and nutrients movements and transformation.

Microbial degradation plays vital function in the bioremediation of contaminated soil, sediment and ground water sites. However, some of such contaminants are compounds of organo-phosphorus, steroids, alkanes, polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and chloroethenes (Koukkou, 2011). Microorganisms are used for *in situ* degradation of domestic, agricultural and industrial wastes as well as sub-surface pollutants in soils, sediments and marine environments. The contaminant composition determines the strength of any of the microbes employed to degrade toxic wastes. Since most of the sites typically habour a combination of different pollutants types, the most

efficient and appropriate method is the introduction of co-metabolism which employs the breakdown of the contaminants by different bacterial groups, species and strains to achieve a mineralized environment (Koukkou, 2011). The monitoring of the composition of the indigenous and added bacteria is very important so as to access the activity level and to permit modifications of the nutrients and other conditions for optimizing the bioremediation processes (Koukkou, 2011).

Based on its composition, it is noteworthy to affirm that crude oil may be said to be the most complex mixture of organic compounds that occurs in the world. Its components include compounds such as alkanes, aromatics, resins and asphaltenes (Płaza *et al.,* 2008). Recent advances in ultra-high-resolution mass spectrometry has identified about 17,000 different chemical components and the term petroleomics has been coined to express this newly uncovered complexity (Marshall and Rodgers, 2004; Head *et al.,* 2006). Hydrocarbon is toxic and pollution of the aquatic ecosystem by it is some of the causes of major ecological concerns. Spillages of crude oil in aquatic environments are poorly containable and mitigation is difficult. Most of the oil can be removed by hydrocarbon degrading actions of bacterial population known as hydrocarbonoclastic bacteria (HCB). These organisms can help remediate the ecological damages caused by oil pollution of aquatic and terrestrial habitats. HCB also have potential biotechnological applications in the areas of bioplastics and biocatalysis (Koukkou, 2011). Mechanical removal of contaminants like hydrocarbons from the ecosystem relies on expensive, slow and inefficient methods (Mandri and Lin, 2007). Therefore, the purpose of this work was to determine the crude oil degradation potentials of bacterial communities from humic freshwater Ecosystem of Eniong River sediment.

MATERIALS AND METHODS

Study Area

The study area is a humic freshwater ecosystem of Eniong River, a branch of the Middle course of the Cross River located in South-Eastern coast of the Niger Delta, Akwa Ibom State, Nigeria between latitude 05° 15¹ and 56.0¹¹ N and longitude 05° 12¹ N and 05° 054¹¹ E (figure 1).

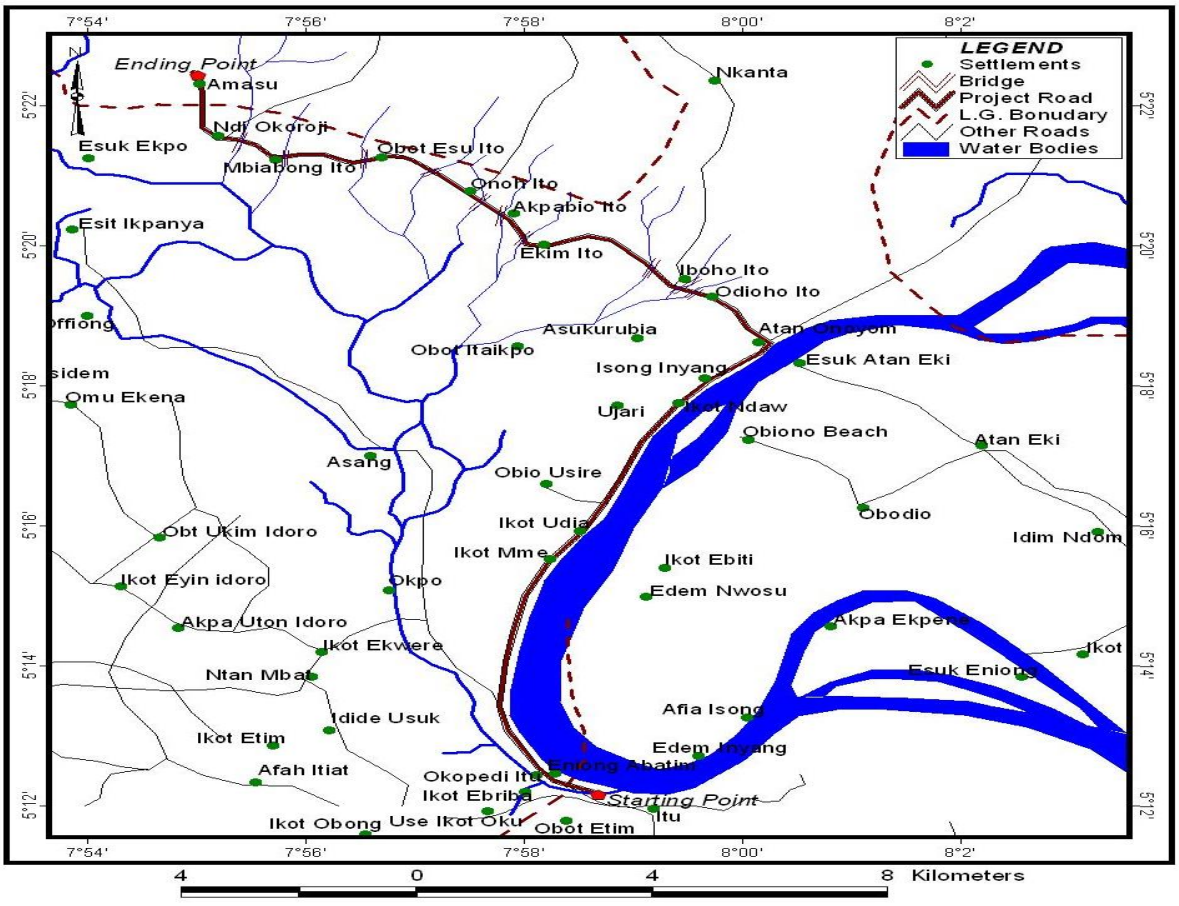

Figure 1: The middle course of Cross River showing the location of the humic freshwater ecosystem of Eniong River (Egbomuche *et al.,* 2019).

Sample Collection and Preparation

Benthic sediment samples were collected from three (3) different sample stations which are designated ST_3 (Upstream), ST_2 (Midstream) and ST_1 (Downstream) of the River as previously described by Nweke *et al.* (2007). A total of nine (9) sediment samples were collected for bacteriological analysis. Benthic sediment samples were aseptically collected using Eckman sediment grab into ninety five percent (95%) ethanol-sterilized plastic containers and accordingly transported to the Microbiology laboratory of Michael Okpara University of Agriculture (MOUAU) within twenty four hours (24 h) of collection for further analysis. Samples were kept at lower temperature by being placed in ice packed coolers. On arrival to the laboratory, the samples were immediately transferred into the refrigerator where they were stored at 4ºC until required (Essien and Udosen, 2000).

Isolation of Diverse Groups of Bacteria from Sediment Samples of Eniong River

Ten-fold serial dilution of the sediment samples was carried out with known volume of sterile distilled water before enumeration of densities of the different bacterial groups. Serial dilution of sediment samples was done according to the method of Cheesbrough (2006). About 10g of sediment samples were carefully measured and introduced into conical flasks containing 90ml of sterile distilled water (Cheesbrough, 2006). These were shaken for even distribution and thereafter one milliliter (1.0ml) of the aliquot was aseptically transferred into sterile test tubes containing nine milliliter (9.0ml) of diluents to give a dilution of 10^{-1}. This was repeated until a tenth (10^{-10}) dilution factor was actualized.

Determination of Densities of Different Bacteria Groups in Eniong River Sediment Ecosystem

The densities of heterotrophic and oil degrading bacteria were determined using standard analytical techniques. These included the estimation of the densities of aerobic and oil-degrading bacteria.

Densities of Heterotrophic Bacteria from Eniong River Sediment

The counts of total heterotrophic bacteria (HB) in the samples were respectively determined by the pour plate techniques using Nutrient agar (NA) as the analytical media. One mililitre (1.0 ml) of the serially diluted sample was inoculated into empty petri dishes and about 15.0 ml of melted nutrient agar (NA) which has been cooled to 45ºC was poured into the culture plate and allowed to solidify. The discreet colonies were counted after 24 hours incubation at room temperature.

Densities of Sulphate Reducing Bacteria from Eniong River Sediment

The modified Bacto Sulfate API medium plus agar was adopted for the enumeration and isolation of sulphate reducing bacteria (SRB). The number of sulphate reducers was determined by the pour plate technique at 28ºC±2ºC after 7 days of incubation using compounded media as analytical medium. The medium comprises of Bacto yeast extracts-1g; Ascorbic acid-0.1g; Sodium lactate-5.2g; Magnesium sulphate-0.2g; Dipotassium phosphate-0.01g; Ferrous ammonium sulphate-0.1g; Sodium chloride-10g; Agar agar-7.5g and 1000ml of distilled water. 1ml each of the serially diluted samples was pour plated and incubated anaerobically in duplicates at room temperature in an anaerobic jar for a period of 7 days. The colonies were counted and recorded appropriately (Essien and Udosen, 2000).

