# Determination of the Bacterial Community Structure in a Crude oil-inundated Tropical Soil using Next Generation Sequencing Technique

**ABSTRACT**

**Aim:** To identify the community composition of a crude oil impacted soil in Gbarain kingdom of Bayelsa State, Niegria

**Study design:** A crude oil-impacted soil sample (0-10 cm depth) was collected from Etelebuo-Ogboloma, flow station in Yenagoa L.G.A. of Bayelsa State and taken to the laboratory for various microbiological analyses.

**Place and duration of study:** The study was carried out at the Environmental Microbiology Laboratory, University of Port Harcourt, Nigeria, for 35days.

**Methodology**: Cultural morphology of the isolates was studied based on their physical appearance such as colour, shape, size, elevation and margin. While catalase test, oxidase test, indole test, motility test, Methyl-Red Voges-Proskauer's (MRVP) test and citrate utilization test were used for the biochemical identification of the isolates. DNA Extraction from crude oil polluted soil sample was performed using ZYMO soil DNA extraction Kit (Model D 6001, Zymo Resaerch, USA) following the manufacturer's instructions. DNA sequencing was performed by the Next Generation Sequencing Technique to determine the nucleotide sequence of all microorganisms present in the soil sample using sequencing primer -16S: 27F: 5'-GAGTTTGATCCTGGCTCAG-3' and 518R: 5'-ATTACCGCGGCTGCTGG-3'.

**Results:** Five different bacterial genera were isolated and identified using the cultural techniques, and they include *Acetobacter* sp*., Pseudomonas* sp., *Arthrobacter* sp., *Bacillus* sp. and *Micrococcus* sp. However, the molecular characterization revealed that the soil was mainly dominated by the Alphaproteobacteria (54.64%), followed by the  Actinobacteria (9.67%), Gammaproteobacteria (6.55%) , Betaproteobacteria (2.27%) and Bacilli (0.95%) as well as Clostridia (0.34%); as the most dominant class of bacteria. A total of one hundred and four (104) diverse bacterial species were identified, in the overall metagenomics.

**Conclusion:** This study has shown the bacterial community composition of the crude oil polluted soil obtained from Gbarain Kingdom. These findings are fundamental in understanding the biological fate of crude oil in these oil rich regions.

37    *Keywords*: Metagenomics*; bacterial* composition*;* Gbarain Kingdom*; crude oil.*

38    1.0 INTRODUCTION

39    Crude oil spill has remained a major source of environmental pollution in the Niger Delta area of
40    Nigeria. These spills impact on the environment at varying degrees, depending on the volume spilled
41    and physicochemical properties of the oil. The deleterious effects of crude oil contamination on the
42    flora and fauna of the impacted mediausually result in biodiversity loss, as exposure to crude oil
43    fractions reduces bacterial population inthe affected media, leading to loss in species diversity [1].

44    Bioremediation has remained an effective impact mitigation strategy (alongside other physical and
45    chemical methods of site remediation).   The process of bioremediation involves the interplay of
46    various factors meant to achieve an efficient breakdown of these highly complex crude oil products in
47    the environment, in which case the synergistic activities of microbial consortium are required; where
48    hydrocarbonoclastic and hydrocarbon degrading microorganisms work together [2]. *Bacillus,*
49    *Pseudomonas, Rhodococcus, Arthrobacter and Corynebacterium* spp. are a group or bacterial
50    consortium having the ability to degrade hydrocarbons [3]. However, for bioremediation to be very
51    effective, the involvement of well adapted species to the prevailing environmental/site conditions is
52    very necessary with the following hydrocarbonoclastic bacteria;*Arthrobacter, Flavobacterium*,
53    *Sphingomonas* (a novel *Pseudomonas* sp), and *Pseudomonas* spp.,well known for their adaptation to
54    petroleum contaminated environments [4]. The use of well adapted species helps in facilitating a
55    better and faster rate of hydrocarbon biodegradation in the contaminated site. However, not all
56    microorganisms found in the environment can degrade a particular hydrocarbon due to genetic
57    factors. Various authors have reported the efficacy of these adapted species. For instance, John and
58    Okpokwasili (2012) [5] conducted a plasmid curing experiment and established that *Nitrosomonas*
59    *and Nitrobacter* degraded crude oil by the aid of crude oil degrading plasmid. Also, *Staphylococcus*
60    sp. has been shown to have the ability to utilize drilling fluid base oil and is therefore, a good agent for
61    the remediation oil from drilling fluid [6]. Fungal species such as *Candida* sp. and
62    *Schizosaccharomyces pombe* have been also found to be involved in the biodegradation of
63    hydrocarbon [7; 8].

