16S RNA Survey Of Anaerobic Microcosms Of A Niger Delta Wetland Impacted With High Levels Of Polyaromatic Hydrocarbons

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Abstract: Sediment samples were collected at georeferenced sites from the Egbara stream of Ejamah Ebubu community in the Niger Delta, Nigeria. In vitro experiments were set up to assess the potential for bioremediation of the polyaromatic hydrocarbons (PAHs) impacted sediment under anaerobic conditions, in the presence of indigenous microorganisms. The experiment consisted of added consortium, non added consortium and autoclaved controls in 100 ml serum bottles. Results show that all serum bottles containing the medium to enrich sulphate reducing bacteria and total anaerobic bacteria tested positive. DGGE fingerprinting for 16S rRNA gene and dsrA gene revealed different profile for various consortia and sampling points. Real time qPCR analysis revealed differences with various consortia, in the order; bacteria abundance in added consortium greater than non added consortium greater than autoclaved control. Test with oil recorded 16S rRNA gene copy number in the range $3.35 \times 10^7 – 4.93 \times 10^{11}$ (µg/µl), test without oil ranged from $7.76 \times 10^6 – 3.79 \times 10^8$ (µg/µl), test with autoclaved killed cells ranged from $5.60 \times 10^2 – 2.48 \times 10^6$ (µg/µl). The dsrA gene fragments in the test with oil ranged from 6.03×10^3- 1.03×10^9(µg/µl), test without oil ranged from $8.06 \times 10^4 – 5.61 \times 10^6$ (µg/µl), test with autoclaved killed cells ranged from $6.21 \times 10^1 – 1.06 \times 10^5$ (µg/µl). It is recommended that sulphate be utilized for bioremediation of anoxic sites; sulphate reducing bacteria of contaminated sites be studied for optimum utilization of their potentials.

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1. Introduction

Polyaromatic hydrocarbon (PAH) is a common term for high molecular aromatic hydrocarbons. They consist of two or more fused benzene or aromatic rings in linear, angular, or cluster arrangements (Wilson and Jones., Muiller, et al, 1996). PAHs are classified as either alternant or non-alternant, the former include molecules that are derived from benzene by fission of additional benzenoid rings (e.g naphthalene, phenanthrene, pyrene), the later contains rings with fewer or more than six carbon atoms in addition to the six-membered benzenoid rings (e.g acenaphthylene, fluorene, fluoranthene) (Harvey, 1998).

Polyaromatic hydrocarbons are not easily degraded by microorganisms but they provide more detailed compositional information that can be used to identify both the source and degree of degradation of spilled crude oil (Bence and Burn, 1995; Page *et al.*, 1995; Douglas *et al.*, 1996). PAHs are of most concern owing to their toxicity, low volatility, resistance to microbial degradation and affinity for sediment (Coates *et al*, 1997). Research has shown their interference with hormone system and their potential effects on reproduction, as well as their ability to depress immune function (Chaloupka *et al.*, 1993).

Hydrocarbons represent an abundant class of organic compounds on a global scale. The natural accumulation of hydrocarbon or oil contaminated sites are characterized by absence of molecular oxygen. Polyaromatic hydrocarbons typically had been thought to be recalcitrant to biodegradation without oxygen. Recent studies however, (Annweiler, et al., 2000; Annweiler, et al., 2001; Coates, et al., 1996a; Rockne, et al., 2000) had demonstrated PAH degradation under sulphate reducing and nitrate reducing conditions. While it has been shown that polyaromatic hydrocarbon are degraded anaerobically; little is known about the microorganisms responsible for this activity. Mineralization of hydrocarbons under anoxic conditions would play an important role in such environments.

The collective genomes of the soil microflora is referred to Metagenome (Osburne *et al* 2000). Analyzing prokaryotic 16S rRNA gene sequences is part of a standard method for assessing microbial diversity. Metagenomics can be combined with

Molecular Geo-referencing (MGR) to give a holistic picture of the state of that environment. Geo-coordinate samples analyzed at a given time would indicate the distribution of molecules of potential concern (MOPC) with time.

The objectives of our study were (i) To use molecular genetic methods to characterize microorganisms prevalent under anaerobic conditions in a typical Niger Delta wetland ecosystem impacted with crude oil (ii) To verify that anaerobic utilization of crude oil containing high level of PAHs is obtainable under conditions tested in the laboratory (iii) To determine the role of non-oxygen terminal electron acceptors in the metabolism of crude oil containing high level of PAHs.