Densities of Oil-Degrading Bacteria from Eniong River Sediment

The vapour phase transfer method described by Okpokwasili and Okorie (1988) was adopted. The inocula were cultured on mineral salt medium (MSM) and sterile Whatman filter paper soaked in crude oil, which served as the carbon and energy sources supplied from the lid of the inoculated plates. The set up as well as the controls (without crude oil) were incubated for about 7 days at room temperature. For the 10^{-3} dilutions, 0.5 ml 100µg/ml cycloheximide/ 50µg/ml benomyl were added to the mineral salt agar to suppress fungal growth. Colony forming units per gram (CFU/g) on the MSM plates were enumerated and the number of crude oil degrading bacteria (ODB) obtained.

Maintenance of Pure Cultures of Bacteria Isolates

Distinct cultures of the representative colonies of the different bacteria isolates observed on the culture plates were purified by repeated sub-culturing on freshly prepared NA medium. The medium used and conditions originally used for their isolation were employed. Stock cultures of the pure bacteria isolates were maintained on NA slants in McCartney bottles and incubated appropriately before preservation in the refrigerator for further usage at 4ºC.

Characterization and Identification of Bacteria Isolates

The bacteria isolated from humic freshwater sediment were characterized by using standard procedures as described by Cowan (1985) and Holt *et al*., 1994). The tests that were conducted include Gram's stain, spore stain, motility test, catalase test, urease test, coagulase test, oxidase test, citrate test, sugars utilization test, methyl red and voges-proskauer (MR-VP test) etc.

Determination of Hydrocarbonoclastic Potential of Bacteria Isolates from Eniong River Sediment

The vapour phase transfer method described by Okpokwasili and Okorie (1998) was employed. Bacterial isolates obtained were inoculated into oil agar (mineral salt medium) and sterile Whatman filter paper soaked in crude oil, which served as source of carbon and energy was supplied from the lid of the inoculated plates. The set up as well as the controls (without crude oil) was incubated for about 7 days at room temperature. Visible bacterial growth was regarded as evidence of ability to degrade hydrocarbons. Colony forming units per gram (CFU/g) on the oil agar plates were enumerated and the number of crude oil degrading bacteria obtained by subtracting the number of CFU/g in the control (without crude oil) from those in test (with crude oil) cultures (Okpokwasili and Okorie, 1998). This was graded as strong (+++), moderate (++), weak (+) and no (-) degrading potential as described by Ekundayo and Obire (1987).

Effects of Environmental Factors on Bacteria Utilization of Hydrocarbon

Effects of temperature

The effects of temperature on bacterial growth on 1% (v/v) (99ml of mineral salts medium + 1ml of crude oil + Isolate) crude oil biodegradation were studied at 30°C, 37°C and 40°C. Duplicate inoculation for all three different temperature studies was performed at the same time using the same batch of medium, bacterial inoculum and crude oil to minimize variations. The growth was measured as colony forming unit per milliliter (CFU/ml) (Ghazali *et al*., 2004).

Effects of pH

The influence of medium pH on growth of bacterial strains was investigated at pH 6.0, 8.0 and 9.0. The medium contains (99ml of mineral salts medium + 1ml of crude oil + 1ml of Isolate) and control (100ml of mineral salts medium + 1ml of Isolate). The initial pH of basal medium was adjusted to the desired pH using 0.1 or 1 M of either HCl or NaOH. The same incubation periods and temperature were applied. The study was conducted in duplicate and growth was measured as CFU/ml (Ghazali *et al*., 2004).

Effects of agitation

The study of agitation during incubation on growth and crude oil (1% v/v) utilization by bacterial strains was carried out using orbital shakers (Shelab) at 160 and 180 rpm. Incubation was conducted at 28±2°C and all inoculum preparation, inoculation and sampling times (0, 2, 4, 6, 8 and 10 days) were identical to the procedures described for the other studies (Ghazali *et al*., 2004). The medium composition was (99ml of mineral salt medium without agar + 1ml of crude oil + Isolate) and the control (99ml of mineral salt medium without agar + Isolate). The growth was measured as (TVC, OD and pH).

RESULTS AND DISCUSSION

Enumeration of different bacterial groups in Eniong River sediment (freshwater ecosystem)

The humic sediment of Eniong River harbours remarkable bacteria loads which are presented in Figures (2-4) below. The results showed that the heterotrophic bacterial counts ranged from 5.92 $\log_{10}$ cfu/g downstream, 5.95 $\log_{10}$ cfu/g midstream and 5.97 $\log_{10}$ cfu/g upstream with a mean count of 5.95±0.41 $\log_{10}$ cfu/g (Figure 2). Sulphate reducing bacteria counts ranged from 5.20 $\log_{10}$cfu/g midstream to 5.79 $\log_{10}$ cfu/g downstream with a mean count of 5.51±0.62 $\log_{10}$cfu/g (Figure 3) while hydrocarbon utilizing bacteria counts ranged from 4.36 $\log_{10}$cfu/g midstream to 4.6 $\log_{10}$cfu/g downstream with an average count of 4.48±0.95 $\log_{10}$ cfu/g (Figure 4).

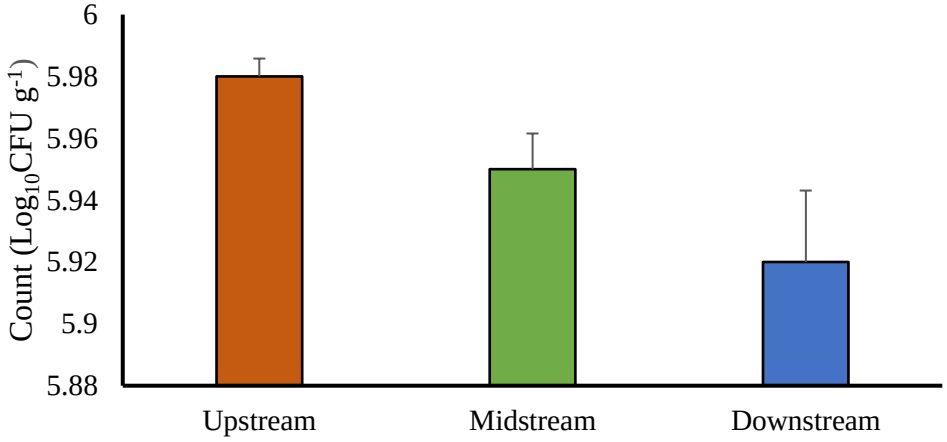

Figure 2: Heterotrophic bacteria counts in upstream, midstream and downstream of Eniong River

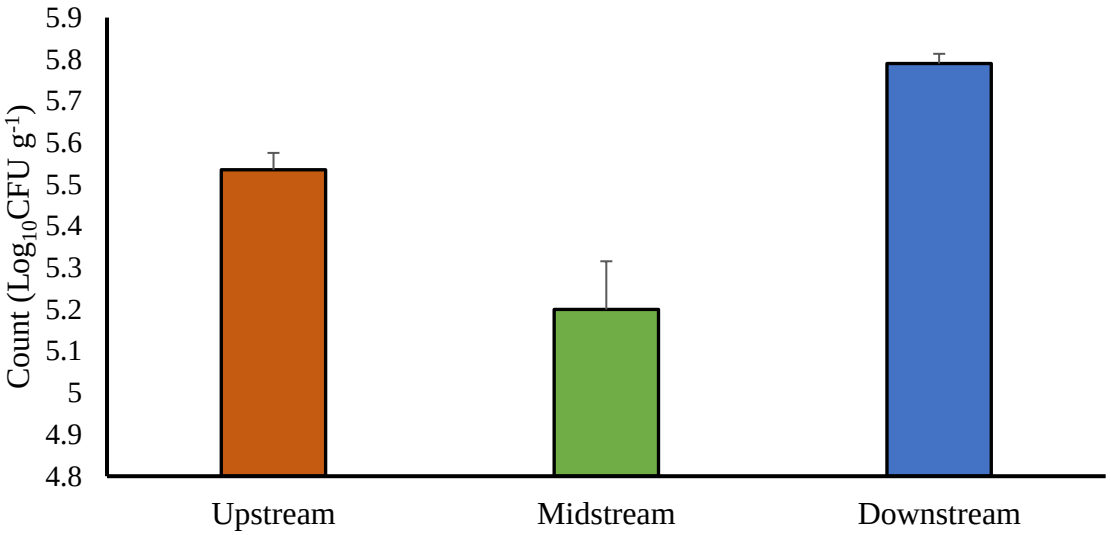

Figure 3: Sulphate reducing bacteria counts in upstream, midstream and downstream of Eniong River

