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The Molecular Succession of Bacterial Community of Crude Oil Polluted Soil and Water Samples from the Niger Delta, Nigeria

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Authors' contributions

This work was carried out in collaboration between the two authors. Author OFO designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author BB supervised the research work and read through the manuscript. Both authors read and approved the final manuscript.

Research Article

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ABSTRACT

A study was conducted on molecular succession of bacterial community to show their response to oil spill. Crude oil contaminated soil and water samples were obtained from Agbada-Aluu, Obite, and Bonny in Rivers State, Nigeria. The PCR-DGGE method was used to generate complex molecular profile analysis of the polluted sites microbiota. The growth dynamics of the bacteria was determined by the degrading activity, bacterial population and the pH of the culture media (Bushnell-Haas broth supplemented with 2% v/v crude oil). On day 35, Bonny soil had the highest bacterial load of (17.67 ± 1.25) $x10^4$ Cfu/mL. The highest bacterial population for the mixed cultures of each sample was observed on day 7. The bacteria in Obite soil demonstrated the greatest ability to degrade crude oil with degrading activity of 246.28 ± 3.4 Units/Hr on day 7 while the lowest degrading activity of 0.9 ± 1.2 Units/Hr was obtained with Obite water and Bonny soil on days 28 and 35 respectively. The pH values (6.8 - 8.2) were slightly basic throughout the incubation period of 35 days. The PCR-DGGE showed that there were shifts in the bacterial community during the 35 days of incubation, showing that the oil affected the bacterial community. The DNA bands that showed dominancy throughout the incubation period indicates the bacteria that played a major role in the degradation of the oil which is vital for their metabolism.

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1. INTRODUCTION

Hydrocarbon-degrading microorganisms are widely distributed in the ecosystem but their characterization is difficult to achieve, mainly because their ability to metabolize hydrocarbons depends largely on a particular substrate. With the advent of molecular biology tools in 1980's, the abundance and distribution of microorganisms in natural environments can be studied. In addition, the study of the relation between the community structure and the ecosystem functions is possible [1].

Microbial community compositions can be analyzed by different methods. Since the introduction of PCR-DGGE of ribosomal DNA by Muyzer *et al*. [2] to monitor microbial community ecology, DGGE has become an established tool in the hands of the scientists for the investigation of microbial diversity.

Denaturing Gradient Gel Electrophoresis (DGGE) is an ideal molecular method for monitoring microbial community ecology. Denaturing gradient gel electrophoresis (DGGE) is a molecular fingerprinting method that separates polymerase chain reaction (PCR)-generated DNA products. The band pattern on the polyacrylamide gel forms a genetic fingerprint of the entire community being examined [3]. This gel electrophoresis technique relies on variation in genetic sequence of a specific amplified region to differentiate between species within microbial communities [4]. Templates of differing DNA sequence from a given reaction but of similar size are often generated. With agarose gel electrophoresis seperation, a single, non-descriptive DNA band can be viewed. This does not show the different bacterial population in a particular community. This limitation is however, overcome by using DGGE, the principle of which is based on the denaturing of the double-stranded PCR at different concentrations resulting in a pattern of bands on polyacrylamide gel. Each band theoretically represents a different bacterial population present in the community. Denaturing Gradient Gel Electrophoresis (DGGE) has a broad scope of utilities in scientific research, with the breadth of PCR primers available, DGGE can be used to investigate broad phylogenies or specific target organisms such as pathogens or xenobiotics degraders with a quite high limit of detection (around 10^4). It could be used also to link geographical origin to microbial ecology [5].

Molecular methods involve the separation of PCR amplicons on the basis of DNA nucleotide sequence differences, most often the 16S rRNA gene (the highly conserved gene). Since 16S rRNA is ubiquitous in all bacteria [6], the PCR primers are designed with the aim to target different taxonomic groups (from kingdom to genus). However, the 16S rRNA gene provides phylogenetic comparisons of microbial communities because of its variability [7].