2. Material and Methods

2.1. Source of Samples

Large, kilogram quantities of Sediment were collected from Egbara stream (Ewenta Egbara) in Eleme, Rivers State, Nigeria. Composite sediment samples were collected at depths of 20 cm to 40 cm. The location of sampling points was marked using Geo-positioning system. (Model GPSMAP76, Am, serial no: 91090835). The GPS reading of the sampling points are: A (N04^0 45' 23.8" E007^0 09' 47.0"), B1 (N04^0 46' 19.9" E 007^{0}09' 33.5"), B2 (N04^0 46' 10.5" E007^{0}09'28.3") C1 (N04^{0}46' 22.4" E 007^{0}08' 49.8") C2 (N04^{0}46' 22.2" E 007^0 08' 51.6") C3 (N04^{0}46' 23.9" E 007^{0}09'07.0").

2.2. Microcosm Set-up

Enrichment cultures were prepared by inoculating samples from low polluted points and high polluted points. In order to monitor biodegradation of polyaromatic hydrocarbon and gene abundance of rrs gene for total bacterial and drsA gene for sulphate reducing bacterial the following treatment conditions were simulated; test with oil, test without oil, test with autoclave killed cells and control. The set up were designated as (SLTo, SLTw/o, SLTk, SHTo, SHTw/o. SHTk) for sulphate enrichment, (NLTo, NLTw/o, NLTk, NHTo. NHTw/o, NHTk) for nitrate enrichment and (MLTo, MLTw/o, MLTk, MHTo, MHTw/o, MHTk) for mixed culture.

About 1g of sample was added to 9ml of resazurin reduced solution, all components in g/L (Sodium thioglycolate: 0.124, Ascorbic acid: 0.1, Resazurine: 4 ml, Marine water: 1L) Then 5ml of the mixture was added to Postgate E (KH_2PO_4: 0.5, NH_4Cl: 1, Na_2SO_4:1, $CaCl_2.6H_2O$: 1, $MgCl_2.6H_2O$: 1.83, Sodium Lactate (50%p/v): 7ml, Sodium acetate: 2, Yeast extracts: 1, Ascorbic acid: 0.1, $FeSO_4.7H_2O$: 0.5, Agar: 1.9, Resazurin: 4ml, Marine water:1L) for sulphate reducing bacteria (SRB) and Total heterotrophic anaerobic bacteria (THANB). While 5ml of the reduced solution was also added to the composition (Glucose: 5, Universal peptone: 4, Yeast extracts: 1, Resazurin: 4 ml, Sodium thioglycolate: 0.124. distilled water: 1L) for nitrate reducing bacteria (NRB) using 50 ml serum bottles and incubated for 30 days. It was then washed twice by centrifuging, re-suspended in 10 ml Bushnell-Haas medium (Magnesium sulphate: 0.2, Calcium Chloride: 0.02, Monopotassium Phosphate: 1, Diammonium Hydrogen phosphate: 1, Potassium Nitrate: 1, Ferric Chloride: 0.05, distilled water: 1L) and transferred to 90 ml Bushnell-Haas medium. About 1 ml of crude oil was added to test with oil. test with autoclave killed cells and control.

2.3. DNA extraction

The total community DNA was extracted from 0.5 g of sediment from the low contaminated point and high contaminated point. Total genomic DNA from enrichment culture grown with Bushnell-Haas medium was extracted using a Fast DNA®SPIN kit (MP Biomedicals, Inc) according to the manufacturer's instructions (Heuer and Smalla, 1997).

2.4. PCR-DGGE

The amplification of specific regions of the gene encoding the 16S rRNA was performed using the primers U968*f* GC (5′ CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAA CG CGA AGA ACC TTA C 3′) and L1401*r* (5′ GCG TGT GTA CAA GAC CC 3′) (Heuer and Smalla, 1997). PCR was performed in a thermocycler (Mastercycler Gradient, Eppendorf, Hamburg, Germany) with the following conditions: initial denaturation at 94°C for 3 min; 35 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min and a final extension at 72°C for 10 min.

The DGGE gels (40-70% urea and formamide) were prepared with a solution of polyacrylamide (6%) in Tris-acetate (pH 8.0). Electrophoresis was performed in Tris-acetate-EDTA buffer at 60°C at a constant voltage of 75 V for 16 hours. The DGGE gels were stained with SYBR Green (Molecular Probes) and visualised using a Storm 860 Imaging System (GE Healthcare).

2.5. Quantitative Real - Time PCR (*rrs* and *dsrA* gene)

Quantitative real time PCR (qPCR) assays were carried out for total bacterial community and sulphate reducing bacteria (SRB) populations. The ABI Prism 7500 (Applied Biosystem) detection system was used following manufacturer's recommendations.

