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Molecular Identification of Non-Cultured Bacteria in Soils of Three Communities in the Niger Delta Area of Nigeria

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Abstract
Nucleic acid analytical methods were used for species identification of bacterial diversity not culturally identified in soils of three communities flow stations owned by an oil exploration company operating and flaring gas for about 45 years now in the Niger Delta area of Nigeria (Ubi, Riri and Aboma flow stations). Surface soil samples were collected from mini pits using sterile bottles at 50, 100 and 150 meters from the flare barrier of each community flow station while the control sample was taken outside the flaring environment at a distance of 2 to 2.5 kilometers during the dry and wet seasons. The bacterial properties were first assessed using the cultural methods after serial dilution method which revealed the presence of the following bacteria in both seasons *Bacillus*, *Staphylococcus*, *Pedicocccus, Corynebacterium, Pseudomonas,* and *Kurthia spp*. However, the bacteria cultured appeared on the plate as monocultures. Bacterial DNA extraction was done using Norgen Bacterial Genomic DNA extraction kits for the molecular analysis to ascertain the true identity of the isolates not properly identified using cultural methods and it revealed the presence of the following bacteria, *Staphylococcus epidermidis, Bacillus atrophaues, Bacillus licheniformis. Streptococcus auginosus* that were identified by comparison with database sequences. Molecular evaluation of prominent bacteria in these study soils would enhance better catalogue and understanding of microbial biodiversity for possible bioremediation measures as it will identify some beneficial bacteria which cannot be identified using the cultural methods and biochemical tests.
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INTRODUCTION

Human technological and scientific advancements have caused environmental changes that are impossible to evaluate and fully comprehend. Our ability to change the environment has increased faster than the ability to predict the effect of that change. Pollution of the environment is one of the major effects of human technological advancement (Kalita *et al.,* 2012). This results when a change in the environment harmfully affects the quality of human life including effects on animals, microorganisms as well as soil ecosystem (Marinescu *et al.,* 2010). Soil microorganisms, Prokaryotic and Eukaryotic spend all or part of their lives in the soil environment. The chemical composition of the soil affects the types and size of its microbial population in the soil.

The activities of large communities of microbes influence chemical and growth rate changes in naturally managed and engineered ecosystems. As such, giving meaning to the constituents of a community of interacting population of microbes which is substantial for severe progress in the field (Konokpa, 2009).

Microorganisms are actively involved in many soil processes altogether and are not limited to soil carbon sequestration, nitrogen and phosphorus cycling and detoxification of inorganic and organic pollutants. Soil microbes regulate processes through their survival strategies at the organismal level, competitive and synergistic interactions at the community level and feedback control mechanisms at the ecosystem level (Wei Shi, 2015).

There are various impacts of the organic content, texture, pH, and where the soil was collected on soil microbial diversity. Soil samples collected from a particular location but at different depths in a core can be completely different. These factors and more affect the microbial load and consequently the yields of DNA and RNA that can be obtained. A good number of physical and bio-chemical activities are responsible for the distribution of trace elements in soils, sediments and natural water which are also contaminants (Polizzo, 2011).

There are noticeable effects resulting from oil exploration and gas flares on host Communities farmlands that may in turn affect the microbial quality of the farmland which may result in low fertility of the soil. Microorganisms show variation in their activities and in their relationship with other faunas and floras. This is due to their differences in structure and functions in the microbial communities that it was almost difficult to differentiate between the biological and agricultural ecosystems. In recent times there is increase in microbial identification in the natural environment using cultural method. But currently the use of the Nucleic acid analyses method to identify a whole microbial community will resolve and provide answers to questions that have been unanswered using standard cultural methods. The 16s and 18s regions of the ribosomal genes' sequences are the universally accepted phylogenetic markers for the study of microbial systematic and ecological surveys from the natural environments in recent research. Ribosomal molecules have highly conserved sequence domains interspersed with hyper variable regions and these variables domain differentiate one microbe from

another and therefore, can be used as molecular markers to discriminate among taxa. The molecular technique enables us to analyze the soil microbial community with more accuracy than cultural methods. This is because microbiological advances are closely linked with molecular biological technique (Nakatsu, 2006).

The use of technology that helps to identify the microbial community means creating a technology that 'works for the microorganism management, so that they work for us. It is an epitome of a win-win' situation since creating a win-win-technology' requires that the knowledge gained from microbial-ecology research could be translated to a practical setting.