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a single, non-descriptive DNA band can be viewed. This does not show the different bacterial population in a particular community. This limitation is however, overcome by using DGGE, the principle of which is based on the denaturing of the double-stranded DNA linked by a GC clamp PCR at different concentrations resulting in a pattern of bands on polyacrylamide gel. Each band theoretically represents a different bacterial population present in the community.

2. MATERIALS AND METHODS

2.1 Sampling Sites

The sampling sites for the oil-contaminated environmental samples (water and soil) were obtained from three different flow stations (Agbada-Aluu shell, Obite, and Bonny) in Rivers State. The water samples were collected aseptically into screw-capped containers while the soil samples were collected into sterile cellophane bags. Uncontaminated sample was collected and used as control.

2.2 Isolation, Purification and Quantification of Total Genomic DNA

Total genomic DNA was extracted from 24hr old culture nutrient broth. The culture was centrifuged at 9,500g for 10 min. The bacterial pellet was re-suspended in 100µl TE solution (buffer). Twenty milliliters of lysozyme (100mg/µl), 1µl RNase (10mg/µl), 25µl proteinase K (20mg/ml) and 10µl 10% SDS were added. The mixture was incubated at 60ºC for 1hr. Thereafter, 1ml phenol:chloroform:isoamyalcohol (25:24:1) mixture was added and the tubes inverted many times. The mixture was centrifuged for 3 min and the water phase was carefully transferred to a new eppendorf tube. The phenol extraction was repeated two more times. The DNA was then precipitated by the addition of equal volume of cold isopropanol and the tube was kept at 4ºC for 5 min. It was centrifuged and the supernatant was discarded. DNA pellets were suspended in 100ml of TE buffer. 3M Sodium acetate (NaAc) solution (100µl) and twice the new volume of cold absolute ethanol were added and the tubes were kept at -20ºC for 20 min, after which the tube was centrifuged and the pellets were rinsed in 70% ethanol. The residual ethanol was evaporated by keeping the tube at room temperature (22ºC) for 15 min. The pellets were air dried and suspended in 100ul of TE buffer. The concentration of the DNA was measured using a Spectrophotometer (Nanodrop). The DNA extracts and a standard marker **-** Lambda (λ) PstI molecular size marker (kbp) were electrophoresed on a 1% agarose gel.

2.3 Determination of 16S *rRNA* Amplification

A portion of 16S bacterial gene of the rDNA was amplified by PCR from the total extracted soil DNA using two primers:

(i) E9F: GAGTTTGATCCTGGCTCAG between 7-26 bases
(ii). U1510R: GGTTACCTTGTTACGACTT between 1490-1512 bases. The reaction mixture contained: 27.75µl distilled sterile H_2O, 5µl reaction buffer (10x), 5µl dNTPs, 5µl of each Primer, 0.250µl Taq Polymerase (Dream), 2µl Template DNA. DNA amplification was performed in a PCR thermal cycler using the following programme: 5 min at 95ºC, 30 cycles of 30s at 94ºC, 30s at 52ºC and 1 min at 72ºC, followed by 10 min at 72ºC and then held at 4ºC. The PCR product was analyzed on a 1% TAE agarose gel.

2.4 Determination of Nested PCR-Denaturing Gel Gradient Electrophoresis (341F-GC and 534R)

The primary PCR products as template DNA (80*p*g)) was amplified using primers 341F-GC and 534R in a nested PCR-DGGE. Thermocycling conditions were the same as those used for the E9F and U1510R amplification of bacterial 16S rRNA genes.

