African Journal of Biotechnology Vol. 6 (4), pp. 393-399, 19 February, 2007
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2007 Academic Journals

Full Length Research Paper

Bioluminescent hydrocarbonclastic bacteria of the Niger Delta

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Accepted 3 October, 2006

Utilization of three petroleum hydrocarbons (Mobil SAE 40 Engine Oil, Diesel and Bonny light Crude Oil) by four bioluminescent bacteria (*Vibrio harveyi, V. fisheri, Photobacterium leiognathi* and *P. Phosphoreum* isolated from the Bonny estuary in the Niger Delta, Nigeria was investigated. Microbial utilization was monitored for an 8-week period using mineral salt medium containing the hydrocarbon sources as sole carbon source. Results showed that bioluminescent bacteria were widely distributed in the brackish and marine waters of the Niger Delta, representing 7.5-18.72% and 0-2-5% of the total heterotrophic bacteria of the marine and brackish water systems, respectively. A hydrocarbon loss of 100% by week 7 for all four-test organisms was observed. These results indicated that the bacteria were capable of utilizing the hydrocarbon sources as sole sources of carbon and energy. Increased phosphate concentrations (0.03-0.05 g/ml) in marine aquatic systems were also observed to stimulate increases in bacterial population and the intensity of luminescence of the bacteria. The study revealed that increasing phosphate levels in phosphate depleted marine waters would encourage the growth of hydrocarbonoclastic bioluminescent bacteria which could serve as a potential tool for the remediation of petroleum polluted marine systems of the coast of the Niger Delta. Physiochemical analyses of water from the Bonny estuary (marine) and from Isaka (brackish) environments revealed that phosphate levels in the marine system was 0.04 mg/l while in the brackish environment no phosphate was recorded (0 mg/l).

Key words: Hydrocarbonclastic, Bioluminiscent, Brackish, Marine, Remediation.

INTRODUCTION

Bioluminescence is the chemical emission of light by organisms (Lang and Lange, 1997). It is a widespread but randomly distributed natural phenomenon, occurring more commonly among animals than plants. (Lange and Lange, 1997). Bioluminescence has been reported among microorganisms and has been used as an index of various characteristics, including ability to utilize organic compounds (Breitung et al., 1996; Thomulka and Lange, 1995). Recent studies by Breitung et al. (1996) have shown the bioremediation of 2, 4, 6- trinitrotoluene (TNT) – contaminated soils by two different erated comp-ost systems using the inhibition of bioluminescence of *Vibrio fsicheri* as an index of the toxicity of the mineralized TNT.

In the Niger Delta, increasing petroleum exploration and transportation has introduced large amounts of hydrocarbons into the area (Odokuma and Dickson 2003 a, b). This has led to wide spread contamination of its creeks swamps soil ground water estuaries and rivers (Odokuma and Okpokwasili, 1993). These contaminants present several environmental problems. They may affect physiological processes, population and behavioural profiles of organisms (Teh and Lee, 1974).

In Nigeria, separation of water and crude oil in Rivers state takes place at Bonny oil terminal which has resulted in relatively large amounts of crude often being spilled into the estuary. Physical and chemical methods employed in the remediation of affected ecosystems have not

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been effective in restoring normalcy to such environments. Chemicals used in remediation have led to the formation of persistent organic pollutants (POPs) (Gilek et al., 1997). The microbiological option has been preferred as the most environmentally friendly option (Okpokwasili, 1992; Odokuma and Dickson, 2003 a,b). Bioaugumentation, Biostimulation and Remediation by Natural Attenuation (RENA) have been suggested as more acceptable alternatives (Odokuma and Okpokwasili, 1992; Odokuma and Dickson, 2003 a b).