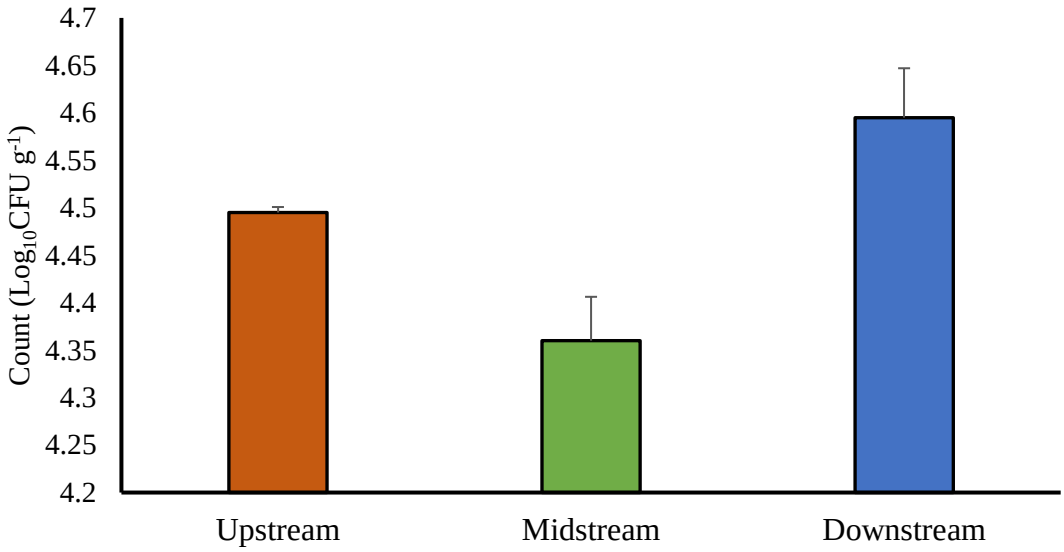

Figure 4: Hydrocarbon utilizing bacteria in upstream, midstream and downstream of Eniong River

Identification of diverse species of bacteria isolated from Eniong River sediment humic freshwater ecosystem
Results of the characterization studies of the isolates from the humic freshwater sediment ecosystem are presented in Table 1. These involve 19 genera and 25 species of bacteria and 1 genus and 1 species of Actinomycetes.

Table 1: Morphological, cultural and biochemical characteristics of isolates from Eniong River sediment ecosystem

Serial Number	Morphology	Gram Reaction	Catalase	Citrate	Coagulase	Indole	Motility	Oxidase	Methyl Red	Voges Proskauer	Urease	Glucose	Sucrose	Lactose	Mannitol	Maltose	Nitrate Reduction	H_2S Production	Gas Production	Probable Organisms
1	Rod	-	-	-	-	+	+	+	+	+	-	A	A	-	A	A	-	-	-	*Vibrio cholera*
2	Rod	-	-	+	-	+	+	+	+	-	-	A	-	-	A	A	-	-	-	*Vibrio parahaemolyticus*
3	Rod	-	+	-	-	+	-	-	+	-	-	A	-	-	A	A	+	-	+	*Escherichia coli*
4	Rod	-	+	+	-	-	+	+	+	-	-	AG	-	-	A	A	-	+	+	*Salmonella typhi*
5	Rod	-	+	-	-	-	-	-	+	-	-	A	-	-	A	A	+	-	+	*Shigella sonnei*
6	Rod	-	+	+	-	-	+	-	-	+	+	A	+	V	+	+	+	-	-	*Enterobacter agglumerans*
7	Cocci	+	+	+	+	+	-	+	+	+	+	+	+	A	A	A	+	-	-	*Staphylococcus aureus*
8	Cocci	+	+	+	+	-	+	+	+	+	+	+	+	-	A	A	-	-	-	*Staphylococcus epidermidis*
9	Rod	-	+	-	-	+	+	+	+	-	+	AG	+	-	-	+	+	+	+	*Proteus vulgaris*
10	Rod	+	+	+	-	-	+	-	-	+	+	A	A	A	A	A	+	+	+	*Bacillus cereus*
11	Rod	-	+	+	-	-	+	+	-	+	-	-	-	-	+	-	+	-	-	*Pseudomonas aeruginosa*
12	Rod	-	+	-	-	-	+	+	-	+	+	AG	A	A	AG	-	-	+	+	*Desulfovibrio vulgaris*
13	Rod	-	+	+	-	-	+	+	-	+	-	A	A	A	A	A	+	+	-	*Desulfuromonas* sp.
14	Rod	-	-	+	-	-	-	-	-	+	-	AG	A	A	A	AG	+	+	+	*Desulfobacter* sp.
15	Cocci	+	+	-	-	-	-	+	+	+	-	A	A	AG	AG	A	-	-	+	*Micrococcus* sp.
16	Rod	-	+	+	+	+	-	-	+	+	-	AG	A	A	AG	A	+	-	+	*Burkholderia* sp.
17	Rod	+	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	*Lactobacillus casei*
18	Rod	-	+	+	-	-	+	+	-	-	-	+	-	-	-	+	-	-	-	*Sphingomonas* sp.
19	Rod	+	-	-	-	-	+	+	-	-	+	+	-	+	-	-	+	-	-	*Streptomyces griseus*
20	Rod	-	+	+	-	-	+	-	-	+	-	+	-	-	+	-	+	-	-	*Serretia marcescens*
21	Rod	+	+	+	-	-	+	+	+	-	-	+	+	+	+	-	+	+	+	*Bacillus subtilis*
22	Rod	+	-	-	-	-	+	+	-	-	-	+	-	-	-	+	-	+	-	*Clostridium botulinum*
23	Rod	+	-	-	-	-	-	-	-	+	-	+	+	+	+	+	+	+	+	*Clostridium perferingens*
24	Rod	+	+	-	-	-	-	+	-	-	+	-	-	+	+	-	+	+	-	*Nocardia* sp.
25	Rod	+	+	-	-	-	-	-	+	+	+	-	-	-	+	+	-	-	-	*Mycobacterium* sp.
26	Rod	-	+	+	-	-	-	+	-	-	-	+	+	+	-	+	+	-	-	*Flavobacterium aquatile*

Key:
A: Acid, G: Gas, AG: Acid and Gas, + Present, - Absent

Hydrocarbonoclastic potentials of isolates from Eniong River sediment

The oil-degrading potentials of bacteria isolated from the humic freshwater sediment ecosystem are reported in Table 2. The screening tests revealed that a number of the bacteria (14 out of 19 species) encountered in the black water ecosystem have the ability to utilize hydrocarbons. Among the isolates, *Bacillus subtilis*, *Micrococcus* sp., *Bacillus cereus* and *Pseudomonas aeruginosa* showed very strong crude-oil degrading potentials. This was followed by *Staphylococcus epidermidis*, *Enterobacter*, *V. cholera* and *Burkholderia pseudomallei* which exhibited moderate to weak crude-oil degrading capabilities. *Salmonella typhi*, *Streptomyces* sp., *Sphingomonas* sp., *Shigella* sp., *Escherichia coli* and *Nocardia* sp. all exhibited weak crude-oil degrading capabilities. However, isolates such as *Lactobacillus*, *Proteus vulgaris*, *Staphylococcus aureus*, *Enterococcus faecalis and Klebsiella* showed no observable effect on crude-oil utilization.

Table 2: Crude oil degrading potentials of bacteria isolated from Eniong River sediment

SN	Isolate	Test (T)	Degrading potentials
1	*Pseudomonas aeruginosa*	300	+++
2	*Bacillus cereus*	300	+++
3	*Bacillus subtilis*	80	+++
4	*Micrococcus* sp.	60	+++
5	*Burkholderia pseudomallei*	20	++
6	*Vibrio cholera*	50	++
7	*Staphylococcus epidermidis*	100	++
8	*Enterobacter* sp.	25	++
9	*Escherichia coli*	24	+
10	*Sphingomonas* sp.	4	+
11	*Shigella*	30	+
12	*Nocardia*	18	+
13	*Salmonella typhi*	10	+
14	*Streptomyces* sp.	4	+
15	*Proteus vulgaris*	-	-
16	*Enterococcus faecalis*	-	-
17	*Staphylococcus aureus*	-	-
18	*Klebsiella* sp.	-	-
19	*Lactobacillus casei*	-	-

Key:Test (T): Crude oil+Mineral salt+ Agar +Isolates
Strong: +++
Moderate: ++
Weak: +
No effect: -

Effect of Environmental Factors on Bacterial Utilization of Crude Oil

Effect of temperature on bacterial utilization of crude oil

Table 3, 4 and 5 show the effects of temperature at 30ºC, 37ºC and 40ºC. The results revealed the best growth was obtained by both *Pseudomonas aeruginosa* ($2.60x10^2$cfu/g) and *B. subtilis* ($1.60x10^2$cfu/g) at 30ºC Table 3. This was followed by both *B. subtilis* ($3.0x10^1$cfu/g) and *B. cereus* ($3.0x10^1$cfu/g) at 37ºC Table 4. The least growth was exhibited by both *Pseudomonas aeruginosa* ($8.00x10^o$cfu/g) and *Shigella* ($2.00x10^o$cfu/g) at 40ºC Table 5.