2.5 Determination of Molecular Succession of Bacterial Community

Soil (5g) and water (5ml) each was separately suspended in 100ml Bushnell-Haas broth (BHB) [8] supplemented with 2% (v/v) crude oil. Each sample was incubated at 30ºC on an orbital shaking incubator at 200 rpm for a period of 7 days. During the incubation, optical density (600nm) was taken every day. A 10ml from this culture was inoculated into a fresh 100ml BHB containing 2% (v/v) crude oil. Incubation and OD reading were repeated. Four such transfers were made and every time the enriched population was plated on Bushnell–Haas agar containing oil, it was incubated for 1-3 days at 30ºC. Colonies of bacteria that grew on BHA supplemented with crude oil were counted and streaked repeatedly onto nutrient agar plates, incubated at 37ºC overnight (16h) until a single pure colony was obtained. The pure bacterial isolates were stored in 15% glycerol at -80ºC for long term preservation. At 7 days interval, genomic DNA extraction, amplification of 16S rRNA, denaturing gradient gel electrophoresis (DGGE) and electrophoresis were carried out on the mixed cultures.

2.6 Measurement of Degradative Activity of the Samples

The Bushnel-Haas broth supplemented with 2% (v/v) crude oil was inoculated with each sample, incubated for 35 days at 30ºC during which the optical density of each cultured medium was quantified with spectrophotometer at 600nm and the degradative activity (Unit/mL/h) was calculated.

2.7 Determination of pH

The pH was measured with the aid of Jenway 3510 pH meter after calibrating it with buffer solutions of pH 4, 7 and 10.

2.8 Statistical Analysis

The statistical analysis was performed using Microsoft office Excel 2007 for calculating mean, standard deviation and standard error.

3. RESULTS

The genomic DNAs extracted from the mixed cultures of soil and water samples used for the bacterial diversity are presented in Plate 1. The PCR amplified the genomic DNA targeted to amplify the 16S rRNA of each mixed culture is presented in Plate 2. The amplification produced a DNA molecule with a molecular weight of 1.5kb for all the mixed cultures. The bacterial populations of the oil polluted samples for the 35 days incubation period is presented in Fig. 1. The bacterial populations ($x10^4$) ranged from 30.33 ± 3.9 to 54 ± 4.3 Cfu/mL for samples 1w (Agbada-Aluu water) and 2s (Obite soil) on day 7; 13.67 ± 1.25 to 20 ± 2.45 Cfu/mL for samples 1s (Agbada-Aluu soil) and 2s (Obite soil) on days 14 and 21.

Samples 1s (Agbada-Aluu soil) and 2w (Obite water) had the lowest bacterial population while 2w (Obite water) and 2s (Obite soil) had the highest bacterial population of 15 ± 0.94 and 18.33 ± 2.1 Cfu/mL on day 28. On day 35, sample 3s (Bonny soil) had the highest bacterial load (17.67 ± 1.25 Cfu/mL). The highest bacterial population for the mixed cultures of each sample was observed on day 7.

Oil-degrading activity of the bacterial community of the oil polluted environmental samples during incubation period of 35 days is illustrated in Fig. 2. The bacteria present in the mixed cultures degraded the crude oil, although at various levels. There was a sharp decrease in the degrading activity of the bacteria between 7 and 14 days after which there was a gradual reduction over the period of time. The bacteria in sample 3s (Bonny soil) demonstrated the greatest ability to degrade crude oil with degrading activity of 246.28 ± 3.4 Units/h on day 7 while the lowest degrading activity of 0.9 ± 1.2 Units/h was obtained with samples 2w (Obite water) and 3s (Bonny soil) on days 28 and 35 respectively.