Within the Bonny estuary in Nigeria, bioluminescent bacteria have been found to exist, though more prevalent in Bonny area than Isaka area. Thomulka and Lange (1995) have employed the bioluminescent bacterium *V. harveyi*, in the detection of organic bio-hazardous chemicals in contaminated soils and aquatic systems. Peter et al. (1995) used the luminescence of *Photobacterium phosphoreum* as a sensitive marker for monitoring the toxicity of coke plants. Lange and Lange (1997) have discussed exhaustively the use of bioluminescence as a marker in the detection of environmental contaminations in water and waste water systems. This study was carried out to examine the hydrocarbonoclastic potentials of bioluminescent bacteria within the Bonny River. The bacterial utilization of three-hydrocarbon substrates Engine oil, Diesel and Bonny light crude oil has been suggestive of the hydrocarbon degradation potentials of these bacteria.

MATERIALS AND METHODS

Study area description

The Bonny estuary, which begins at Bonny and extends up to Isaka Port Harcourt, is located within the Niger Delta. The estuary contains a busy harbour and is the discharge point from many effluents from industries located in Port Harcourt. The Bonny terminal where a lot of petroleum activities occur is also located at the entrance of the estuary.

Sample collection

Surface water samples for microbiological analysis were collected at 3 locations around Bonny and at one location at Isaka beach, Port Harcourt in sterile 1 L plastic bottle. The samples for the first three locations were pooled together. Each sample was transferred into sterile 25 ml plastic containers. Water samples for physiochemical analyses were collected with clean 1.5-litre amber coloured reagent bottles. All samples were then taken to the laboratory in ice-chests and analysed within 24 h of collection.

Chemical reagent and hydrocarbon substrates

The crude oil Bonny Light was obtained from Shell Petroleum Development Company (SPDC) Port Harcourt, Nigeria. Diesel and Super Visco Static SAE 20 W50SG/CD premium multigrade engine oil were obtained from African Petroleum (AP) Plc Lagos, Nigeria. All chemical reagents employed in this study were of analytical grade and were purchased from Sigma Chemical Company St Louis. Mo USA.

Microbiological analyses

Estimation of the total heterotrophic aerobic bacterial count was performed by inoculating 0.1 ml of appropriate dilution of water sample on nutrient agar plates using the spread plate technique (Odokuma and Okpokwasili, 1993; APHA, 1998; Odokuma and Otokunefor 2003) Colonies were enumerated after 48 h incubation at room temperature (28 ± 2^0C).

Isolation and estimation of bioluminescence bacteria was performed by inoculating 0.1 ml of estuarine water in Modified Zobell Agar (MZA). The MZA had the following compound; 1 g; Fe_3P0_4, 0.01 g; bacteriological agar, 15 g; estuarine salt solution (Estuarine colonies were enumerated after 24 h incubation at room temperature 28 ± 2^0C). For isolation of bioluminescent bacteria, the growth after 24 h incubation was examined in a dark room, and positions of luminescence marked colonies from the positions marked (up to a maximum of 2 colonies per plate) were picked, under dim illumination, and transferred to fresh plates for purity checks and identification the isolates were presumptively identified through their cultural, morphological characteristics. The isolates were further characterized via a battery of biochemical tests using criteria in Krieg and Holt (1994).

Effect of phosphate levels on bacterial growth

The effect of phosphate levels on the intensity and duration of bioluminescence was studied using MZA containing different levels of Fe_3P0_4 (0.00, 0.02, 0.03, 0.04 and 0.05 g). Plates inoculated and incubated as described above were observed at 24 h intervals. Luminescence of culture was rate "+" to "+++" depending on intensity.

Water pH was determined using PYE UNICAM PW 9418 pH meter fitted with a combined glass pH and reference electrode (APHA, 1998). Phosphates were determined using the Molybdate method (APHA, 1998). Sulphates were determined by the Turbidometric method (APHA, 1998). Nitrate was determined by the Phenoldisulfonic acid method (APHA, 1998). Chloride was determined using the Argentometric method (APHA, 1998). Dissolved Oxygen (DO) and Biochemical Oxygen Demand (BOD) were determined by the Azide Modification of the Winkler method (APHA, 1998). Ammonium ion was determined using the Phenate method (APHA, 1998). Total hydrocarbon levels of test systems were determined by employing the Photometric method (American Petroleum Institute. 1980). Ten milliliter of the sample was mixed with 10 ml of carbon tetrachloride solution. This mixture was stirred and allowed to stand. The carbon tetrachloride phase was decanted into a clean conical flask. Enough Na_2SO_4 (anhydrous) was added and shaken vigorously to remove all traces of water that may have been present in the mixture. The resultant clear solution was analyzed spectrophotometrically at 420 nm using carbon tetrachloride solution as blank.