The soil microbial community varies in their association at the various levels of the biological organization. The chemical composition of the soil affects the types of microorganisms and size of the microbial population in that soil environment and crude oil exploration in the Niger Delta which cannot be talked about without mentioning gas flaring that is the most notorious activity of the oil companies. It is one major activity in the Niger Delta capable of changing the chemical composition of the soil and as such affect microbial diversity in the community. Therefore, this research aims at assessing the impact of gas flare on the bacterial diversity using cultural and molecular methods of identification for non-culturable bacteria.

MATERIALS AND METHODS

The study was set up in three facilities belonging to an undisclosed oil company with geographical coordinates of Latitude 05 14'51 20" N and Longitude 05 35' 58: 63''E, located in Rivers State Latitude 05 29' 10.3'N and Longitude 06 14'39.5'E located in Delta State and Latitude 04 37'46.6N and Longitude 06 15'00.6E located in Bayelsa State in the Niger Delta area of Nigeria. The real names of the facilities are not disclosed for legal reasons, therefore, Ubi, Riri and Aboama were used in this study.

Soil Sample Collection

Sampling was done twice, in dry season (late November) and raining season (early August) of the following year. Soil samples were taken from 0-15 cm depth at 50, 100 and 150 m intervals from the flare barrier while the control was taken from 2km radius of each of the study communities. Thus 12 soil samples were taken from each facility, resulting in 36 soil samples for each season and a total of 72 for both dry and wet seasons.

Apparatus used during the Study

A measuring tape was used to measure the distance. A hand trowel was used to collect soil samples into sterile bottles.

Bacterial Analysis

Determination of bacteria properties of the soil samples was carried out visibly by counting microbial colonies after culturing and 24 hours incubation. The streaking method was used to isolate the individual bacteria cultures as the samples were cultured on fresh plates. Sub-culturing of bacteria culture was done by pouring freshly prepared Nutrient agar plate into already sterilized petri dishes and allowed to solidify. The different isolates were picked with sterile wire loop and streaked on the Nutrient agar and incubated for 24 hours and used for further studies. The bacteria cultures were sub cultured by carefully taking a fresh growing portion with a sterilized needle into fresh NA plates.

Characterization and Identification of Bacteria Isolates

Cultural characteristics were observed on nutrient agar plates for bacteria isolates. These include size; shape, surface, opacity, texture, elevation and pigmentation were determined by visual observation. Gram staining reaction was carried out as described by Harold, (2004) and motility test following the procedure by (Chakraborty and Pal, (2011). Indole test as described by Barrow and Feltham (2003) and Carbohydrate fermentation test (Buchana, and Caibbons. 1994) were also carried out.

Determination of Microbial Load

The microbial load of each sample was determined visibly by counting the total number of colonies on each culture plate after 24 hours incubation. The microbial load/ml was determined by the formula of Cheesbrough (2002) as:

$$\text{Count/ml} = \frac{\text{Number of counted colonies on plate x volume plated x dilution factor}}{\text{Number of counted colonies on plate x } 1/10/10^3 \text{ cfu/ml}}$$

Molecular Identification of Bacterial Isolates

Molecular analysis for bacteria isolates was done to identify some of the bacteria which the cultural method and biochemical tests carried out could not identify. The bacteria to be identified using molecular methods were isolated and sub-cultured using the cultural technique.

Bacterial DNA Extraction was done using Norgen Bacterial Genomic DNA extraction kits.

The bacterial ITS1 region of the 16SrRNA was isolated using the General multipurpose primers (EUBf92: GCACAAGCGGTGGAGCTGTGG and EUBr1386: GCCGGGAACGTATTCACCG) were used for the PCR amplification. The products gotten from the PCR amplification were purified using the Invitrogen PCR amplification kit. Gene enrichment was done using the RDT 1000 Raindance Sequence Enricher. Agarose Gel Electrophoresis of PCR Products was done by using 2% of Agarose Gel Electrophoresis containing 05µg/ml Ethidium Bromide (EtBr). The DNA sequencing was done using the Next Generation Sequencer (NGS) (solid 5500xl plat). The nucleotide sequences obtained from the various bacteria isolates were analyzed by searching and comparing with other sequences from the National Center for Biotechnology Information (NCBI) website. A blast search was conducted on all the nucleotide sequences and the identities of the isolates were revealed (Stach *et al.,* 2001).

RESULTS

Enumeration and Identification of Bacteria in Soils with Distances from Flare in the Dry and Wet Seasons of study area.