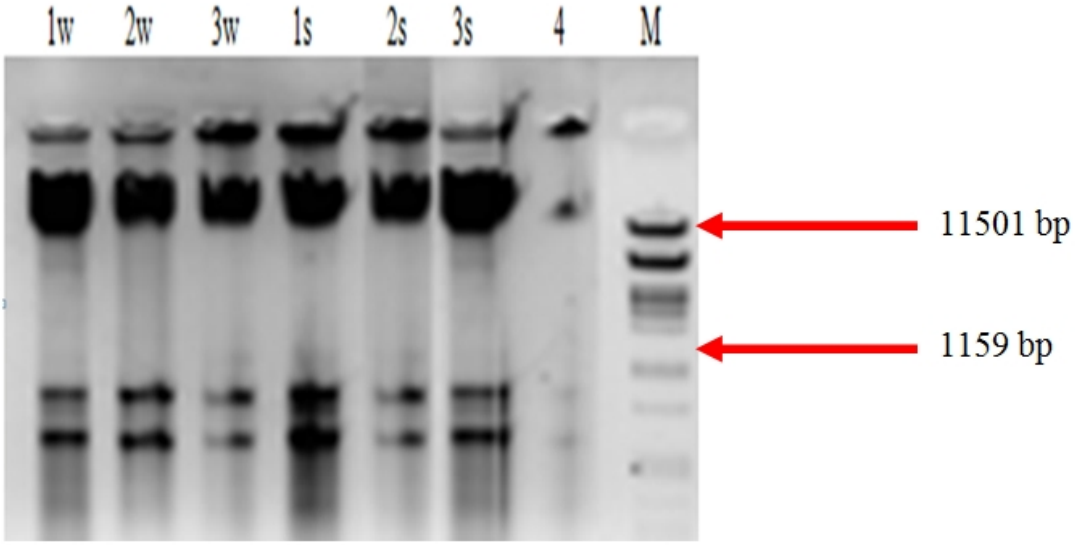


Plate 1. Genomic DNA extracted from the mixed cultures obtained from oil polluted environmental samples

Legend:

Lanes 1w - 3w are DNAs from water samples
Lanes 1s - 3s are DNAs from soil samples.
Lane 4 is positive control. (*E.coli*)
Lane M contained *Pst*I molecular weight marker.

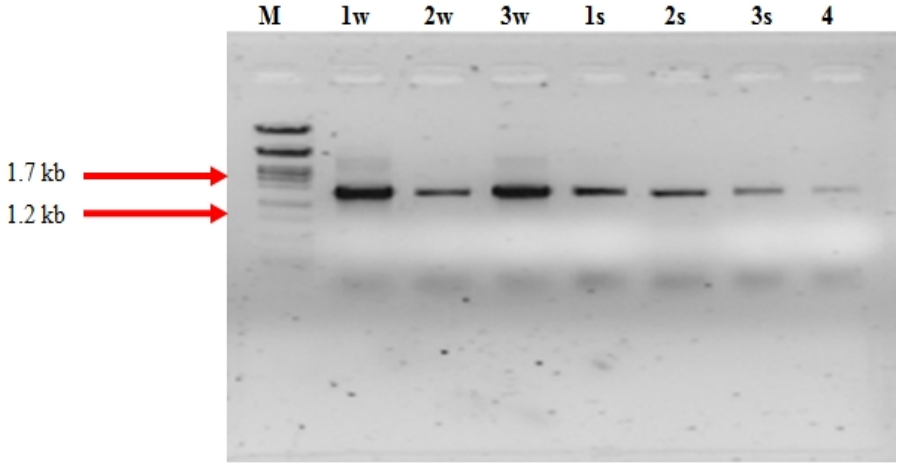


Plate 2. Genomic DNA amplified with primers targeted at the 16S rRNA of mixed cultures

Legend:

Lanes 1w - 3w are DNAs from water samples
Lanes 1s - 3s are DNAs from soil samples.
Lane 4 is positive control. (*E.coli*)
Lane M contained *PstI* molecular weight marker