Hydrocarbon (oil and grease) concentration in the sample was extrapolated from a standard curve obtained by preparing various concentrations of the crude oil (0, 1.0, 10 mg/ml) with absorbance (0.01, 0.1 and 0.3) at 420 nm and calculated using the relationship:

$$\% \text{ Crude oil (ppm)} = \frac{\text{Conc. from graph x TVSE}}{\text{Volume of sample (ml)}}$$

Where TVSE = Total volume of solvent extract, 10 ml.

Disappearance of crude oil from the set up was monitored throughout the study period and was calculated using the formula: Total % loss of crude oil = (wt of crude oil at Day O - wt of crude oil at Day of Analysis)/ wt of crude oil at Day O. Net % loss = % loss at

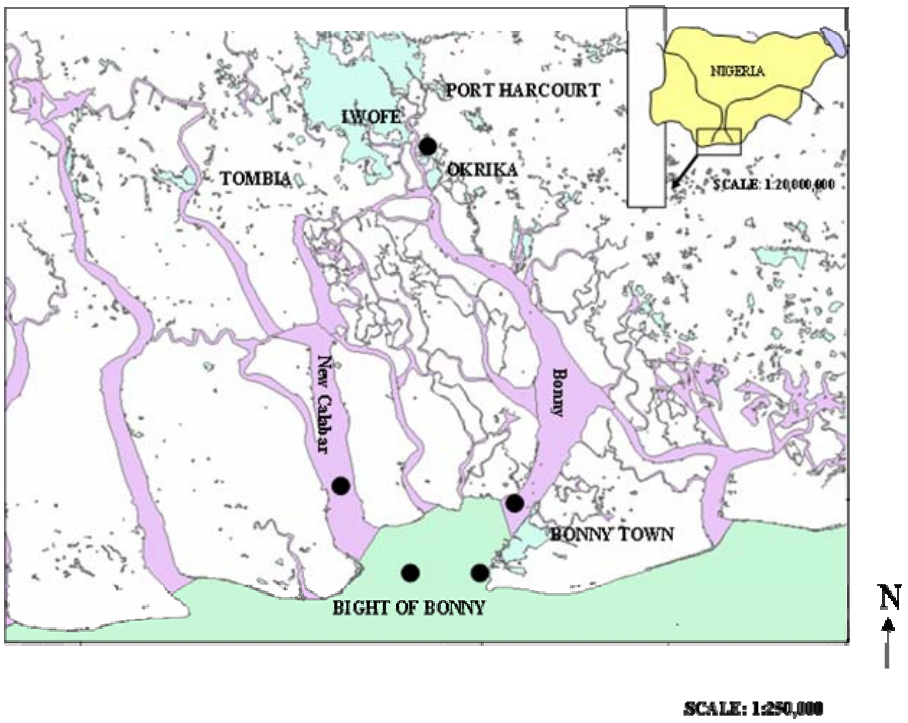

Figure 1. Map of the Bonny estuary showing sampling stations.

Table 1. Physicochemical characteristics of water samples.

Parameter	Bonny area	Port Harcourt
pH	7.95	7.92
NH_4^+ (mg/L)	0.0	0.09
$N0_3^-$	ND	0.644
$S0_4^{2-}$ (mg/L)	1.50×10^4	4.93×10^2
$P0_4^{3-}$ (mg/L)	8.29	ND
Cl^- (mg/L)	6.91	1.19×10^4
DO (mg/L)	6.02	6.44
BOD (mg/L)	5.3	3.22