64    Recent advancement in bioremediation studies relies on molecular methods. These methods are
65    useful in characterizing microorganisms associated with biodegradation of crude oil contaminated
66    sites or media. The advantages of these molecular techniques are owed to their ability to identify
67    autochthonous bacterial group *in situ* and also provide a rapid as well as efficient non-culture
68    dependent methods of studying the bacterial composition (structure and function) of a site undergoing
69    bioremediation [9].

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71    Several molecular techniques are available to study microbial communities in nature, especially the
72    non-culturable ones which have not been identified in the laboratory via cultural approach.
73    Comparative studies and analysis involving culture dependent and molecular metagonomic approach
74    have revealed that only about 1% of the total microorganisms are amenable to culture [10]. Currently,

75   nucleic acids, lipids, and proteins are harnessed to provide primary information that aid in the
76   identification of these uncultured bacterial groups. Whole genomes or selected genes analysis which
77   includes 16S for prokaryotes and 18S rRNA for eukaryotes are molecular techniques that are non-
78   culture dependent. Analytical studies of these genomes help to group microorganisms into three (3)
79   primary categories: two prokaryotic groups such as bacteria and archaea as well as one eukaryotic
80   group (eukarya) [11]. The development of techniques to characterise or identify functional diversity as
81   well as phylogenic differences has been of enormous importance in microbiology. Approaches such
82   as partial community analysis and whole community analysis are methods used to probe into bacterial
83   genetic properties which help in identifying microbial population based on their structure as well as
84   functional attributes.Modern molecular techniques have made way for an increased understanding of
85   bacterial diversity as well as functionality during crude oil degradation.
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87   Sequence-based and function-based sequencing are two conventional paths metagenomic screening
88   has followed. The analysis of genome sequence data that has been recovered from the environment
89   is motivated by many objectives, which include the establishment of gene inventories and natural
90   product discovery [12].
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92   Various researchers have used different molecular techniques to characterise and identify functional
93   genes in hydrocarbon degradation. However, the study of microbial diversity is somewhat complex
94   and requires several approaches, used in combination. This will provide more useful information
95   regarding diversity of the organisms. Owing to the paucity of information regarding the bacterial
96   community composition of hydrocarbon impacted sites in Gbarain kingdom, Bayelsa State, Nigeria,
97   this study was therefore carried out to identify the community composition using metagenomics and
98   culture dependent techniques. Data from this investigation could provide useful information for
99   potential bioengineering of the species through targeting specific genes of interest for efficient
100  bioremediation of crude oil contaminated sites.
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102  **2.0  MATERIALS AND METHODS**

103  **2.1 Soil Sample Source and Collection**

104  A crude oil-impacted soil (0–10 cm depth) was collected from Etelebuo-Ogboloma, flow station in
105  Yenagoa L.G.A. of Bayelsa State using an Elderman auger into a Ziploc bag. Thereafter, the samples
106  were kept in an ice box and immediately transported to the laboratory for microbiological analyses.