To quantify the gene abundance of rrs gene, a PCR protocol reaction containing 1x Go Taq qPCR Master Mix 2x (Promega), 200 nM of each primer, 0.5 µl BSA (1 mg/ml), 5.5µl H_2O and 2ng DNA was performed. The oligonucleotide primers used were 357F (5'- CTA CGG GRS GCA-3') and 529R (5'-

CGC GGC TGC TGG CAG -3') modified from Muyzer *et al*, (1993). PCR condition consisted of an initial denaturation step at 94^0C for 3 minutes, followed by 30 – 40 cycles of 95^0C for 1 minute, 55^0C for 1 minute and 72^0C for 45seconds.

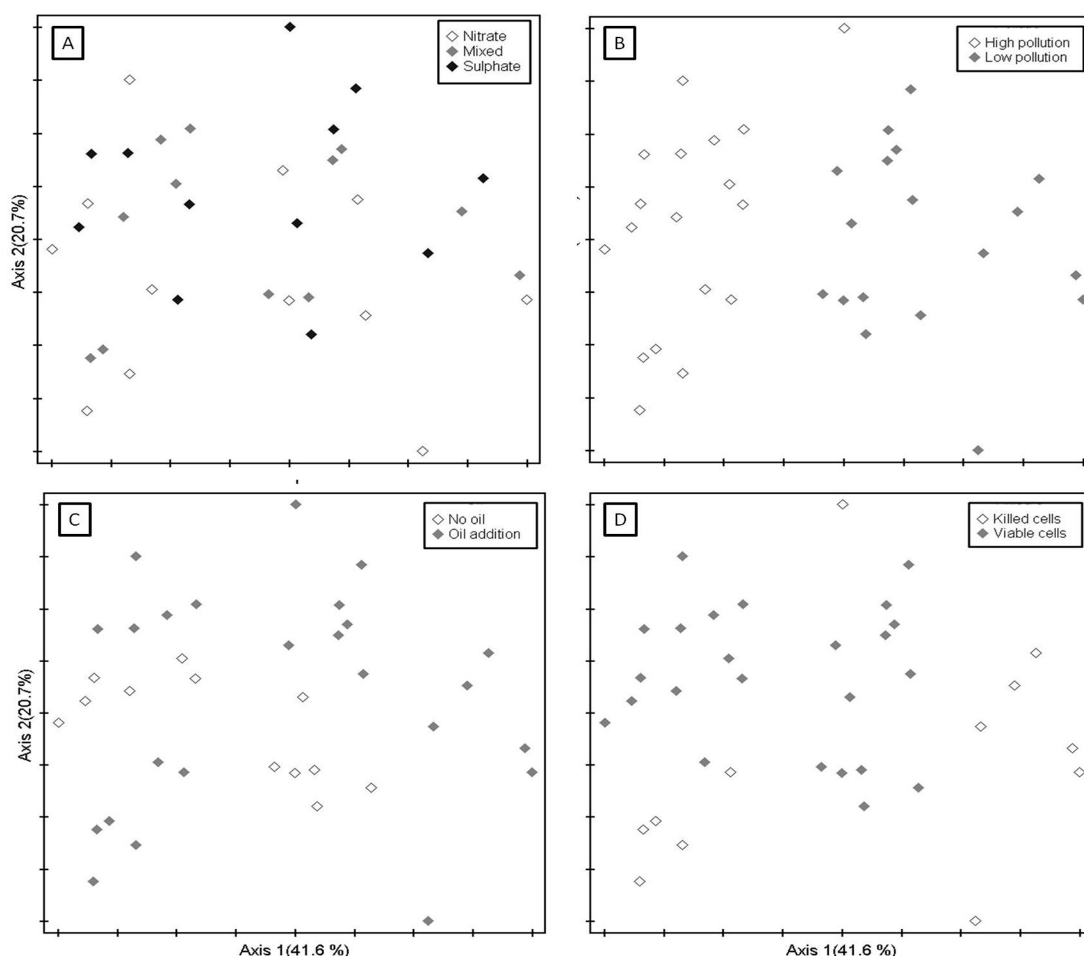

Figure 1**:** NMS ordination of bacterial communities based on DGGE analysis (n=2) according to (A) nutrient enrichment, (B) pollution level, (C) oil addition and (D) cells viability.

A q-PCR was also used to quantify SRB population, using primer targeting the dsr gene that encodes the dissimilatory sulphite reductase enzyme that is present in all sulphate reducers, the ABIPrism 7500 (Applied Biosystems) detection system was used following manufacture's recommendation.