Results are means of triplicate determinations
ND – not detectable

Day O - % loss at Day of Analysis

Hydrocarbon utilization tests

Tests were carried out by inoculating 1.0 g of confirmed isolate (*Vibrio harveyi., V. fisheri, Photobacterium leiognathi* and *P. phosphoreum*) into mineral salt broth medium containing 5 ml of hydrocarbon (Mobil SAE 40 engine oil or diesel or Bonny light crude oil) in 1 L of mineral salt broth (MSB). The MSB medium had the following composition: NH_4CL (0.5 g); K_2HPO_4 (0.5 g); Na_2HP0_4 (1 g); sterile estuarine water 250 ml and distilled water 750 ml. These were contained in 2 L Erlenmeyer flasks with cotton wool plugs. The bioreactors were incubated at room temperature (28±2^{0}C) for 56 days. At weekly intervals (0, 7, 14, 21, 28, 35, 42, 49 and 56 days) each isolate (0.1 ml) was inoculated onto solid plates of mineral salt agar containing 0.5% of the hydrocarbon and incubated for 14 days at room temperature (28±2^{0}C) and enumerated. The log of the counts was then plotted against time (weekly interval).

RESULTS

In Figure 1 the sampling area and the station are presented. The Bonny River empties its contents through the Bonny estuary into the Bight of Bonny. The Bight of Bonny is that part of the Atlantic Ocean on the southern fringes of Nigeria.

The physicochemical characteristics of the water samples presented in Table 1 showed that the chloride content was high and ranged from 11900 to 16000 mg/L. The pH of the area was slightly alkaline (7.92 – 7.95). The BOD ranged from 3.22 to 6.91 mg/L. The DO ranged from 6.44 to 8.29 mg/L. Phosphate levels were low (0.00 + 0.04 mg/ L) and were only detected within the Bonny area. Nitrate levels were also low (0.00 to 0.64 mg/L and were only detected at Port Harcourt. Ammonia levels were low 0.06 to 0.09 mg/L. Sulphate levels were high 493 to 1500 mg/L.

The effect of phosphate levels on the growth of bioluminescent bacteria (Table 2) revealed that phosphate levels, of 0.03 to 0.05 g/ml had in significant effect on growth ($p>0.05$) of the bacteria. However it increased the intensity of luminescence of these bacteria. Reduced phosphate levels (0.00 to 0.02 g/ml) decreased the intensity of luminescence.

Table 2. Effect of phosphate levels on growth of bioluminescent bacteria.

Phosphate level (g/ml)	cfu/ml after 24 h incubation	Luminescence intensity	Luminescence duration (h)
0.00 (control)	4.0×10^2	+	48
0.01	3.2×10^2	+	72
0.02	2.9×10^3	++	72
0.03	4.1×10^2	+++	72
0.04	3.3×10^3	+++	72
0.05	3.8×1010^2	+++	72

Table 3. Aerobic heterotrophic bacterial counts* along Bonny estuary.

Location	Station Code	Total heterotrophic bacteria (THB) (cfu/ml)	Bioluminescence bacteria (BB) (cfu/ml)	Ratio (%) of BB to THB
Bonny	A	1.2×10^5	1.8×10^4	15.0
Bonny	B	1.6×10^5	3.0×10^4	18.7
Bonny	C	1.6×10^5	1.2×10^4	7.5
Port Harcourt	D	1.2×10^5	3.0×10^3	2.5

***Means of triplicate determinations. Sampling stations A-C were in the Bonny area while station D in the Isaka area.

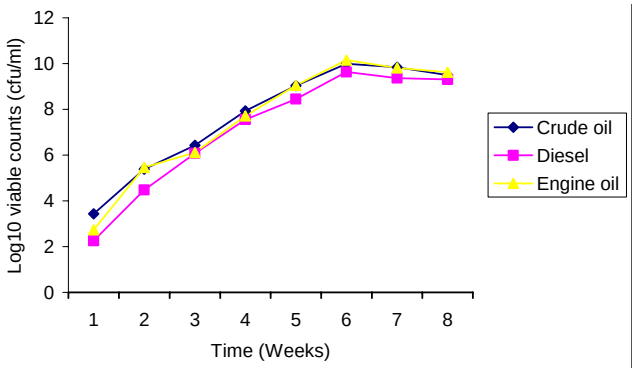

Figure 2. Growth of *Vibro harveyi* in mineral salts medium amended with petroleum hydrocarbons.