Table 3: Effect of temperature at 30ºC on bacterial utilization of crude oil

SN	Isolates	T(cfu/g)	C(cfu/g)
1	*P. aeruginosa*	$2.60x10^2$	$7.00x10^2$
2	B. subtilis	$1.60x10^2$	$6.00x10^2$
3	*B. cereus*	$2.00x10^1$	$5.00x10^2$
4	*Micrococcus* sp.	$1.80x10^1$	$5.00x10^1$
5	*Shigella* sp.	$1.80x10^1$	$4.00x10^1$

Key:
Test (T): Crude oil+Mineral salt+ Agar +Isolates
Control (C): No Crude oil+Mineral salt +Agar+Isolates

Table 4: Effect of temperature at 37ºC on bacterial utilization of crude oil

SN	**Isolates**	**T** (cfu/g)	**C** (cfu/g)
1	B. cereus	$3.00x10^1$	$6.00x10^2$
2	*B. subtilis*	$3.00x10^1$	$5.00x10^2$
3	*P. aeruginosa*	$2.10x10^1$	$2.00x10^2$
4	*Micrococcus* sp.	$1.60x10^1$	$4.00x10^1$
5	*Shigella* sp.	$1.20x10^1$	$2.00x10^1$

Table 5: Effect of temperature at 40ºC on bacterial utilization of crude oil

SN	**Isolates**	**T** (cfu/g)	**C** (cfu/g)
1	*B. subtilis*	$2.00x10^1$	$7.00x10^1$
2	*B. cereus*	$1.40x10^1$	$5.00x10^1$
3	*Micrococcus* sp.	$1.00x10^1$	$3.00x10^1$
4	*P. aeruginosa*	$8.00x10^o$	$3.00x10^1$
5	*Shigella* sp.	$2.00x10^o$	$2.00x10^1$

Effect of pH on bacterial utilization of crude oil

Table 6, 7 and 8 summarized the effects of pH on bacterial utilization of crude oil. The results revealed the best growth was exhibited by *B. cereus* ($1.20x10^2$cfu/g) and *Micrococcus* sp. ($1.0x10^2$cfu/g) at pH of 6 (Table 6). This was followed by *P. aeruginosa* ($9.20x10^1$cfu/g) at pH 6 (Table 7). The least growth was exhibited by *Shigella* ($3.00x10^o$cfu/g) at pH 8 (Table 8). In general, there was a reduction of growth at pH 9 showing that microbes prefer a near neutral pH, but this however depends on the type of microbe.

Table 6: Effect of pH 6 on bacterial utilization of crude oil

SN	Isolates	T (cfu/g)	C (cfu/g)
1	*B. cereus*	$1.20x10^2$	$5.00x10^3$
2	*Micrococcus* sp.	$1.00x10^2$	$5.00x10^2$
3	*P. aeruginosa*	$9.20x10^1$	$5.00x10^2$
4	*B. subtilis*	$2.00x10^1$	$1.20x10^2$
5	*Shigella* sp.	$1.00x10^1$	$1.12x10^2$

Table 7: Effect of pH 8 on bacterial utilization of crude oil

SN	Isolates	T	C
1	*P. aeruginosa*	$1.50x10^1$	$8.00x10^2$
2	*B. susbtilis*	$1.50x10^1$	$5.00x10^2$
3	*Micrococcus*	$5.0x10^1$	$1.00x10^2$
4	*B. cereus*	$5.0x10^o$	$2.0x10^2$
5	*Shigella* sp.	$3.00x10^o$	$1.10x10^2$

Table 8: Effect of pH 9 on bacterial utilization of crude oil

SN	Isolates	T (cfu/g)	C (cfu/g)
1	*P. aeruginosa*	$4.00x10^1$	$2.00x10^2$
2	*Micrococcus* sp.	$2.50x10^1$	$1.00x10^2$
3	*B. subtilis*	$2.00x10^1$	$5.00x10^2$
4	*Shigella* sp.	$1.10x10^1$	$1.08x10^2$
5	*B. cereus*	$1.00x10^1$	$2.0x10^1$

Effect of Agitation at (160 rpm and 180 rpm) on Bacterial Growth and Utilization of Crude Oil

The results of agitation on bacterial growth and utilization of crude oil are presented in Figures (5-23). The best growth was exhibited by *Pseudomonas aeruginosa* (log_{10}6.93cfu/ml) at day 10 and 180 rpm (Figure 10), *Bacillus subtilis* (log_{10}6.70cfu/ml), *Bacillus cereus* (log_{10}6.65cfu/ml) and *Micrococcus* sp. (log_{10}6.37cfu/ml) equally recorded high growth at day 10 and 180 rpm (Figure 10) respectively. The least growth was however recorded by both *Escherichia coli* (log_{10}2.86cfu/ml) at day 0 and 160 rpm (Figure 5) and *Streptomyces* (log_{10}2.88) at day 2 and 160 rpm (Figure 6).

The best growth which was shown by reduction in pH of media was shown by both *Pseudomonas aeruginosa* (pH 6.35) and *Bacillus cereus* (pH 6.37) at day 12 and 180

rpm (Figure 17). This was followed by *Pseudomonas aeruginosa* (pH 6.39) at day 6 and 180 rpm (Figure 14). The least growth was recorded by *E. coli* (pH 6.95) at day 4 (160 rpm) (Figure 13), *Serratia mercescin* (pH 6.92) at day 2 (160 rpm) Figure 12, *Serratia mercescin* (pH 6.92) at day 0 (160 rpm) Figure 11 and *E. coli* (pH 6.90) at day 2 (160 rpm) Figure 12. These are illustrated in Figures 11-17.

The profile that shows the relationship between cell number and pH are indicated in (Figures 18-23). Here, there is a steady increase in cell numbers and decrease in pH of the medium as incubation periods increase from day 1 to day 10 in (Figures 18 – 23)

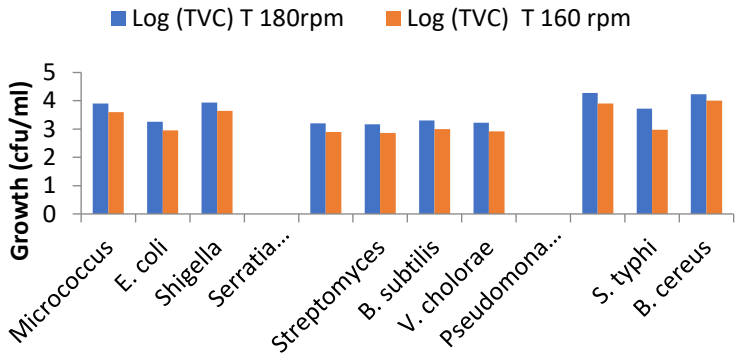

Figure 5: Viable cell counts (cfu/ml) in crude oil supplemented culture of isolates agitated at 180 (rpm) and 160 (rpm) at day 0

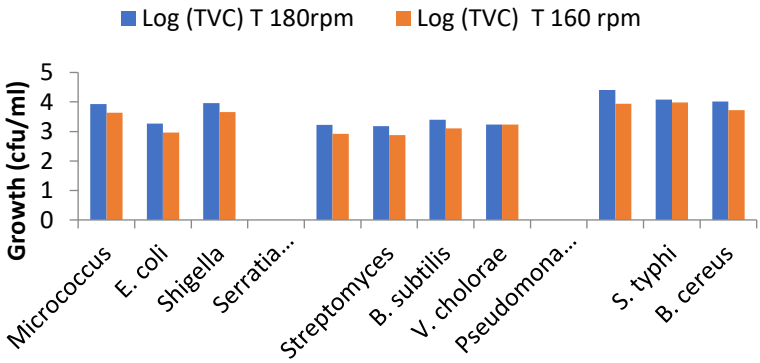

Figure 6: Viable cell counts (cfu/ml) in crude oil supplemented culture of isolates agitated at 180 (rpm) and 160 (rpm) at day 2