To quantify the *dsrA* gene abundance, we performed a PCR protocol containing 1x GoTaq qPCR MasterMix 2x (Promega), 200 nM of each primer, 0.5 µl BSA (1 mg/ml), H_2O q.s.p. and 2 ng DNA. Oligonucleotide primers used were DSR1F (5'-ACS CAC TGG AAG CAC GGC GG-3') and DSR-R (5'-GTG GMR CCG TGC AKR TTG G-3') (Kondo *et al*., 2004). PCR conditions consisted of an initial denaturation step of 95°C for 5 min, 35 cycles of 95°C for 1 min, 57°C for 1 min and 72°C for 45 s. All samples were used in triplicates and H_2O was used as the negative control. To both reactions (*rrs* and *dsrA* gene) efficiencies and melting curves were determined and analysed using ABIPrism 7500 Detection System (Applied Biosystems).

F. Data analysis

All analyses were performed using the program STATISTICA 7 (StatSoft). All data were tested for normality and homogeneity. Analysis of variance (ANOVA) and the Tukey tests were used to ascertained the significance of the differences between groups. Non-metric scaling (NMS) with PC-ORD statistical package V5 (MjM Software, Gleneden Beach, OR) was used to analyze the difference between bacterial community profiles. The DGGE band profiles were digitalized and inserted into the data matrices by applying Bionumerics v6.0 package (Applied Maths), according to the manufacturer's instructions. The matrices were ordered by NMS (Kruskal, 1964, Mather, 1976) employing a Bray-

Curtis distance matrix. To confirm the existence of the groupings generated by NMS analysis we performed a Multi-Response Permutation Procedure (MRPP) was to tests the hypothesis that no difference exists between two or more groups of entities (Biondini, *et al.*, 1985).

3. Results

Bacterial communities profile

The bacterial communities (figures 1A-B-C-D) changes according to the levels of pollution, oil contamination and cell viability. The factor which had the most effect is the level of pollution.

The *rrs* and *dsrA* gene abundance

The rrs gene copies number for test with oil recorded $3.35 \times 10^{7} – 4.93 \times 10^{11}$ (µg/µl), test without oil ranged from $7.76 \times 10^{6} – 3.79 \times 10^{8}$ (µg/µl), test with autoclaved killed cells ranged from $5.60 \times 10^{2} – 2.48 \times 10^{6}$ (µg/µl) (figures 2 A-B-C). The *dsrA* gene copies number in the test with oil ranged from 6.03×10^{3}- 1.03×10^{9}(µg/µl), test without oil ranged from $8.06 \times 10^{4} – 5.61 \times 10^{6}$ (µg/µl), test with autoclaved killed cells ranged from $6.21 \times 10^{1} – 1.06 \times 10^{5}$ (µg/µl) (figures 3A-B-C).