107  **2.2 Culture-dependent Isolation, characterization and identification of hydrocarbon utilizing**
108            **bacteria**

109  **2.2.1** Method of isolation of bacteria from soil

110  The soil sample was collected as explained in 2.1 above and diluted using 10-fold serial dilution,
111  which was carried out to a dilution of $10^{-5}$ by weighing 1 g of the soil sample into a sterile test tube
112  containing 9ml of sterile physiological saline.
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114    The heterotrophic bacteria were isolated by spreading 0.1 ml of the diluted soil sample on nutrient
115    agar plates (Petri dishes) and incubated in an incubator at $25^0C$ for 24hours. In the same manner, the
116    hydrocarbon utilizing bacteria were isolated by culturing the diluted soil samples on mineral salts agar.
117    A Whatman's filter paper was saturated with crude oil and placed on the lid of each glass Petri dish
118    using sterile forceps. The crude oil served as the sole source of hydrocarbon (that is carbon and
119    energy source for the hydrocarbon utilizers). The inoculated mineral salts agar plates were inverted
120    and placed over the lid containing the saturated filter paper, and incubated at room temperature for
121    seven (7) days.

**2.2.2 Preparation of pure bacteria cultures**

123    Representative colonies of the different morphological types that appeared on the plates after
124    incubation were carefully picked with a sterile inoculating loop and sub-cultured to obtain pure
125    cultures. This was done by streaking aseptically, onto freshly prepared nutrient agar plates. After
126    incubation the purity of the isolates were checked by subjecting them to the Gram staining technique.

**2.2.2 Cultural morphologyand biochemical characterization of isolates**

128    Cultural morphology of the isolates was studied based on their physical appearance such as colour,
129    shape, size, elevation and margin. Isolated colonies were further purified by sub-culturing and
130    identified using biochemical tests and microscopy [13].

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**2.3 Molecular Analyses**

**2.3.1 DNA extraction from soil sample**

134    DNA extraction from crude oil polluted soil sample was performed using ZYMO soil DNA extraction Kit
135    (Model D 6001, Zymo Research, USA) following the manufacturer's instructions. According to this
136    method, genomic DNA was extracted by weighing out 0.25 grams of soil sample using an analytical
137    balance (Ohaus, Germany). The sample was then added into a ZR Bashing Bead ™ lyses tube
138    followed by the addition of 750 µl lyses solution to the tube.  The content of the 2 ml tube was
139    disrupted by mixing in a vortex mixer at maximum speed for 5 minutes. The ZR Bashing Bead ™
140    lyses tube was centrifuged in a micro centrifuge at ≤ 10,000 x $g$ for 1 minute. After this, 400 µl of the
141    filtrate was added to a Zymo-Spin ™ IV spin filter in a collection tube and centrifuged at 7,000 rpm for
142    1 minute. This was followed by the addition of 1,200 µl of DNA binding buffer to the filtrate in the
143    collection tub after which 800 µl of the mixture from above was added to a Zymo-Spin ™ IIC column
144    in a collection tube and centrifuged at 10,000 x $g$ for 1 minute. Flow through from the collection tube
145    was discarded and this particular step was repeated with the remaining filtrate. This was followed by
146    the addition of 200 µl of DNA pre-wash buffer into the Zymo-Spin ™ IIC Column in a new collection

147    tube and centrifuged at 10,000 x *g* for 1 minute, and then 500 µl DNA wash buffer was added to the

148    Zymo-Spin ™ IIC column and centrifuge at 10,000 x *g* for 1 minute. The Zymo-Spin ™ IIC column

149    was transferred into a clean 1.5 ml micro centrifuge tube and 100 µl DNA elution buffer was directly

150    added to the column matrix.  This was centrifuged at 10,000 x *g* for 30 seconds to elude the DNA.

151    The eluded DNA was transferred into a filter unit of Zymo-Spin ™ IV-HRC Spin Filter in a clean 1.5 ml

152    micro centrifuge tube and centrifuged at exactly 8,000 x *g* for 1 minute.  The filtered DNA was then

153    used for PCR and DNA sequencing.

