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Analysis of Bacterial and Archaeal 16S rRNA Gene in Soil Obtained from a Petroleum Refinery Effluent Site in Nigeria Using Real-Time PCR

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Abstract: The microbial community in environments exposed to effluents released from industrial processes such as petroleum refining are usually adapted to utilising and degrading these bye-products. Soil was sampled in an area adjacent to the water body containing refinery effluent released from the refinery in Nigerian National Petroleum Company (N. N. P. C), Kaduna. The samples were obtained at two depths, 17 – 20 cm and 37 – 40 cm respectively. Genomic DNA was extracted from these samples in triplicates and the 16S rRNA gene was amplified using the primers, 518F and 907R in 20µL reaction mixtures. The data obtained after the Q-PCR run was analysed using MxPro, Q-PCR software. The final number of target genes was an average of triplicate measurements from three independent DNA extractions from each soil sample. The average 16S gene copy number in the samples was in the range, 3.11E+07 – 1.23E+08 gene copies per gram of soil for bacteria and 8.13E+06 – 5.76E+07 gene copies per gram of soil, for archaea. Sampling depths of 17 – 20 cm had relatively higher gene copy number as opposed to depths of 37 - 40 cm. Soils closer to the surface are typically richer in nutrients and oxygen thus favouring bacterial growth. The 16S rRNA gene is highly conserved and very useful in phylogenetic studies of bacterial populations. However, in order to screen for specific activities such as degradation of toxic compounds by bacteria in soil, detection of functional genes is necessary.

Keywords: Soil, Bacteria, Archaea, 16S rRNA, Real-Time PCR, Nigeria

1. Introduction

Petroleum is a source of fossil fuel globally; Nigeria is a major oil producing country with crude oil accounting for most of the nation's exports. One of the three petroleum refineries in the country is located in the capital city of Kaduna, in Kaduna state (Coordinates: 10°31′23″N 7°26′25″E). The refinery is located in a region of the city called Sabon tasha. The process of refining petroleum leads to the formation of bye-products which eventually get discharged as effluents into the environment. Microbial populations in soils and aquatic systems receiving these effluents typically get adapted to these complex compounds and are able to biotransform and/or biodegrade them. Sulphate reducers, hydrocarbon degraders, acidophiles, represent some of the groups of microorganisms in such environments associated with the release of industrial effluents. Metagenomics plays a key role in identifying these bacteria as well as distinguishing their functional diversity. Culture dependent methods do not provide as much comprehensive information since most of these bacteria do not grow on laboratory media; as such their actual population size is not captured. Several studies have been carried out on soil bacterial populations by assessing the number of 16S

ribosomal gene copies and compared with the number of a specific functional gene copy [1, 4].

The aim of this study is to quantify the soil bacteria and archaea present in a petroleum refinery effluent site using real time PCR.

2. Methodology

Sampling site and Soil sample

The Petroleum refinery is located in the north-western part of Nigeria, in the southern part of the state of Kaduna. The effluent is released after treatment in the refinery into a stream that flows into the Romi river after about some distance. The water from the stream is used by local farmers in irrigating the farms close to the stream. Core samples were collected from the bank of the stream at 17 – 20 cm, and 37 – 40 cm, and kept in clean polythene bags; samples for molecular analysis were stored at -4°C while physicochemical analyses were carried out immediately.

Soil properties

The soil samples were analysed for textural class, physicochemical characteristics using standard methods.

DNA extraction and Real-Time PCR conditions

Total chromosomal DNA was extracted from the soil samples in triplicates following the procedure in the Soil DNA Extraction kit, MP Biomedicals. The bacterial and archaeal strains used as standards are *Anaerolineae thermophila* DSM 14523 and *Methanococcoides methylutens* DSM 2657 respectively. The primers for the 16S rRNA bacterial genes are 518F [7] and 907R [8] while the Archaeal primers are Arch – 0025 – AS17F and ARCH – 00344 AS20R [12].