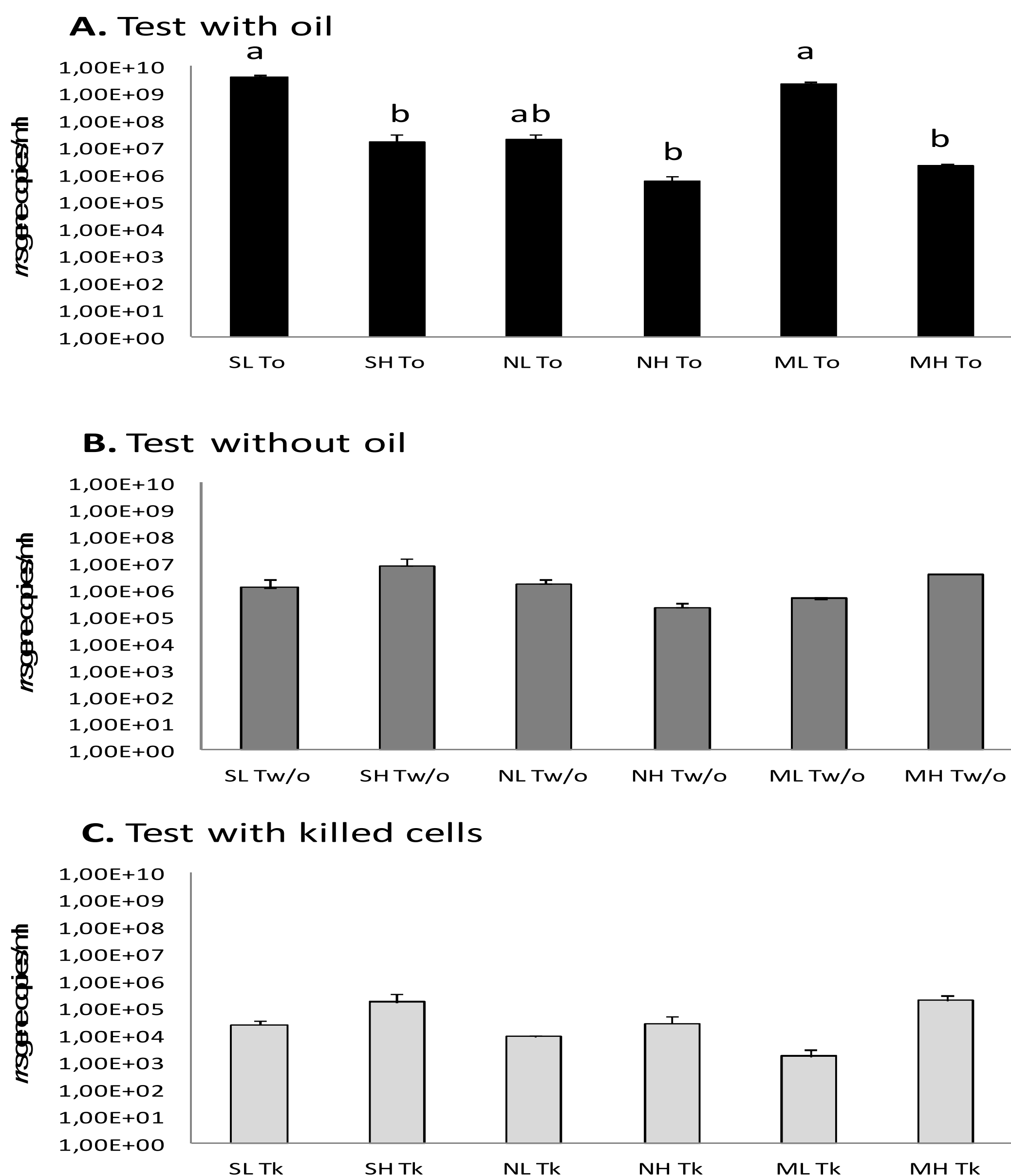

Figure 2. Abundance of rrs gene according to different kinds of test.