154    **2.3.2 DNA Sequencing**

155    All sequencing analysis was carried out at Inqaba Biotechnical Pty Ltd, South Africa. DNA sequencing

156    was performed by Next Generation Sequencing Technique to determine the nucleotide sequence of

157    all microorganisms present in the soil sample using sequencing primer -16S: 27F: 5'-

158    GAGTTTGATCCTGGCTCAG-3' and 518R: 5'- ATTACCGCGGCTGCTGG-3'. The sequencing was

159    carried out using an automated PCR cycle-Genome Sequencer™ MiSeq (Illumina). Analysis and

160    alignment was performed using Vecton NTI suit 9 (InforMax, Inc.). Overall bioinformatics analysis was

161    done using NCBI-BLAST-2.2.24 and CLC bio Genomics workbench v7.5.1, for every sample set:

162    every read was BLASTed and the result file saved.  The top 5 hits for every BLAST result (that is,

163    species name) was counted and a record was kept of how many times each species appeared as a

164    hit. The number in the last column is the number of times a read hit/matched that species. The

165    frequency (i.e count/total number of reads) and absolute count of each species were reported and

166    used to name the specific organism.

167    Sequencing Codons were finally saved in fasta format and output of results showing the kingdom,

168    phylum, class, order, family, genus, species present in the sample given. The percentage of each

169    variable was also enumerated. The names of species of culturable and non culturable organisms

170    present in the sample, the corresponding accession number in NCBI data base, the number of hits

171    and the e score were also given.

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173    **3.0  RESULT AND DISCUSSION**

174    **3.1 Colonial Morphology of Isolates**

175    The cultural morphology was employed in the identification of the various isolates. The cultural cum

176    morphological characteristics examined included colour, shape, size, elevation and margin (Table 1).

177    **Table 1: Colonial Morphology of bacterial isolates obtained from crude oil contaminated soil.**

| S/N | Isolate code | Colour | Shape | Size (mm) | Elevation | Margin |
|-----|-----|-----|-----|-----|-----|-----|
| 1 | A | Brown | Circular | 3.0 | Flat | Irregular |
| 2 | B | Cream | Circular | 3.5 | Flat | Entire |
| 3 | C | Cream | Circular | 3.2 | Flat | Entire |
| 4 | D | Yellow | Circular | 1.0 | Flat | Entire |
| 5 | E | Cream | Circular | 1.4 | Convex | Entire |
| 6 | F | Brown | Circular | 5.0 | Flat | Regular |
| 7 | G | Yellow | Circular | 1.0 | Flat | Entire |
| 8 | H | Cream | Circular | 1.5 | Convex | Entire |
| 9 | I | Yellow | Circular | 1.0 | Flat | Entire |
| 10 | J | Cream | Circular | 3.5 | Flat | Entire |
| 11 | K | Cream | Circular | 1.2 | Convex | Entire |
| 12 | L | Yellow | Circular | 1.0 | Flat | Entire |
| 13 | M | Brown | Circular | 3.0 | Flat | Irregular |
| 14 | N | Cream | Circular | 1.2 | Convex | Entire |
| 15 | O | Cream | Circular | 4.0 | Flat | Entire |
| 16 | P | Brown | Circular | 3.0 | Flat | Irregular |
| 17 | Q | Brown | Circular | 4.5 | Flat | Regular |
| 18 | R | Cream | Circular | 3.5 | Flat | Entire |
| 19 | S | Cream | Circular | 3.2 | Flat | Entire |
| 20 | T | Yellow | Circular | 1.0 | Flat | Entire |
| 21 | U | Cream | Circular | 3.5 | Flat | Entire |
| 22 | V | Yellow | Circular | 1.0 | Flat | Entire |
| 23 | W | Cream | Circular | 1.2 | Convex | Entire |
| 24 | Y | Cream | Circular | 3.0 | Flat | Entire |

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191 **3.2 Culture based identification of Isolates**

192 Five different bacterial genera (*Acetobacter* sp*., Pseudomonas* sp., *Arthrobacter* sp., *Bacillus* sp. and
193 *Micrococcus* sp.) were isolated and identified (Table 2). Most of the organisms isolated were gram-
194 negative rods except *Bacillus* sp. and *Micrococcus* sp. that were gram-positive rod and cocci,
195 respectively.

196 As shown in Table 2, all the organisms isolated were indole negative and also showed an inability to
197 produce hydrogen sulphide ($H_2S$) and gas as well as an inability to ferment lactose. *Bacillus* sp. was
198 the only spore former isolated. While the rest isolates were urease negative, only *Acetobacter* sp. was
199 urease positive.