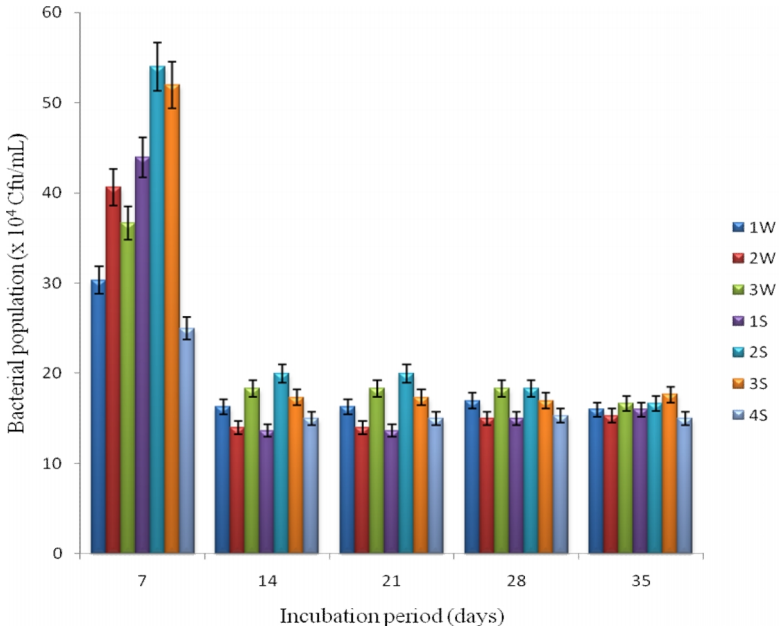


Fig. 1. Total load of succeeding oil-degrading bacterial in the oil polluted samples incubated for 35 days

Legend:

1w – 3w Water samples
1s – 3s Soil samples
4s - Unpolluted sample

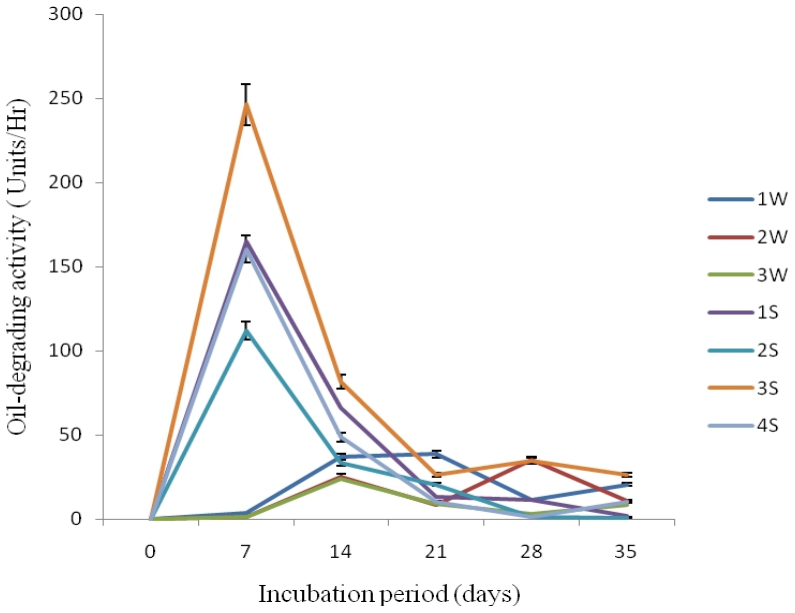
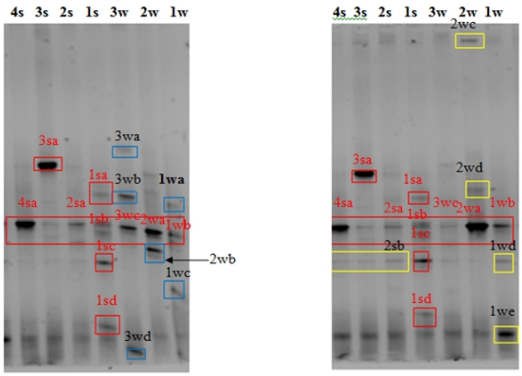


Fig. 2. Oil-degrading activity exhibited by the bacterial community of the oil polluted soil and water samples for an incubation period of 35 days