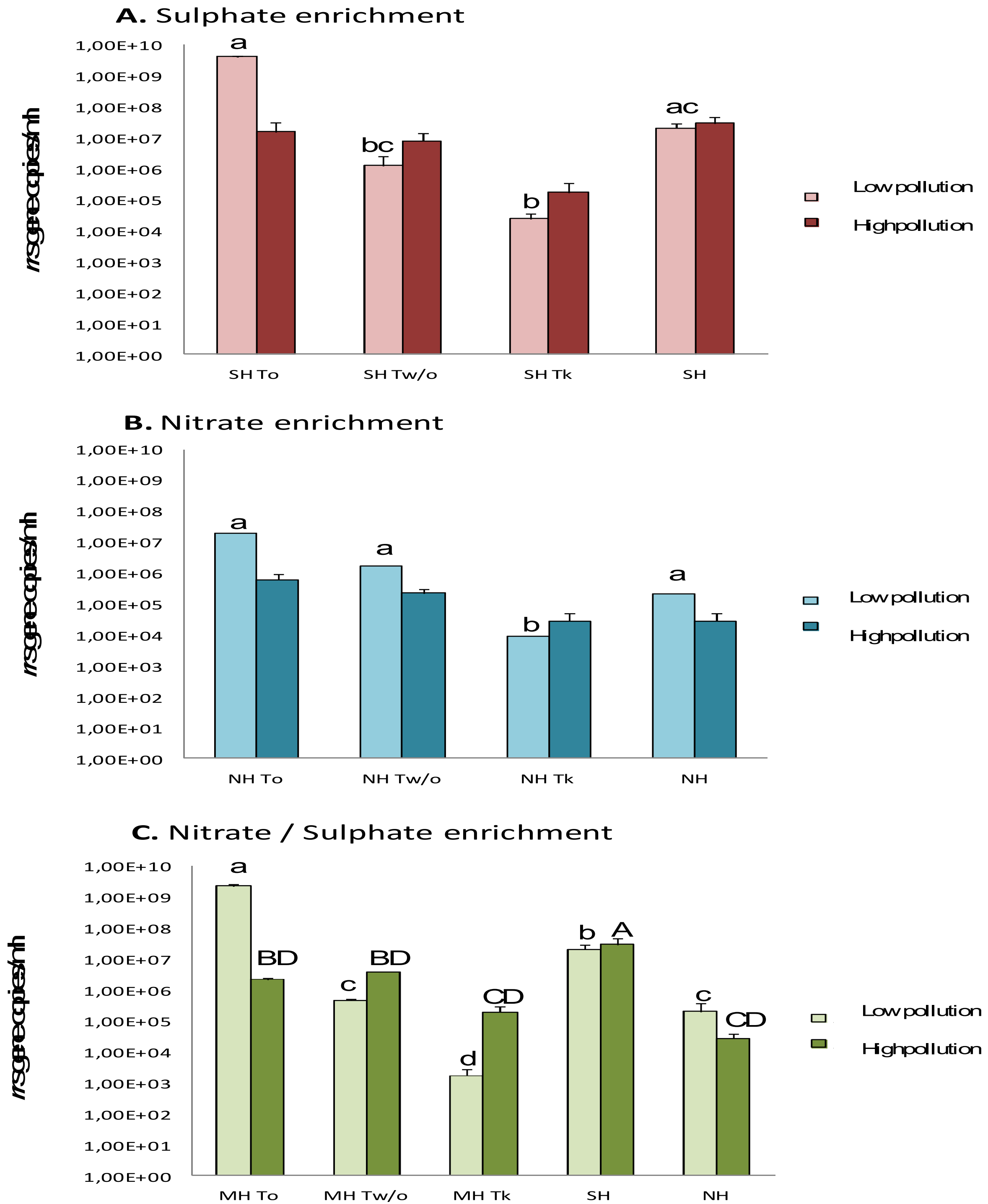

Figure 3. Abundance of *rrs* gene according to different enrichments.

Lowercase letters were used to show significant difference among the treatments of low pollution samples. Capital letters were used to show significant difference among the treatments of high pollution samples.

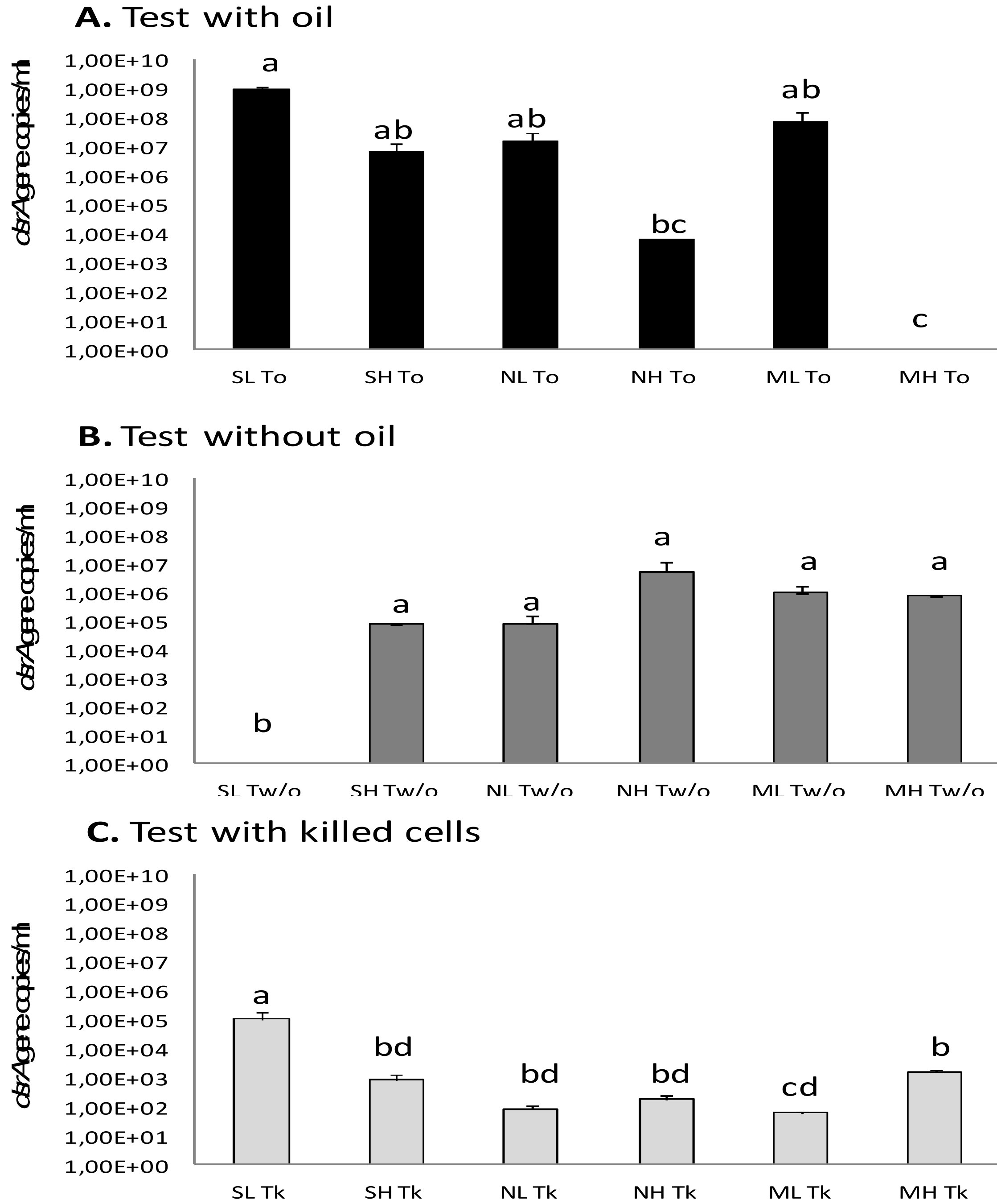

Figure 4. Abundance of *dsrA* gene according to different tests.