200 **Table 2: Morphological and biochemical characteristics of bacteria isolated from crude oil**
201 **contaminated soil**

| S/N | Isolate Code | Gram reaction | Cell morphology | Oxidase | Citrate utilization | Methyl Red | Voges Proskauer | Catalase | Indole | Urease | Motility | Endospore | $H_2S$ production | Spore test | Gas production | Glucose | Lactose | Maltose | Sucrose | Mannitol | Probable organism |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | A | - | Rod | - | - | - | - | + | - | - | + | - | - | - | - | A | - | - | + | - | *Arthrobacter* sp. |
| 2 | B | + | Rod | - | - | - | + | + | - | - | + | + | - | + | - | AG | - | - | - | - | *Bacillus sp.* |
| 3 | C | + | Rod | - | - | - | + | + | - | - | + | + | - | + | - | AG | - | - | - | - | *Bacillus sp.* |
| 4 | D | - | Rod | + | - | + | + | - | - | - | + | - | - | - | - | AG | - | - | - | - | *Pseudomonas* sp. |
| 5 | E | + | Cocci | + | + | + | - | + | - | - | - | - | - | - | - | A | - | - | + | - | *Micrococcus* sp. |
| 6 | F | - | Rod | - | + | - | - | - | - | + | + | - | - | - | - | AG | - | + | - | - | *Acetobacter* sp. |
| 7 | G | - | Rod | + | - | + | + | - | - | - | + | - | - | - | - | AG | - | - | - | - | *Pseudomonas* sp. |
| 8 | H | + | Cocci | + | + | + | - | + | - | - | - | - | - | - | - | A | - | - | + | - | *Micrococcus* sp. |
| 9 | I | - | Rod | + | - | + | + | - | - | - | + | - | - | - | - | AG | - | - | - | - | *Pseudomonas* sp. |
| 10 | J | + | Rod | - | - | - | + | + | - | - | + | + | - | + | - | AG | - | - | - | - | *Bacillus sp.* |
| 11 | K | + | Cocci | + | + | + | - | + | - | - | - | - | - | - | - | A | - | - | + | - | *Micrococcus* sp. |
| 12 | L | - | Rod | + | - | + | + | - | - | - | + | - | - | - | - | AG | - | - | - | - | *Pseudomonas* sp. |
| 13 | M | - | Rod | - | - | - | - | + | - | - | + | - | - | - | - | A | - | - | + | - | *Arthrobacter* sp. |
| 14 | N | + | Cocci | + | + | + | - | + | - | - | - | - | - | - | - | A | - | - | + | - | *Micrococcus* sp. |
| 15 | O | + | Rod | - | - | - | + | + | - | - | + | + | - | + | - | AG | - | - | - | - | *Bacillus sp.* |
| 16 | P | - | Rod | - | - | - | - | + | - | - | + | - | - | - | - | A | - | - | + | - | *Arthrobacter* sp. |
| 17 | Q | - | Rod | - | + | - | - | - | - | + | + | - | - | - | - | AG | - | + | - | - | *Acetobacter* sp. |
| 18 | R | + | Rod | - | - | - | + | + | - | - | + | + | - | + | - | AG | - | - | - | - | *Bacillus sp.* |
| 19 | S | - | Rod | - | - | - | + | + | - | - | + | + | - | + | - | AG | - | - | - | - | *Bacillus sp.* |
| 20 | T | - | Rod | + | - | + | + | - | - | - | + | - | - | - | - | AG | - | - | - | - | *Pseudomonas* sp. |
| 21 | U | + | Rod | - | - | - | + | + | - | - | + | + | - | + | - | AG | - | - | - | - | *Bacillus sp.* |
| 22 | V | - | Rod | + | - | + | + | - | - | - | + | - | - | - | - | AG | - | - | - | - | *Pseudomonas* sp. |
| 23 | W | + | Cocci | + | + | + | - | + | - | - | - | - | - | - | - | A | - | - | + | - | *Micrococcus* sp. |
| 24 | Y | + | Rod | - | - | - | + | + | - | - | + | + | - | + | - | AG | - | - | - | - | *Bacillus sp* |