Legend:

1w – 3w Water samples
1s – 3s Soil samples
4s - Unpolluted sample

There were shifts in the bacterial community during the 35 days of incubation as illustrated on Plates 3 and 4. The DGGE analysis of the bacterial community revealed common distinguishable DNA bands marked with red colour (1wb, 2wa. 3wc, 1sb, 2sa and 4sa) on day 7 (Plate 3A). These bands were also observed on day 14 (Plate 3B). The bands marked with blue colour (1wa, 1wc, 2wb, 3wa. 3wb and 3wd on day 7 disappeared on day 14. The intensity of the bands in the first batch culture for samples 2s (Obite soil) and 3w (Bonny water) were reduced on day 14. Some new bands (yellow colour) appeared on day 14 (1wd, 1we, 2wc, 2wd, 2sb). Comparing the bands on days 28 and 35, it was observed that the bands that were common in the bacterial community were gradually fading out with the exception of bands 4sa on days 28 and 3sa on days 28 and 35. Notable new bands (1wf, 1wg, 1wh, 2we and 2wf) were observed on day 28 day while 1se, 2sc, 2sd, 2se, 4sb, 4sc for samples 1s, 2s and 4s were observed on day 35. The intensity of some of the bands was more prominent than others which for some, faded out. The pH of the cultured samples during the 35 days of incubation ranged between 6.8 and 8.2 (Fig. 3). In each of the culture, there was a gradual increase during the incubation.

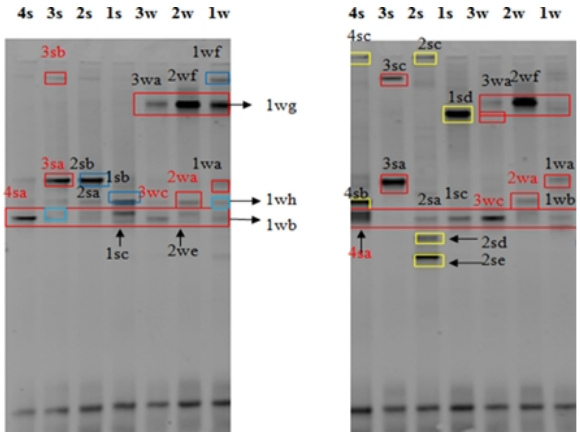


A: 7 days incubation **B: 14 days incubation**

Plate 3. The DNA bands of community bacteria in oil polluted soil and water samples incubated for a period of 7 to 14 days

Legend:

a) 1w - 3w (serial number of water samples); 1s - 3s (serial number of soil samples); a - d (serial number of DNA bands);
b) Initial bands: 1w (a-b); 2w (a-b); 3w (a-d); 1s (a-d); 2sa; 3sa; 4sa
Disappeared bands: 1wa; 1wc; 2wb; 3wa; 3wb; 3wd
New bands: 1wd; 1we; 2wc; 2wd; 2sb



C: 28 days incubation **D: 35 days incubation**

Plate 4. The DNA bands of community bacteria in oil polluted soil and water samples incubated for a period of 28 to 35 days

Legend :

c)	Initial bands	1wb; 3wc; 1sb; 1sc; 2sa; 2sb;3sa;
	Reappeared bands	1wa; 3wa
	Disappeared bands	2wb; 2wc; 2wd; 1sa; 1sd
	New bands	1wf; 1wg; 1wh; 2we; 2wf; 3sb
d)	Initial bands	1wa; 1wb; 2wa; 2wf; 3wa; 3wc; 1sc; 3sa
	Reappeared bands	1wa; 3wa
	Disappeared bands	2we;
	New bands	1sd; 2sc; 2sd; 2se; 3sc; 4sb; 4sc

The band 3sa was consistent throughout the incubation period of 35 days. Band 2wa was observed to be dominant on days 7 and 14 and disappeared on days 28 and 35 while new bands (2wf) emerged on days 28 and 35 for 2w. The band 1wf became prominent on day 28. The DGGE analysis shows that there were noticeable changes in the bacterial community both in the oil-polluted and unpolluted samples (4s).