The protocol used was adapted from [13]. Ten-fold serial dilutions of the standards was carried out (10^{-1} – 10^{-8}) and pipetted into the wells in triplicates. 20µL reaction mixtures containing 1µL of soil DNA, 20pmol. µL of each primer, 10 µL 5 X SYBR Green qPCR SuperMix (Invitrogen, U. S. A.) and BSA 0.5µL were used for the amplification. Also included in the run is a no-template control (NTC) which is a mixture of all the PCR agents without any DNA. The qPCR runs were carried out: annealing temperature of 95°C for 7 mins, 55°C for 30 secs, 72°C for 1 min, 60°C for 30 secs, 55°C for 30 secs, and 95°C for 30 secs. The resulting data after the qPCR run was analysed using MxPro, QPCR Software, version 4.10d (Stratagene, Agilent Technologies Division, Germany), the final number of target genes, was an average of triplicate measurements from three independent DNA extractions made from each soil sample.

The concentration of DNA in the standards (Archaeal and Bacterial) was measured using the Qubit Fluorometer (Quant-It Assay Kit) and Molecular Probes (Invitrogen, U. S. A.), the concentration of the Archaeal and Bacterial DNA contained in the standards were used in the calculation of gene copy number.

3. Results

Figure 1. *A photograph of Romi River receiving effluent from the petroleum refinery in Kaduna; X is the point where the soil samples were obtained.*

Table 1. *Physico-chemical characteristics and textural classification of soil obtained from refinery effluent site.*

Sample	R. E. S (17 -20 cm)	R. E. D (37 - 40 cm)
Textural class	Sandy loam	Loamy
pH (1:2.5 0.01 M CaCl)	4.8	4.9
Oil and Grease content (mg/L)	1 340	770
Moisture content (%)	0.8	0.75
Organic Carbon (mg/kg)	16.36	9.58
Nitrogen (mg/kg)	1.33	1.05
Available Phosphorus (mg/kg)	20.3	5.43

Table 2. *Micro nutrients (mg/kg) of soil obtained from refinery effluent site in Nigeria.*

Sample	R. E. S (17 - 20 cm)	R. E. D (37 - 40 cm)
Copper	[a]13.61	[a]24.11
Manganese	112.46	79.22
Zinc	[a]147.87	[a]2725.53
Iron	20442.91	330.33
Lead	[a]351.42	23.07
Nickel	26.3	10.2
Cadmium	1.8	0.2
Chromium	0.7	0.12

Superscript [a]: Quantity exceeding the recommended intervention values set by Department of Petroleum Resources (2002)

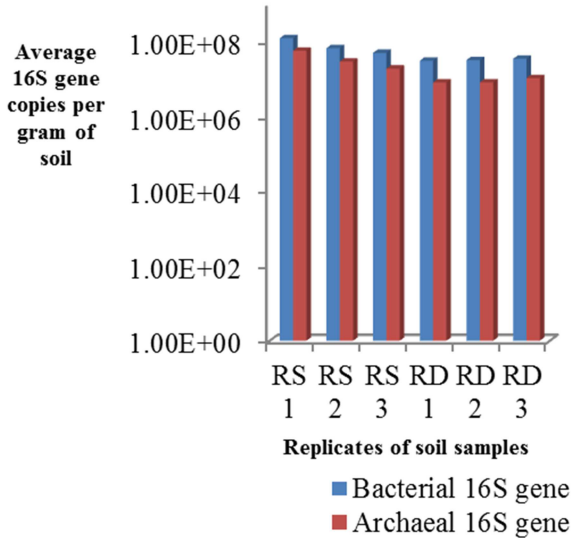

Figure 2. *Analysis of 16S gene in soils obtained from Refinery effluent site in Kaduna state, Nigeria.*