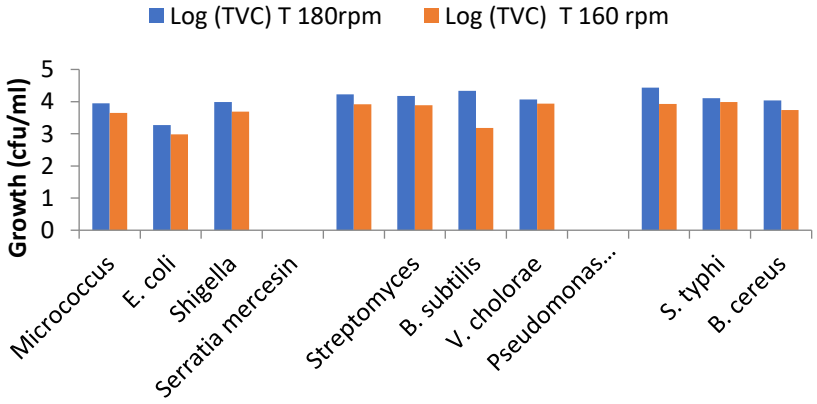

Figure 7: Viable cell counts (cfu/ml) in crude oil supplemented culture of isolates agitated at 180 (rpm) and 160 (rpm) at day 4

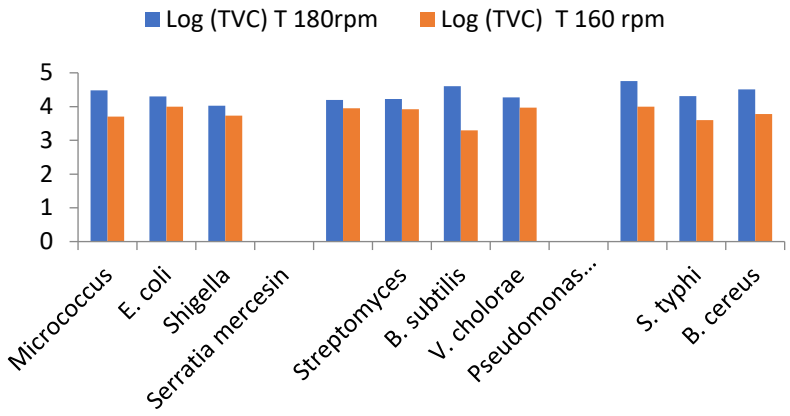

Figure 8: Viable cell counts (cfu/ml) in crude oil supplemented culture of isolates agitated at 180 (rpm) and 160 (rpm) at day 6

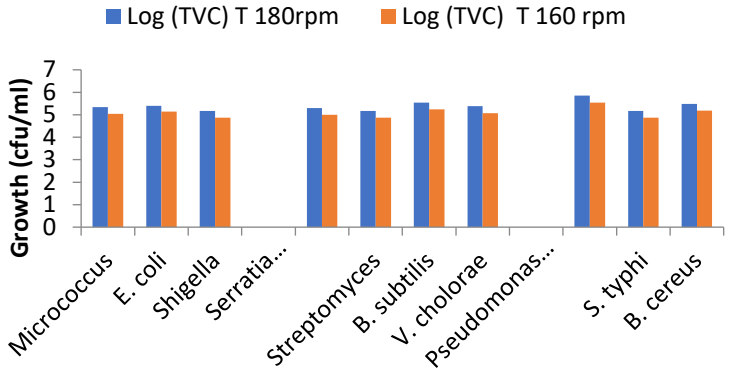

Figure 9: Viable cell counts (cfu/ml) in crude oil supplemented culture of isolates agitated at 180 (rpm) and 160 (rpm) at day 8

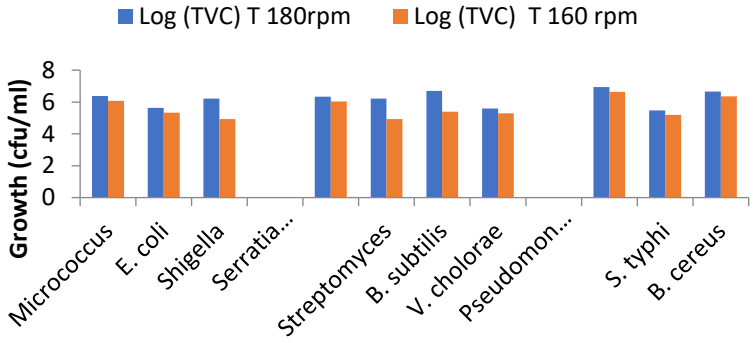

Figure 10: Viable cell counts (cfu/ml) in crude oil supplemented culture of isolates agitated at 180 (rpm) and 160 (rpm) at day 10

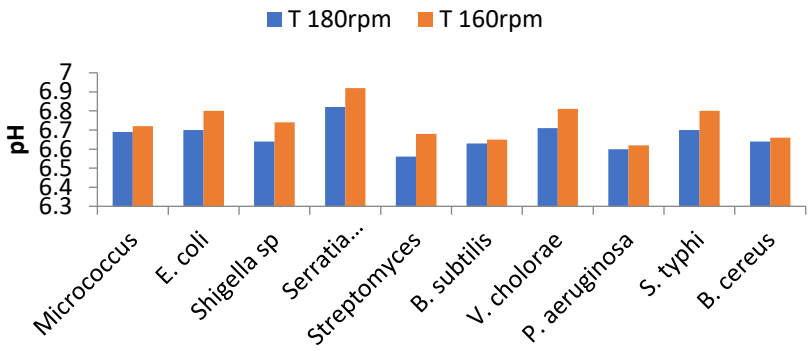

Figure 11: Changes in pH of crude oil supplemented culture of various isolates agitated at 180 (rpm) and 160 (rpm) at day 0

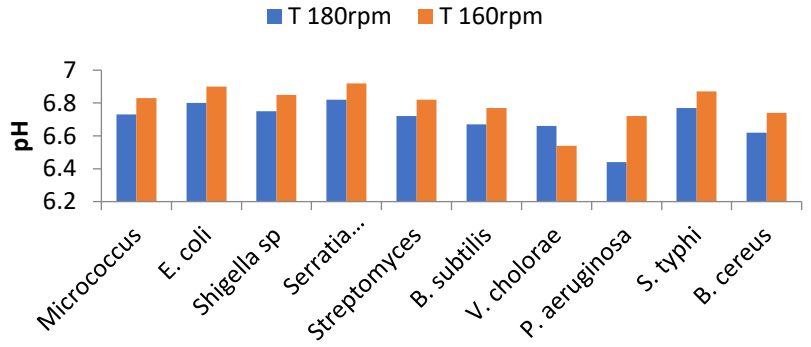

Figure 12: Changes in pH of crude oil supplemented culture of various isolates agitated at 180 (rpm) and 160 (rpm) at day 2

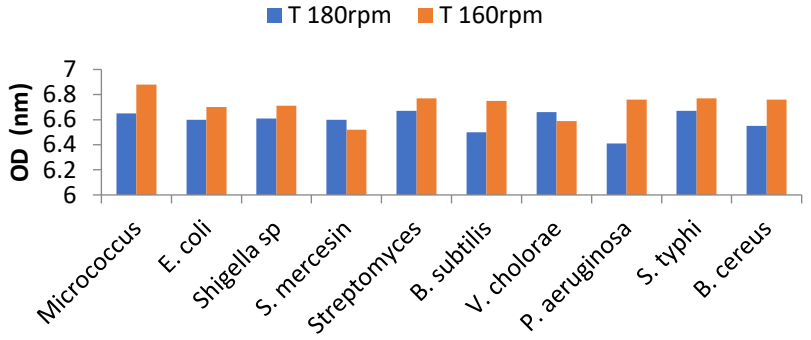

Figure 13: Changes in pH of crude oil supplemented culture of various isolates agitated at 180 (rpm) and 160 (rpm) at day 4

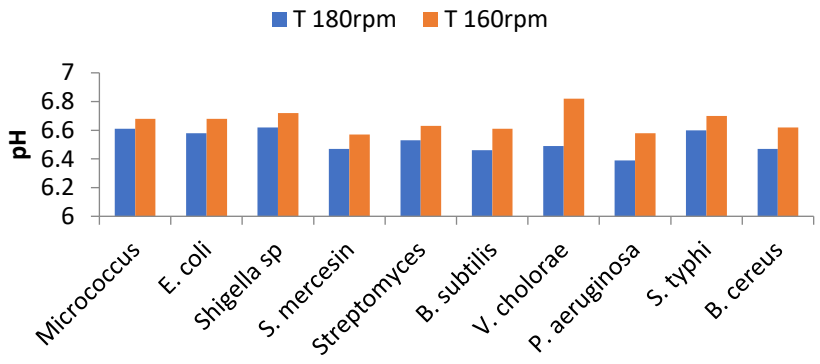

Figure 14: Changes in pH of crude oil supplemented culture of various isolates agitated at 180 (rpm) and 160 (rpm) at day 6