Enumeration and identification of bacteria in soils of Ubi flow station with distances from flare in the dry and wet seasons is presented on Table1, Total bacterial count ranged from 3x 10^6 (cfu) at 50 and 100 m to 10 x 10^6 (cfu) at the control in the dry season. In the wet season, total bacterial count ranged from 5x 10^6 (cfu) at 50 m to 16 x 10^6 (cfu) at 150 m from the flare. Bacteria isolated in the dry season were *Bacillus sp*, ***Staphylococcus***, ***Kurthia sp, Pediococccus sp,*** and ***Corynebacterium sp.*** **and** in the wet season, ***Bacillus sp***, ***Pseudomonas sp,*** **and** ***Corynebacterium sp*** **were isolated while** bacterial species appeared on culture plates as monoculture.

Bacterial count and identification in soils of Riri flow station with distances away from flare in the dry and wet Season is presented on Table 2. Total bacterial count ranged from 3 x 10^6 **(cfu)** at 150 m from flare to 7 x 10^6 **(cfu)** at 50 m from flare in the dry season and in the wet season, 2 x 10^6 **(cfu)** at the control to 6 x 10^6 **(cfu)** at 50 m away from the flare. The bacterial isolated in the dry season were *Bacillus sp* and *Kurthia sp* and in the wet season, *Corynebacterium sp* and *Kurthia sp.*and bacterial isolates appeared on plate as monoculture.

Bacteria count and identification in soils of Aboma flow station with distances away from flare in the dry and wet seasons is presented on Table 3 and shows that Total bacterial count ranged from 3 x 10^6 **(cfu)** at 50, 100 m and control to 8 x 10^6 **(cfu)** at 150 m from flare in the dry season and in the wet season it ranged from 2 x 10^6**(cfu)** at 150 m to 9 x 10^6 **(cfu)** at 100 m and the control. Isolates also appeared on plate as monoculture.

Table:1 Enumeration and Identification of Bacteria in Soils of Ubi Flow Station with Distances from Flare in the Dry and Wet Seasons

Distance	**Dry Season**				**Wet Season**			
	CFU/g	Gram Stain	Cell Type	Bacterial Isolated	CFU/g	Gram Stain	Cell Type	Bacterial Isolated
50m	3x10^6	+ve	Rod	*Bacillus sp*	5x10^6	+ve	Rod	*Bacillus sp*
100m	3x10^6	+ve	Cocci	*Staphylococcus*	6x10^6	-ve	Rod	*Pseudomonas*
		+ve	Rod	*Kurthia sp*				
150m	5x10^6	+ve	Rod	*Bacillus sp*	16x10^6	+ve	Rod	*Bacillus sp*
						-ve	Rod	*Pseudomonas sp*
Control	10x10^6	-ve	Cocci	*Pediococcus sp*	7x10^6	+ve	Rod	*Corynebacterium*
		+ve	Rod	*Corynebacterium*				

CFU- Colony Forming Unit

Table:2 Bacteria Count and Identification in Soils of Riri Flow Station with Distances Away from Flare in the Dry and Wet Seasons

Distance from flare	Dry season				Wet season			
	Characteristics			Bacterial Isolated	Characteristics			Bacterial Isolated
	CFU/g	Gram Stain	Cell Type		CFU/g	Gram Stain	Cell Type	
50 m	**7x10^6**	**+Ve**	**Rod**	***Bacillus sp***	**6xl0^6**	**+Ve**	*Rod*	***Corynebacterium sp***
100 m	**4x10^6**	**+Ve**	**Rod**	***Bacillus sp***	**3x10^6**	**+Ve**	*Rod*	***Corynebacterium sp***
150m	**3xI0^6**	+Ve	**Rod**	***Kurthia sp***	**3xl0^6**	**+Ve**	*Rod*	***Corynebacterium sp***
		+Ve	**Rod**	***Bacillus sp***				
Control	**6x10^6**	**V+e**	**Rod**	***Kurthia sp***	**2x10^6**	+Ve	***Rod***	***Kurthia sp***

CFU- Colony Forming Unit

Table 3: Bacteria Count and Identification in Soils of Aboma Flow Station with Distances Away from Flare in the Dry and Wet Seasons

Distance from flare	Dry season				Wet season			
	Characteristics			Bacterial Isolated	Characteristics			Bacterial Isolated
	CFU/g	Gram Stain	Cell Type		CFU/g	Gram Stain	Cell Type	
50 m	3x10^6	+Ve	Rod	*Kurthia sp*	7xl0^6	+Ve	Rod	***Corynebacterium sp***
100 m	3x10^6	+Ve	Rod	*Kurthia sp*	9x10^6	+Ve	Rod	***Corynebacterium sp***
		+Ve	Cocci	*Staphylococcus*		+Ve	Rod	***Kurthia spp***
150m	8xI0^6	+Ve	Rod	*Bacillus sp*	2xl0^6	+Ve	Rod	***Bacillus spp***
Control	3x10^6	+Ve	Rod	*Kurthia sp*	9x10^6	+Ve	Rod	***Kurthia sp***