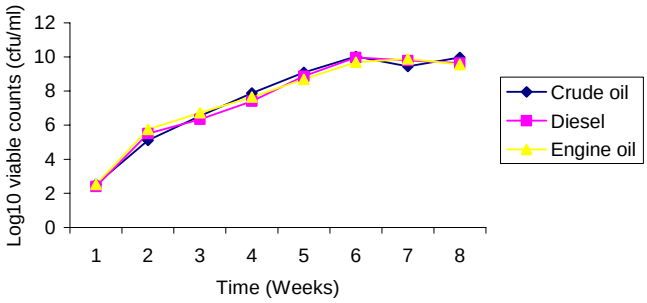

Figure 3. Growth of *Vibro fisheri* in mineral salts medium amended with petroleum hydrocarbons.

In Table 3 the results of the aerobic total heterotrophic bacterial (THB) counts and the bioluminescence bacteria counts (BB) showed a higher ratio of BB to THB in the Bonny area (marine environment) (7.5-18.7) than inland (Isaka) (brackish water environment (2.5).

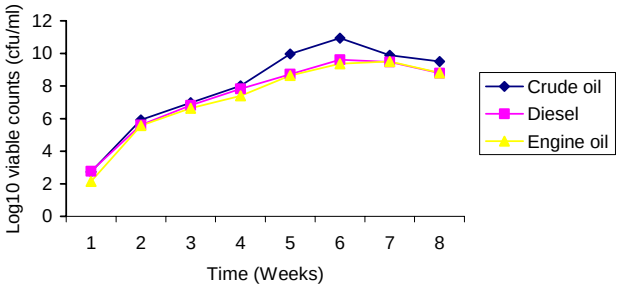

Figure 4. Growth of *Photobacterium leiognathi* in mineral salts medium amended with petroleum hydrocarbons.

The results of the characterization of bioluminescent bacteria isolated from Bonny estuary and Port Harcourt is presented in Table 4. Five species were presumptively identified *Xenorhabdus luminescence*, *P. phosphoreum, V., harveyi., P. leiognathi* and *V. fischeri.* The growth curves of the four bioluminescent bacterial isolates when exposed to the three hydrocarbon sources (engine oil, diesel and Bonny light crude oil) are presented in Figures 2-5. The results indicated that the four organisms *V. harveyi, V. fisheri, P. leiognathi* and *P. phosphoreum* were able to utilize the hydrocarbon sources as sole carbon and energy source. The growth for all four organisms increased from week 1 to week 6 and remained fairly constant at week 8 for all three-carbon sources. One hundred percent hydrocarbon loss was recorded each of the organisms were exposed to each of the three hydrocarbons (Figures 6-9).

Table 4. Characterization of bioluminescence bacteria isolated from the Bonny estuary**.**

Shape	Gram	Pigment*	Oxidase	Catalase	Citrate	Motility	Indole	Urease	Glucose	Latctose	Maltose	Mannnito	Sucrose	HD	Presumptive Identification
C	-	R	-		+	+	+	-	A	-	A	-	-	-	*Xenorhabdus luminiscens*
CB	-		-	+		+	-		AG		A		-	+	*Photobacterium phosphoreum*
R	-		+	+	+	+	+	-	A	-		A	A	+	*Vireo hareyi*
R	-			+	+	+	-		A	-			-	+	*P. leiognathi*
R	-			+	+	+	+	-	A	-		A	A	+	*V. harveyi*
R	-		+	+	+	+	+	-	A	-		A	A	+	*V. harveyi*
R	-		+	+	+	+				-		+		+	*V. fischei*
CB	-		+	+		+	-		AG				-	+	*P. phosphoreum*
CB	-		-	+		+	-		AG				-	+	*P. phosphoreum*
CB	-			+	-	+	-		A	-			-	+	*P. leiognathi*
R	-		+	+	+	+			-	-				+	*V. fischei*
R	-		+	+		+				+			+	+	*V. mediterranei*
CB	-		-	+		+	-		AG		A		-	+	*P. phosphoreum*
CB	-		-	+		+	-		AG		A		-	+	*P. phosphoreum*
R	-		+	+	+	+	+	-	A	-		A	A	+	*V. harveyi*
CB	-		-	+		+	+		AG		A		-	+	*P. phosphoreum*

*Pigmentation on nutrient agar.
R = red, C = cocci, CB = coccobacilli, and R = rods.
Sugar utilization: + = positive, - = negative, A = acid produced, AG = acid and gas produced, HD = hydrocarbon degradation, blank spaces signify tests not done.