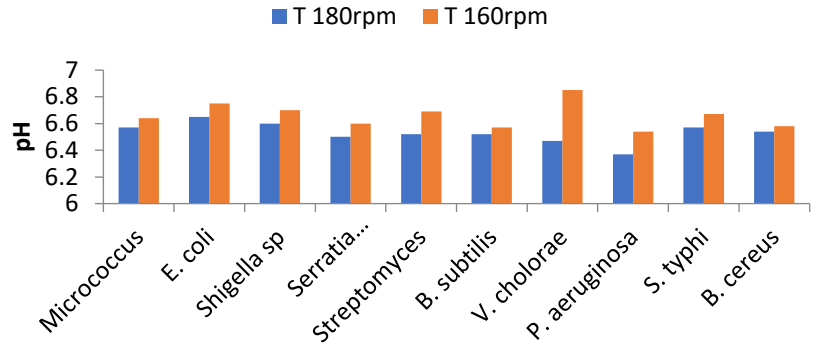

Figure 15: Changes in pH of crude oil supplemented culture of various isolates agitated at 180 (rpm) and 160 (rpm) at day 8

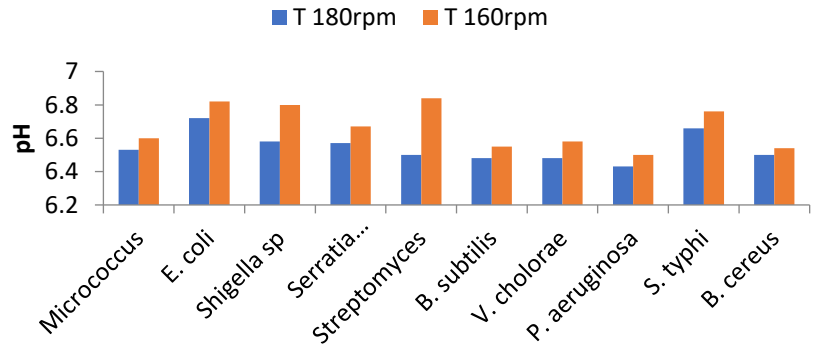

Figure 16: Changes in pH of crude oil supplemented culture of various isolates agitated at 180 (rpm) and 160 (rpm) at day 10

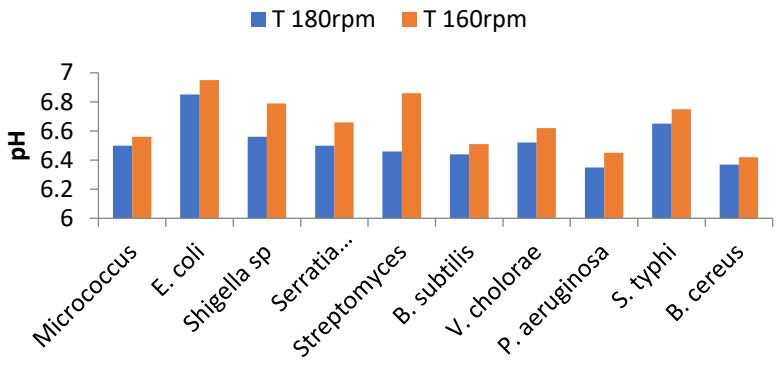

Figure 17: Changes in pH of crude oil supplemented culture of various isolates agitated at 180 (rpm) and 160 (rpm) at day 12

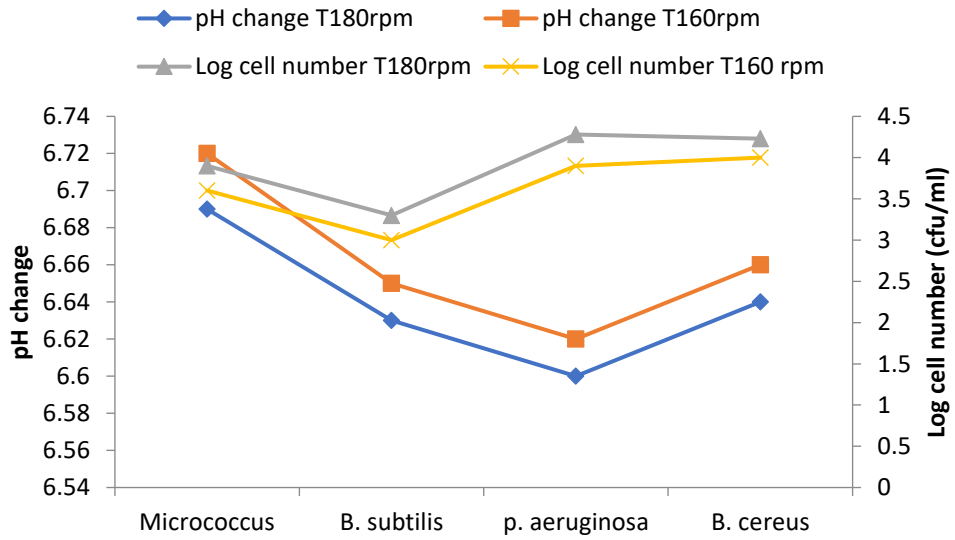

Figure 18: Changes in pH and cell concentration of isolates in crude oil supplemented medium agitated (180rpm and 160rpm) at day 0

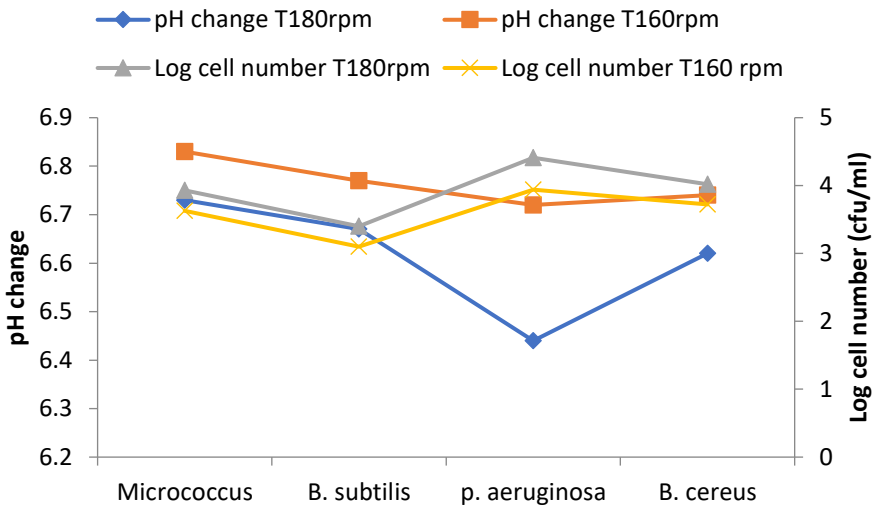

Figure 19: Changes in pH and cell concentration of isolates in crude oil supplemented medium agitated (180rpm and 160rpm) at day 2

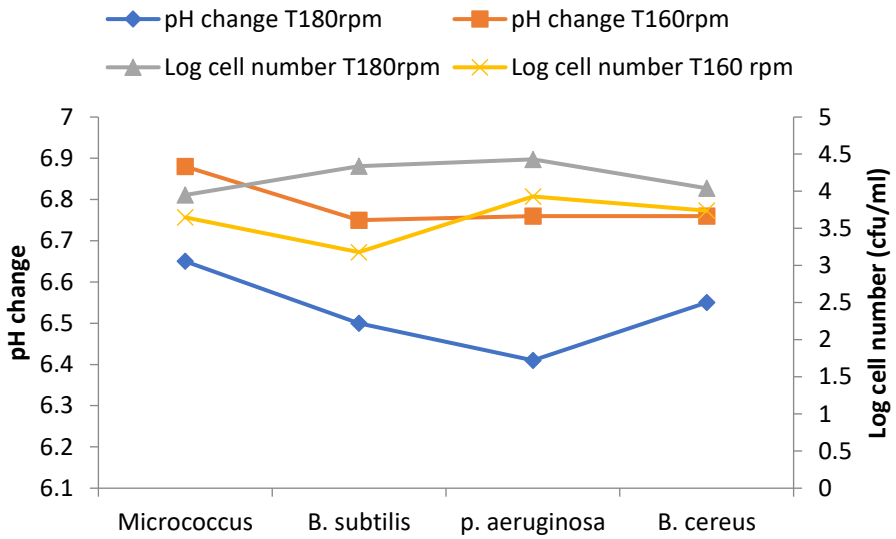

Figure 20: Changes in pH and cell concentration of isolates in crude oil supplemented medium agitated (180rpm and 160rpm) at day 4