4. DISCUSSION

The PCR-DGGE method used in the study was employed to generate complex molecular profile analysis of the polluted sites microbiota. This method was also used by MacNaughton [9]. The distinct DNA bands pattern that were observed constantly during the 35 days of incubation indicates dominancy of the corresponding species in the bacterial community while the DNA bands that disappeared or faded out bands pattern means replacement by other species. The replacement of bacterial species as illustrated by the emergence of new DNA bands during the incubation period demonstrates a dramatic shift in the community structure. It was observed that both oil-polluted and unpolluted samples responded positively, demonstrating a dramatic shift in the community structure.

The intensity of bands in all the polluted samples was high when compared with the unpolluted sample on day 35. This indicates that the growth of some of the bacteria was inhibited when exposed to oil or rather; they were unable to utilize or degrade the oil. In addition to these, the bacteria may find it difficult to compete favourably with their counter parts in the mixed culture. The new DNA bands that emerged or developed during the incubation period is attributed to new set of bacteria that developed then. These organisms could play a major role in the degradation of the oil which is vital for their metabolism. This is in agreement with the assertion of [10]. The profiles obtained are suggested to reflect the composition of the dominant site microbiota, which includes the non culturable fractions [11,10]**.** The data showed the response of the bacterial community in the soil and water polluted with the oil as evident in this microcosm experiment. The decrease in the bacterial population observed in this work over the time is in agreement with the work carried out by Duarte *et al*. [11] It was shown that the oil affected the bacterial community. The growth dynamics of the organisms was determined by the degrading activity and the pH of the culture media. The results showed that the isolates in the mixed cultures grew maximally on the crude oil substrate when supplied as the sole source of carbon and energy. High degrading activity expressed by the mixed culture during the incubation period of 35 days (Fig. 2) is in accordance with the hypothesis that natural microbial degradation of complex oil molecules usually involves more than a single species and that microbial populations consist of strains that belong to various genera [12,13,14]. This suggests the key roles played by each isolate in the degradation process of crude oil [15,16]. It has been postulated that no individual microorganisms has the potential to metabolize all types of hydrocarbon substrates. This is because individual microorganisms do not possess the enzymatic capacities possessed by the overall mixed populations. This is however in contrast to the study carried out by Singh and Lee [17]. It was shown that inoculation with the consortia did not show a higher degradation potential, meaning that competition for the same carbon and energy source by the consortia may affect the performance of the biodegradation process. Thomassin-Lacroix *et al*. [18] has also shown degradation to be efficient when the contaminants belong to a single type of recalcitrant compound. The observation and finding of major shifts in bacterial community structure during enrichment on crude oil substrates can be employed in tracking down the major community responsible for the degradation process.

The pH values (6.8 - 8.2) observed for the 35 days incubation of bacterial community were suitable for bioremediation. This finding is in conformity with Adoki and Orugbani, [19] who reported that neutral pH enable bioremediation activity of bacteria in the soil. The pH values were slightly basic, which favoured microbial degradation of oil. This means that there will be no need to add lime to these mixed cultures when they are employed to degrade oil. In contrast, the pH of the oil spilled soil and water in Agbada- Aluu, Obite and Bonny must be increased for good bioremediation.

5. CONCLUSION

The molecular assessment of bacterial community revealed that the degradation of oil was done in succession and that mixed cultures are responsible for the oil degradation because no individual bacterium possesses the enzymatic capability to degrade all the fractions in the crude oil. The bacteria represented in the band 3sa, (obtained from Bonny soil) which were dominant throughout the incubation period are potential candidates for degradation of crude oil. It could be obtained directly by sequencing the amplicon extracted from DGGE gel.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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