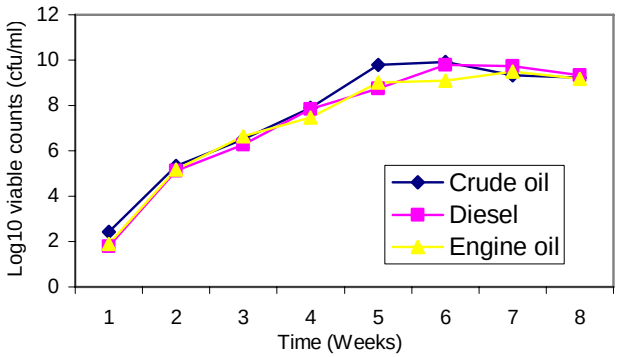

Figure 5. Growth of Photobacterium phosphoreum in mineral salts medium amended with petroleum hydrocarbons

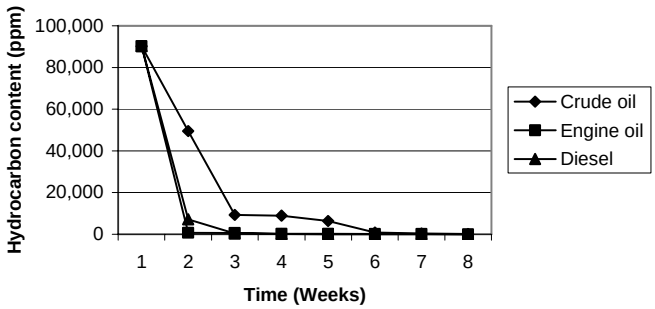

Figure 6. Removal of hydrocarbons by *P. phosphoreum*.