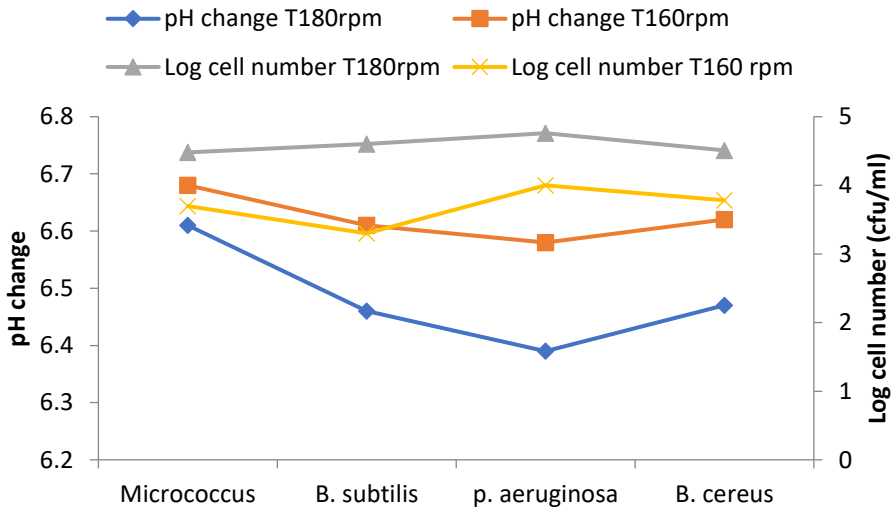

Figure 21: Changes in pH and cell concentration of isolates in crude oil supplemented medium agitated (180rpm and 160rpm) at day 6

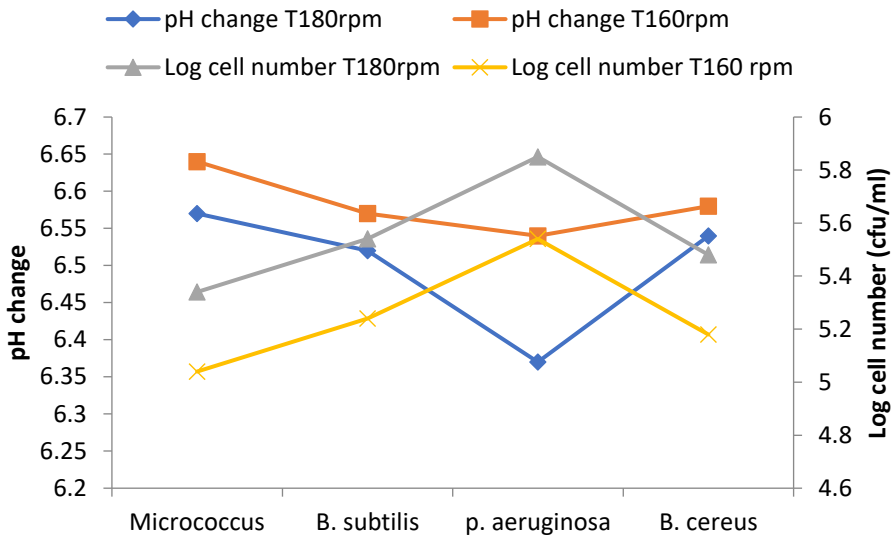

Figure 22: Changes in pH and cell concentration of isolates in crude oil supplemented medium agitated (180rpm and 160rpm) at day 8

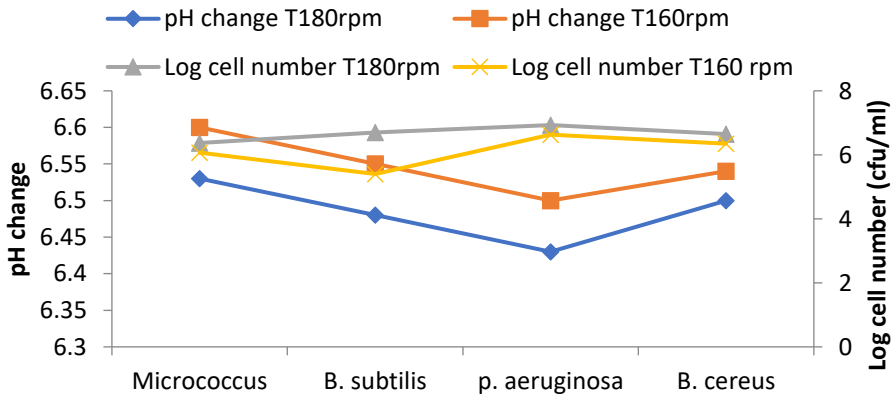

Figure 23: Changes in pH and cell concentration of isolates in crude oil supplemented medium agitated (180rpm and 160rpm) at day 10

Discussion

The best growth was observed by heterotrophic bacteria. Heterotrophic bacteria derive energy from organic compounds. They are widely distributed and could be ether aerobic or anaerobic. Heterotrophic bacteria are omnipresent meaning that they could be found everywhere including food, soil, air and water. They live as parasitic, saprophytic and symbiotic organism with plants and animals. They assist in recycling of substances and decomposition of organic matters such as cellulose, keratin, lignin, chitin and crude oil (Alongi, 2008). The high densities of heterotrophic bacteria obtained from the sediment are in accord with, but slightly lower than values reported in Australia where the numbers ranged from 8.30 $\log_{10}$ cfu/g cells to 10.56 $\log_{10}$cfu/g cells dry weight of sediment (Alongi, 2008). The increase in population levels of heterotrophic microorganisms may be associated with inputs from storm water (Boynton *et al*., 1980; Phelps and Zeikus, 1985). The wide heterotrophic activity of sediment microorganisms is of very considerable importance in the remediation of aquatic system after pollution with hydrocarbons and other organic chemicals. Heterotrophic bacteria are major components of microbial population in the aquatic sediment (Ramamurthy *et al*., 1990).

Sulphate reducing bacteria (SRB) are high in the sediment of humic freshwater ecosystem and mostly occur in seawater sediment rich in decaying organic materials. They are (e.g. *Desulfovibrio* sp.) common in anaerobic environments where they aid in the degradation of

organic materials (Dexter, 2003). SRB are widespread in sewers, wastewater treatment plants and sediments (EPA, 1985) and are often outnumbered by other microorganisms in the sediment because of their slow rate of growth and their preference of carbon as their energy source. The toxic hydrogen sulphide (H_2S) is a waste product of SRB and its rotten egg odour is often used as a marker for the presence of the bacteria in nature (Dexter, 2003). The bacteria are responsible for the sulphurous odour of salt marshes and mud flats. Much of the H_2S reacts with metal ions in the water to produce metal sulphides. These metal sulphides, such as ferrous sulphide (FeS), are insoluble, often black or brown and are responsible for the dark color of sludge (Ernst-Detlef and Mooney, 1993). Sulphate reducing bacteria normally increase with increase in total suspended solids (TSS) concentration (Attal *et al.,* 1992). There exist competitions between SRB and methane producing bacteria (MPB) in anaerobic digesters because of their affinity for substrate of organic origin. However, despite the strict anaerobic requirement of SRB, their presence has been detected in many aerobic environments (Dexter, 2003).

The result equally revealed a moderate population density of hydrocarbon utilizing bacteria (HUB) in the sediment of the ecosystem. Eniong River freshwater ecosystem is continuously exposed to petroleum hydrocarbons owing to navigational activities and this may have enriched the sediment with hydrocarbon utilizing bacteria (HUB). However, the moderate count of the HUB in the sediment may be attributed to the inadequate nutrients concentrations at that depth especially nitrogen and phosphorus which deplete with input of hydrocarbons. Another factor that reduces available metabolic nutrients in aquatic environment according to Xu *et al.* (2004) is heavy leaching caused by tidal inundation and wave action.

Some isolated bacteria grew and produced clear zones on mineral salt agar employed in the determination of hydrocarbonoclastic potentials. The production of clear zones around the colonies was regarded as evidence of ability to degrade crude oil. The present study has proved that the capability to biodegrade crude oil is not restricted to a few bacterial genera. Therefore, a diverse group of bacteria has been reported to possess this ability. The oil-degrading potentials of bacteria isolated from the humic freshwater sediment ecosystem have been reported and the screening tests revealed that a number of the bacteria, 14 out of 19 species encountered in the black water ecosystem have the ability to utilize hydrocarbons. Among the isolates, *B. subtilis*, *Micrococcus* sp, *B. cereus* and *P. aeruginosa* exhibited very strong crude-oil degrading potentials. *Staphylococcus epidermidis*, *Enterobacter* sp., *V. cholera* and *Burkholderia pseudomallei* exhibited moderate to weak crude-oil degrading capabilities. However, isolates such as *Lactobacillus, Proteus vulgaris, Staphylococcus aureus, Enterococcus faecalis and Klebsiella* showed no observable effect on crude-oil utilization. The pollution indicator bacterial species of *Enterococcus faecalis, Staphylococcus aureus, Proteus vulgaris, Klebsiella* and *Lactobacillus casei that* failed to utilize hydrocarbons for growth may not be actively involved in the oxidative degradation of crude oil in humic sediment. The release of hydrocarbon into soil and water environment promotes the growth and proliferation of hydrocarbon utilizing microorganisms, which includes both bacteria and fungi. Incidentally, these hydrocarbon utilizing microorganisms are also the organisms that are responsible for the biodegradation and eventual cleanup of oil spills in our environment (Atlas, 1981; Okpokwasili and Amanchukwu, 1988; Van Hamme *et al.,* 2003; Dollah, 2004; Hamamura *et al.,* 2006). Leahy and Colwell (1990) and Atlas and Bartha (1992), revealed that microbial communities exposed to hydrocarbons become adapted, exhibiting selective enrichment and genetic changes. Ability of microorganisms to utilize petroleum as sole carbon and energy sources has been documented (Okpokwasili and Okorie, 1988; Adekunle and Adebambo, 2007; Al-Nasrawi, 2012). These independent reports by the scientists confirm that microorganisms capable of growth on petroleum play significant and active roles in oil biodegradation in aquatic and terrestrial environments. Although, bacteria have been demonstrated to possess hydrocarbonoclastic potentials, the question of effectiveness or suitability of the genera and species for enhanced oil degradation for cleaning polluted environment has not been properly addressed. If seeding or introduction of bacteria in an ecosystem to clean oil spill is to be effective, then the use of microorganism with proven degradative capability should not be over emphasized.