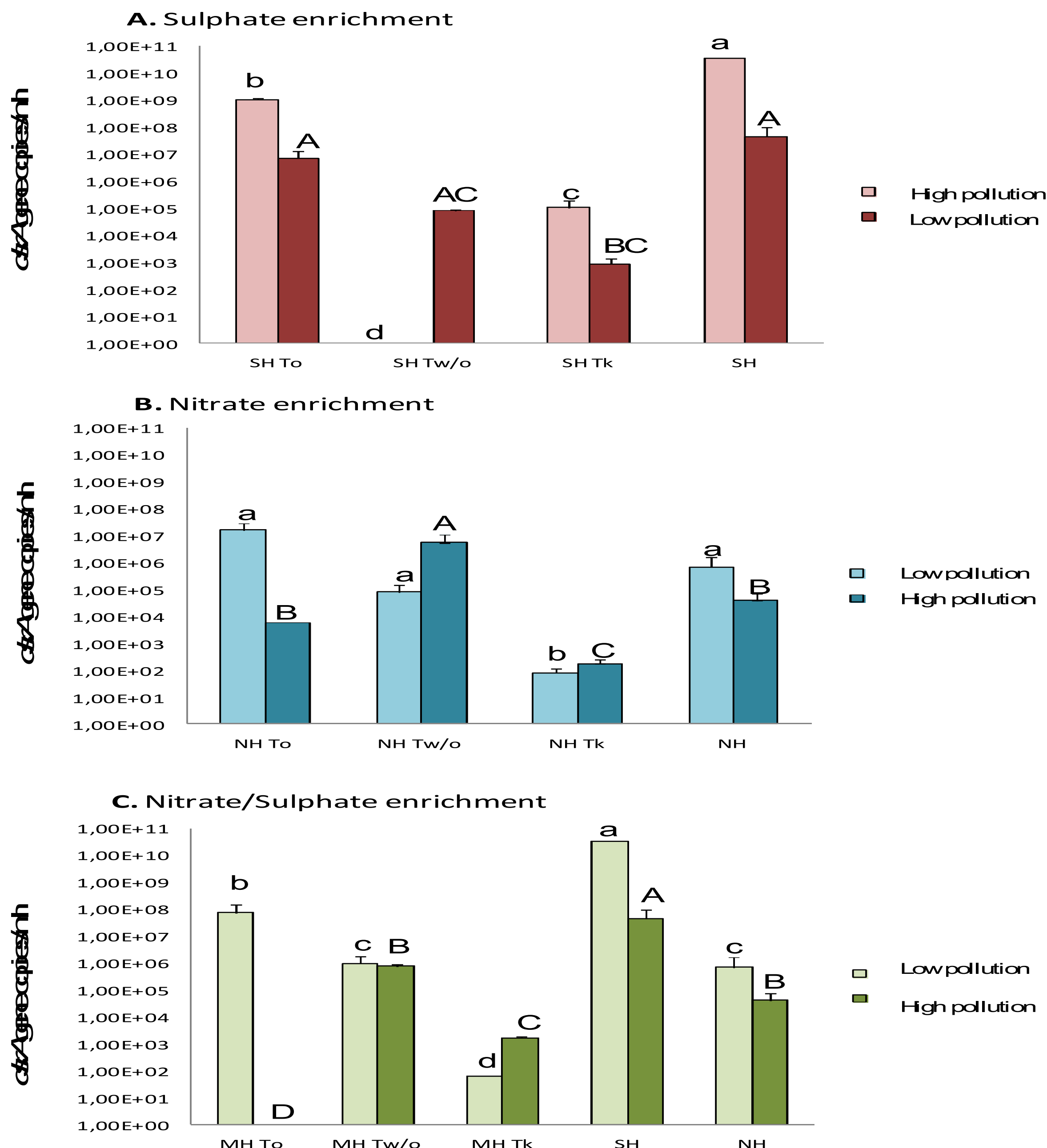

Figure 5. Abundance of *dsrA* gene according to different enrichments.
Lowercase letters were used to show significant difference among the treatments of low pollution samples. Capital letters were used to show significant difference among the treatments of high pollution samples.