CFU- Colony Forming Unit

Molecularly Identified Bacteria Isolates

Molecularly Identified Bacterial Isolates are presented on table 4, these isolates were randomly picked from some of the culture plates and molecularly analyzed. Two strains of *Bacillus licheniformis* were identified with the molecular technique. *Bacillus licheniformis (*DSM 13 = ATCC 14580) and *Bacillus licheniformis.* Actual species of bacteria were identified molecularly.

Table 4: Molecularly Identified Bacteria Isolates

Season	Area	Distances (metres)	Isolated Bacteria
Dry	Ubi	50	*Staphylococcus epidermidisa*
		100	*Staphylococcus epidermidisa*
		150	*Bacillus licheniformis*
	Aboma	50	*Bacillus licheniformis* (DSM13 =ATCC 14580)
		100	*Bacillus licheniformis* (DSM13 =ATCC 14580)
			Staphylococcus epidermidisa
			Streptococcus anginosus
		150	*Bacillus licheniformis*
	Riri	50	*Bacillus atrophaeus*
		100	*Bacillus atrophaeus*
		150	*Bacillus atrophaeus*
		Control	*Bacillus licheniformis*
Wet			
	Ubi	50	*Staphylococcus epidermidis*
		100	*Staphylococcus epidermidis*
		150	*Bacillus licheniformis*
	Aboma	50	*Bacillus licheniformis* (DSM 13 = ATCC 14580)
		100	*Staphylococcus epidermidis* *Bacillus licheniformis* (DSM 13 = ATCC 14580)
		150	*Bacillus licheniformis*
	Riri	50	*Bacillus atrophaeus*
		100	*Bacillus atrophaeus*
		150	*Bacillus atrophaeus*
		Control	*Bacillus licheniformis*

Discussion

Five bacterial species were isolated from the three study flow station at the various distances away from the gas flare stack using cultural methods. Only two species of the five species of bacteria isolated were isolated from each distance in each of the two seasons. In a similar study by Mohamed *et al.* (2015) where cultural and molecular methods were employed to ascertain true identities of the petroleum degradable bacteria in an oil polluted soil, only two bacterial strains were identified, *Staphylococcus* aureus and *Corynebacterium amycolatum using molecular methods. Lactobacillus, Staphylococcus, Citrobacter, Flavobacterium* and *Micrococcus* were the bacteria species isolated from soils of 200 m away from flare where soil samples were collected from the distances of 10 m, 100 m 200 m and a control from the findings of Nwaugo *et al.* (2005). At 10 m from the gas flare, only *Bacillus* and *Pseudomonas* were found using cultural methods. This study, considered the diversity of bacteria in soils of the three flow stations studied. There was no significant impact of seasons on the diversity of the isolated bacteria. However, the wet season had more bacterial load than the dry season. This conforms to the findings of Johannes *et al.* (2011) that high moisture in the soil encourages rapid growth. There was an increase in the bacteria load in respect to increase in distance from the flare in Ubi flow station. Riri and Aboma did not follow any particular trend. This might be as a result of the designs of the flare stacks in these flow stations. This also conforms to the finding of Johannes *et al.* (2011) that high temperature reduces microbial growth and the findings of Abdukareem (2003) that the distribution of pollutants can be attributed to flare stack type. Findings from this work has shown that even with the molecular technique, true identity bacteria species were given. These bacterial isolates also appeared as mono or double cultures on plates in soil samples of distances from the flare. However, Das and Chandran (2011) outlined these bacterial genera isolated from their study as crude oil degraders. The control of 2 km from the flare also having bacterial degrading genera can be attributed to the findings of Anomohanran (2012) which revealed that within a distance of 2.15k in the wet and 2.06 km in the dry seasons from a thermal plant were polluted. This he attributed to some outstanding metrological parameters like rate of dispersal of pollutant gases, Wind speed and direction, closeness to ocean.

CONCLUSION

Findings from this work has shown that molecular technique gave the exact identity of bacteria species isolated from study area. All five molecularly identified bacteria were isolated from the three-study flow station and in both seasons. These bacterial isolates also appeared as mono or double cultures on plates in soil samples with distances from the flare. This means that bacterial diversity reduced in the study areas which can be attributed to some outstanding metrological parameters like rate of dispersal of pollutant gases, Wind speed and direction, closeness to ocean.

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