KEY:
RS: Refinery effluent shallow
RD: Refinery effluent deep

4. Discussion

Soil texture plays a role in the makeup of bacterial community due to factors such as porosity that affect the uptake and assimilation of nutrients by bacteria. However, in this study the soil type was the same for both sampling depths. In a study by [4], the soil type was found to affect the sorption of phenanthrene, a polycyclic aromatic hydrocarbon associated with petroleum contamination as well as processing of other fossil fuels such as coal. The sample had an acidic pH which could also play a role in the diversity of the prokaryotic population. The oil and grease content was significantly higher in the sample closer to the surface (RS). The Carbon to Nitrogen ratio in both sampling depths had relatively similar values at both depths (Table 1). The Carbon to Nitrogen ratio (C: N) in the soil (10:1) and might play a role in the proliferation of these prokaryotes. The importance of moisture content in bacterial multiplication can be seen in sample, TPS which recorded the highest moisture content (3.1%) compared to the other samples. Archaea are known to survive extreme environmental conditions better than bacteria, which could probably explain why they thrived better than bacteria in the Refinery effluent soil. As expected in soils contaminated with industrial effluents, quite a number of heavy metals were found to be present in the soil. However, only three of them were present in high quantities. The presence of these heavy metals in the sampling site implies that there will be potential accumulation of these elements (zinc and lead) by the plants being cultivated in that site, thus suggesting obvious health concerns. Among the heavy metals present in the sampling site, zinc, copper and lead were found in quantities exceeding the recommended limit. However, the deep sample (RD) exceeded the limit set by DPR (2002) for zinc (0 mg/kg) and copper (10 mg/kg) only. The shallow sample (RS) on the other hand, exceeded the limit set by DPR (2002) for zinc (0 mg/kg), lead (210 mg/kg) and copper (10 mg/kg); thus the shallow sample appears to have more heavy metal contamination. The heavy metals reported to be commonly found in contaminated sites are lead, chromium, zinc, cadmium and copper [10].

Real-time PCR is a reliable means of quantifying the 16S gene in prokaryotes because it is sensitive and there is increased reproducibility of results. Fluorescence detection (SYBR Green dye) is the mechanism of quantifying the 16S gene, which is the basis of classifying prokaryotes due to its highly conserved nature. The bacterial (3.11E+07 – 1.23E+08 gene copies per gram of soil) and archaeal (8.13E+06 – 5.76E+07 gene copies per gram of soil) 16S gene copies showed a range showing increase by one order of magnitude respectively. Soil tillage resulting from farming activities enhances the release of organic compounds by plant roots all favour microbial growth due to the resultant increase in nutrients in such soils. This might lead to the relatively consistent number of 16S gene

copies of both bacteria and archaea. Generally, the bacterial and archaeal 16S gene were more abundant in the shallow samples (17 – 20 cm) as opposed to the deep samples (37 – 40 cm). The proximity of the shallow samples (RS) to the surface (17 – 20 cm) and consequently, more access to oxygen and nutrients could have played an important role in the number of 16S gene copies compared to the deep samples (RD). The shallow samples had higher quantities of both macro and micro nutrients as shown in Table 1; thus being a more favourable environment for the bacterial and archaeal communities present. Quantification of 16S rRNA gene and/or functional genes in soils is a reliable and efficient method of assessing the bacterial community structure in contaminated environments [1, 2, 3, 5]. Analysis of the 16S gene copies using the statistical tool, ANOVA, indicated that the bacterial and archaeal gene copies were not statistically significant. A possible reason for this could be the relatively similar 16S gene copies at both sampling depths. However, the bacterial and archaeal gene copies had slightly higher values in samples obtained closer to the surface (17 – 20 cm).

Comparison of the abundance of 16S gene from bacteria and archaea shows the bacterial gene to be more abundant than the archaea by at least an order of magnitude. Figure 2 reveals that the bacterial 16S gene copy was consistently higher than that of the archaea. Bacteria have been reported to be present in larger numbers than archaea in soil environment [9]. Needless to say, their gene copy numbers are almost at par in this study suggesting favourable conditions for the archaeal communities which thrive in extreme or oligotrophic environments. The high quantity of Iron in the soil studied indicates a likelihood of it being used as an electron acceptor by both the bacterial and archaeal communities in circumstances where the oxygen in that environment gets depleted.

The presence of bacterial and archaeal communities in soils related to petroleum refining activities is associated with the presence of functional genes enabling them to utilize the available hydrocarbons present in those ecosystems. Although the role of bacteria in petroleum-hydrocarbon contaminated ecosystems has been documented more than archaeal communities, the latter has been shown to be able to degrade these compounds by fermentative/methanogenic processes [5, 14]. Metagenomics studies of such prokaryote communities expound their metabolic and biodegradative potentials.

5. Conclusion

Soil textural class does not show much variation at depths reaching 40 cm; however, the sampling depth affects the quantity of 16S gene and invariably the number of bacteria and archaea in the location. The availability of nutrients and aeration at depths closer to the soil surface increases the aerobic prokaryotic population. Real time PCR is a very reliable means of assessing the prokaryotic population in a soil ecosystem. The bacterial 16S gene was found to be more abundant than the archaeal 16S gene in this study.

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