The results of the effect of the environmental factors on the bacterial utilization of crude oil were investigated. Among the physical factors influencing biodegradation of hydrocarbon, temperature is the most important, determining the survival of microorganisms and composition of the hydrocarbons (Das and Chandran, 2011). Temperature influences petroleum biodegradation by its effects on the physical nature, chemical composition, rate of metabolism of microorganisms and microbial community composition (Atlas, 1981). Temperature plays a significant role in controlling the nature and the extent of microbial hydrocarbon metabolism which is of special importance for *in situ* bioremediation (Margesin and Schinner, 2001). The temperature of the water in which the oil is spilled determines the rate of hydrocarbon degradation. Hydrocarbon degradation is faster in warm water than cold water because the heat generated within the water body will further enhance the breakdown of the spilled petroleum through natural processes such as evaporation.

This leaves the oil-degrading microbes with a smaller size of hydrocarbon pollutants to clean up (AAM, 2011).

The result of the effect of temperature revealed the best growth was obtained by both *P. aeruginosa* and *B. subtilis* at 30ºC. This was followed by both *B. subtilis* and *B. cereus* at 37ºC. The least growth was exhibited by *Shigella* at 40ºC. Abdualdaim *et al*. (2008) reported an extensive growth in crude oil contaminated water for *B. subtilis*, *Staphylococcus* sp, *B. cereus,* and *Pseudomonas putida* employed in their study. He stated that there is an increment in growth with an increase in temperature from 30ºC to 37ºC but not at 40ºC. In his experiment, he achieved a maximum growth at 37ºC at day 5. In freshwater bioremediation process, 20-30ºC is the ideal temperature while 15-20ºC is recommended for marine water. This is exactly the case in this research where the best growth was exhibited by *P. aeruginosa* and *B. subtilis* at 30ºC. Higher temperatures increase the rates of the metabolism to maximum, typically in the range of 30ºC to 40ºC, but above which the membrane toxicity of hydrocarbons is increased (Bossert and Bartha, 1984). Meanwhile, at low temperatures, the viscosity of the oil increases, the volatilization of toxic short-chain alkanes is reduced and their water solubility is increased, delaying the onset of biodegradation (Atlas and Bartha, 1972).

The result of the effect of pH on bacterial utilization of crude oil revealed the best growth was exhibited by both *B. cereus* and *Micrococcus* sp. at pH of 6. There was a reduction of growth at pH 9 showing that microbes prefer a near neutral pH, but this however depends on the type of microbe. Alexander (1999) reported a similar result where he recorded results of bacterial growth in pH 6.5, 7, 7.5 and 8 which showed that significant growths were observed in medium with pH value between pH 6.5 and 7.5 for all the four isolates (*Pseudomonas putida, Acinetobacter faecalis, Staphylococcus* sp. and *Neisseria elongate*) with the highest growth at pH 7 after 5 days. Tyagi (1991) however reported that, it is important to maintain a pH range of between 6.5 and 8.0 in biological systems. This idea of a neutral pH is also supported by Tano-debrah *et al.* (1999) who reported that most of their isolates grew best around neutral pH. The acidity (pH) of the sediment is an important parameter and has an implication on biodegradation rates. Sediment pH can be highly variable, ranging from 2.5 in mine soils to 11 in alkaline deserts. The rates were found to be highest at neutral pH (Leahy and Colwell, 1990). Most heterotrophic bacteria favour a pH 7.0. Lower pH at around 5.0 (Patrick and De Laune, 1977) as seen in salt marshes reduces oil mineralization but the rates were satisfactory at pH above 6.5 (Hambrick *et al.,* 1980). Therefore, extreme pH of soils would have a negative influence on the ability of microbial populations to degrade hydrocarbons.

The ability of the selected strains to grow on crude oil was tested in mineral salt medium (MSM) in aerobic shaker. Increase in turbidity, total viable cell (TVC) count as well as changes in pH was measured. Relatively high turbidity was observed in less than six days in all the cultures. The oil layers were slowly emulsified and eventually disappeared with incubation.

The best growth was exhibited by *Pseudomonas aeruginosa* at day 10. *Bacillus subtilis, Bacillus cereus* and *Micrococcus* sp. equally recorded very high growth at day 10 respectively. The least growth was however recorded by both *Escherichia coli* at day 0 and *Streptomyces* at day 2.

The best growth which was shown by reduction in pH of media was shown by both *Pseudomonas aeruginosa* and *Bacillus cereus* at day 10. Here, the results are based on the abilities of the diverse bacteria species to utilize crude oil, incubation period and numbers of revolution per minute or agitation in aerobic shaker. It was generally observed that different bacteria utilized crude oil at different rates based on their capabilities. Bacteria equally grew more as a result of the increase in incubation periods. That is to say, bacteria growth increased with increase in incubation periods, although, this depends more on the type of bacteria and their ability to withstand the toxic effects of the crude oil.

It was generally observed that there was a higher growth of bacteria at higher revolution or agitation. This implies that growth at 180rpm is higher than that at 160rpm in all. However, even though there was growth of bacteria in media that contains crude oil and the ones that do not, it was generally observed that there was a lower growth of bacteria in media that contains crude oil than the control which does not. Furthermore, it was consistently observed that *Pseudomonas aeruginosa, Bacillus subtilis, Bacillus cereus* and *Micrococcus* species showed higher growth in almost all the study carried out so far. This could be as a result of their exceptional degradative abilities as observed during biodegradation experiment. The results correspond with that of Hayase *et al.* (2004) where he reported a marked improvement in $cfug^{-1}$ when the cultures were shaken at 100 and 130 rpm. For all the 4 isolates which he employed in the study, the highest growth were recorded when the cultures were incubated with agitation at 130 rpm even when agitation at 100 rpm seems not far behind. The lowest increase in $cfug^{-1}$ was recorded when incubated with no shaking. Hayase *et al.* (2004) also reported best growth to be at 130 rpm. Hydrocarbons are naturally occurring compounds and the ability to utilize it is widely distributed among diverse bacterial populations. In this study, indigenous bacteria with the ability to utilize hydrocarbons were employed to degrade crude oil. The growth profile of the bacteria strain was similar to previous reports (Okerentugba and Ezeronye, 2003; Oboh *et al*., 2006). Their increasing growth dynamics during degradation can be attributed to the constitutive expression of hydrocarbon assimilating capabilities or adaptation of the strains owing to previous

exposure to exogenous hydrocarbons, which may be followed by concomitant development of the ability to use the oil or its catabolic products as carbon and energy source (Adebusoye *et al*., 2007; Omotayo *et al*., 2011).

The reduction in the pH levels may have resulted from organic acids produced in the cultures. This was earlier reported by Okpokwasili and James (1995) and Oboh *et al*. (2006). The observed profuse growth of some bacterial species within the given conditions of this experiment and its presence in most aquatic ecosystem in the environment would suggest a wide versatility in organic matter utilization and genetic flexibility which concomitantly translates to its ubiquity.

The increase in pH of growth medium in this study can be attributed to production of lesser acidic metabolites by bacteria, made possible by initial bacterial attack of the toxic petroleum hydrocarbon. This is because organisms can penetrate soluble fractions of petroleum hydrocarbon thus increasing the surface area available for bacterial attack. Besides, bacteria can grow in environmentally stressed conditions such as low pH and poor nutrient status. The potential of bacteria in the utilization and degradation of petroleum hydrocarbon and eventual oil spill cleanup has been established and polluted soils are vast reservoirs for these groups of beneficial microorganisms.

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