4: Discussion

Polyaromatic hydrocarbons had been of great concern to environmental microbiologists, who continue to investigate the ability of microoganisms to utilize and degrade such from the ecosystem. The cultivable species from a soil sample represent only 1% or less of the total population (Atlas, 1981, Osburne *et al.,* 2000). Interesting the uncultivable species are an important diver group and to profile them we require not only standard microbiology laboratory methods but also genetic molecular methods. Analysis of highly conserved prokaryotic 16S rRNA gene sequences is part of a standard method for assessing microbial diversity (Osburne *et al.,* 2000). This approach circumvents the need to culture microorganisms from environmental samples, and it also provides a relatively unbiased sampling of the genetic diversity of those environments. The

research aimed at charactering microorganisms that are prevalent under anaerobic conditions, verifying anaerobic utilization of crude oil containing high level of PAHs and determining the role of non-oxygen terminal electron acceptors in the metabolism of crude oil.

DGGE gel banding patterns of the various microcosms were evaluated by BioNumeric cluster analysis based on Pearson's correlation coefficient using Gel Compar II ver 4.0 (Applied Maths, Sint – Martein-latern, Belgium, p 0.05) to compare the similarities of the treatments. Treatment setup with oil clustered into two major groups this is based on the sampling points; low contaminated points and high contaminated points. The treatment showed very distinct dark bands, having received 0.1% crude oil; the indigenous microorganisms are ones that had previous exposure to crude oil so can better adapt to the presence of oil (Lee and Levy, 1987; Tagger *et al*., 1983). Several factors affected the bacterial communities present at the site. (Fig. 1B-C-D). The bacterial communities changed according to levels of pollution (Figure 1B), oil contamination (Figure 1C) and cells viability (Figure 1D)) (MRPP tests; 1B = 0.25; 1C = 0.07; 1D = 0.11; $p \leq 0.02$). The major impact which affected the communities profile is the previous exposure of the community to oil as shown in figure 1B. However, there was no observable effect of the nutrient enrichment (Figure 1A) (MRPP tests; A = 0.18 $p < 0.01$).

There were significant different in the abundant of rrs and drsA gene in samples from different test conditions: low or high pollution levels, nutrients enrichment, test with oil or without oil and cell viability. The test with oil had statistical significant effect on rrs gene abundance ($p < 0.01$). It is possible that the sulphate enrichment stimulate the bacterial grown on low contaminated sediments (figure 2A). When the cell were killed (figure 2C) and/or in the absence of oil the number of bacteria cells did not increase or decrease showing clearly that the cells are dead.

All tests setups had effect on dsrA gene abundance at ($p < 0.01$). The result indicated that test with oil (Figure 4A) had sample with low pollution enriched with sulphate having higher abundance of sulphate reducing bacteria than high pollution points; this is noteworthy indicating utilization of the contaminants, nutrients and/or non-oxygen terminal electron acceptors by the indigenous microorganisms (Margensin *et al*., 2003). The dsrA encoding gene was not detected in nitrate and sulphate mix-enriched sample from high polluted points despite physiochemical analysis of the site showing the presence of sulphate at all points; similar result had occurred with other workers (Nolvak *et al*., 2012). Samples with low pollution that were enriched with sulphate from the test without oil (figure 4B) did not reveal the dsrA gene, probably the sulphate reducing bacteria were just stimulated with sulphate in the presence of oil. The test with killed cells (figure 4C) had samples with low pollution that were enriched with sulphate reducing bacteria having a higher abundance of sulphate reducing bacteria however, when nitrate and sulphate were added to polluted samples the abundance of these communities decreased.

The gene copy number of the autoclaved killed treatment indicates that treatment with oil, having an increase in gene copy number, provides a source of energy and substrate which microorganisms present are utilizing, leading to increase in their biomass and the gene copy number.

V. Conclusion

Molecular biology methods are being applied to fundamental anaerobic biodegradation questions to help clarify gross observations and predict degradation potentials for example there is the 16S rRNA gene clone libraries of naphthalene- degrading consortia; real-time PCR targeting of specific phyla known to degrade aromatic and denaturing gradient gel electrophoresis (DGGE) to characterize hydrocarbon- degrading consortia. The DGGE fingerprint of bacterial 16S rRNA gene fragment obtained from extracted DNA genome of sample from Ejamah – Ebubu oil spill site presents distinct bands representing different microbial communities. The community profiles showed no dominant bands and low similarity to each other indicating heterogeneous conditions for catabolic microbes at the site.

Enumeration of 16S rRNA genes gives background information about the total microbial community present at the study site and also enables normalization of functional gene numbers as compared with the entire community. It is known that contamination can boost the growth of indigenous catabolic microbes. The sulphate reductase gene drsA is expressed in high copy numbers in the presence of hydrocarbon under anaerobic conditions. Both sulphate and nitrate serve as terminal electron acceptors.

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