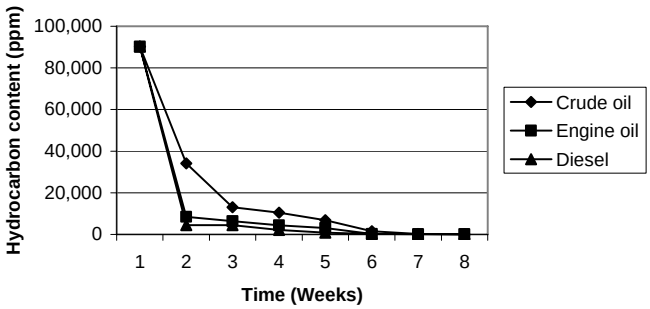

Figure 7. Removal of petroleum hydrocarbons by *V. fischeri.*

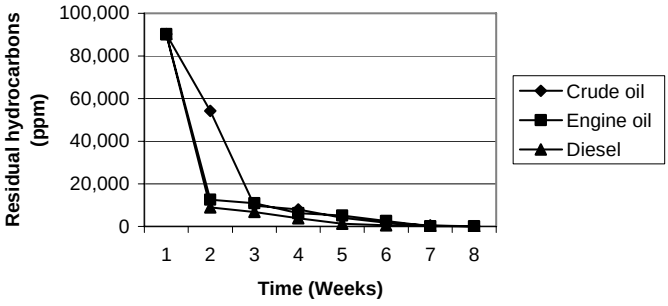

Figure 8. Removal of petroleum hydrocarbons by *V. harveyi.*

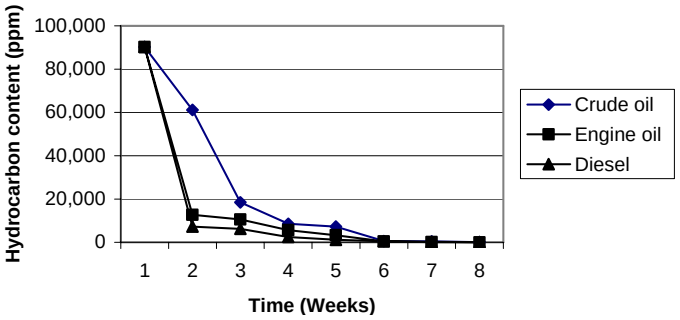

Figure 9. Removal of petroleum hydrocarbons by *P. leiognathi*

DISCUSSION

Physiochemical analyses of the water samples from the marine system of the Bonny estuary and the brackish system of Port Harcourt (Isaka) showed a lot of similarities. The pH, ammonium ion and chloride contents were similar. However, the BOD and the phosphate content of the Bonny estuary were higher than that at Port Harcourt. The higher BOD of the estuary may be due to a higher rate of deposition if biodegradable organic matter in lower course (Bonny estuary) of the Bonny River than at the upper course Port Harcourt. Similar observations have been made by Odokuma and Okpokwasili (1993).

Higher phosphate level (0.04 mg/L) of the marine system of Bonny estuary favour a higher population of bioluminescent bacteria than in the brackish system of Port Harcourt. The ratio (%) of bioluminescent bacteria to THB ranged from 7.5 to 18.70% for Bonny estuary and 0-2.5% for Port Harcourt. However, the study showed that varying the concentration of phosphate within the MZA medium had no significant effect ($p<0.0.05$) on the count of bioluminescent bacteria. However, increased phosphate concentrations 0.02 to 0.05 g/L increased the intensity and duration of luminescence of the test bacteria upon to a limit. These results showed that bioluminescence was phosphate dependent. The inorganic phosphate served as a source of phosphate for the synthesis of adenosine triphosphate (ATP) generation (Lange and Lange, 1997). The bioluminescent bacteria isolated in the present study have been shown to be luminescent by previous workers (Grant and Lange, 1981; Thomulka and Lange, 1995; Breitung et al., 1996; Lange and Lange, 1997). The results revealed that various species of luminescent bacteria existed in the Niger Delta. The growth curves of the four luminescent bacteria were similar. Hydrocarbon loss graphs revealed 100% removal of hydrocarbons after 7 weeks exposure. These results confirmed utilization of the hydrocarbons as sole carbon and energy sources by the test organisms. Microbial hydrocarbon degradation in both terrestrial and aquatic environments have been extensively studied (Higgius and Culbert, 1978; Herbes, 1981; Atlas, 1984). A number of organisms such as *Vibrio* (West et al., 1984), *Acinetobacter, Bacillus* and

Flavobacterium (Atlas, 1981) have been found to degrade hydrocarbons whether in the liquid or volatile state. In order for microorganisms to degrade hydrocarbons in an aerobic and reduced oxygen environment such organisms need to be fermentative, as well as oxidative in their utilization of carbohydrates and related substrates *Vibrio*, for example a hydrocarbon degrader (West et al., 1984) is a facultative anaerobe being able to survive with or without oxygen (Holt, 1984). Under anaerobic conditions, they ferment carbohydrates (sugars) and organic compounds serve as substrates for respiretion in an aerobic environment (Pelczar et al., 1989). Refined petroleum products such as diesel and engine oil containing short chain hydrocarbons of length C_{10}-C_{24} degrade rapidly (Atlas and Bartha, 1998). Diesel being a shorter chain hydrocarbon was expected to degrade more rapidly than engine oil and also crude oil, which was a more complex hydrocarbon. The results supported this explanation. Hydrocarbon losses from engine oil and diesel by the four test organism were greater than that of crude oil within the first 2 weeks of the test.

Conclusion

A number of bioluminescent bacterial species occur in the Niger Delta. The population of these organisms are however greater in the marine environment of the Niger Delta than in the brackish water environment. Results revealed that high phosphate levels in natural aquatic systems might favour high populations of these organisms. Bioluminescent bacteria in the Niger Delta were hydrocarbonoclastic due to increased exposure of bacteria to petroleum and its products resulting from activeties of Petroleum companies in this area. The ability of these organisms to utilize hydrocarbons from crude oil and its refined products could also be exploited in the remediation protocol of polluted marine ecosystems of the Niger Delta.

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