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203 These organisms have been reported by several researchers to be involved in the bioremediation of
204 petroleum hydrocarbons [14; 15]. Eziuzor and Okpokwasili (2009) [16] studied bioremediation of

205    crude oil polluted mangrove soil in Port Harcourt, using NPK as source of limiting nutrients and
206    documented *Acetobacter* sp*., Pseudomonas* sp., *Arthrobacter* sp., *Bacillus* sp., *Alcaligenes* sp*.,*
207    *Flavobacterium* sp., *Citrobacter* sp., *Vibrio* sp. and *Corynebacterium* sp. as the hydrocarbon utilizing
208    population. Their reports were in consonance with the hydrocarbon utilizing bacterial population
209    isolated in this study, from Etelebou- Ogboloma Community (Gbarain Kingdom), an oil rich area in the
210    Niger Delta region.

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212    **3.2 Molecular identification of autochthonous bacterial group**

213    Metagenomics approach was used for *in situ* identification of bacterial population in the hydrocarbon
214    impacted soil sample. The 16s bacterial metagenomic report revealed the taxonomic classification as
215    follows:

216    I.    Kingdom Classification: The report revealed bacteria as the predominant group (99.87%) and
217          protozoa was the next group with 0.09%, followed by fungi and archaea group with 0.02%
218          each.
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220    II.   Phylum Classification: The result showed that the soil had Proteobacteria (63.5%) as the
221          predominant group. This was followed by Actinobacteria (9.67%), Firmicutes (1.29%),
222          Chlamydiae (0.07%) and Ciliophora (0.05%). However, 25.28% of the taxa that belonged to
223          this phylum where unknown whereas others (Bacteroidetes, Chloroflexi, Tracheophyta,
224          Fusobacteria, Cyanobacteria) made up 0.11% of this phylum classification. Also, groups not
225          assigned had 0.04% (Fig. 1).
226    III.  Class Classification: The class taxonomy shows the prevalence of the different groups in the
227          following order:  Alphaproteobacteria (54.64%) > Unknown (25.28%) > Actinobacteria (9.67%)
228          > Gammaproteobacteria (6.55%) > Betaproteobacteria (2.27%) > Bacilli (0.95%) > Clostridia
229          (0.34%) while the rest of the groups (others) made up 0.31% of the population (Fig 2).
230    IV.   Order Classification: Rhizobiales (54.29%) were found to be the more dominant group. While
231          Actinomycetales, Pseudomonadales, Bacillales, Xanthomonadales, Hydrogenophilales as
232          well as other members of the group, were 9.61%, 1.33%, 0.88%, 0.85%, 0.62% and 1.8% of
233          the population, respectively, the unknown group had 30.63% (Fig. 3).
234    V.    Family Classification: The metagenomic analysis of the polluted soil showed that the family
235          level taxonomical groups were mostly of the Methylobacteriaceae family (45.93%). Those that
236          belonged to the Mycobacteriaceae family were8.92% in population whereas, the
237          Bradyrhizobiaceae family made up 8.06% of the population. The family of
238          Pseudomonadaceae accounted for 1.33% of this taxonomical classification.
239          Hydrogenophilaceae and Xanthomonadaceae constituted0.88% and 0.62% of the population,
240          respectively while the unknown group was 30.67% and others made up 3.58% of the
241          population (Fig. 4).

242    VI.    BLAST output result: Data from the basic local alignment search tool (BLAST) showed (in
243           Table 3) the most dominant bacteria to be *Methylobacterium* sp. which constituted 45.90% of
244           the population. The uncultured bacterium was 24.63% and *Mycobacterium* sp., 8.86%. Also,
245           *Bradyrhizobium* sp. was 7.99% whereas, uncultured gamma, *Pseudomonas aeruginosa* and
246           betaproteobacterium accounted for 3.96, 1.29 and 1.05% of the bacterial population
247           respectively.
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249    VII.   Phylogenetic relationships: A phylogenetic tree based on the sequence analysis obtained
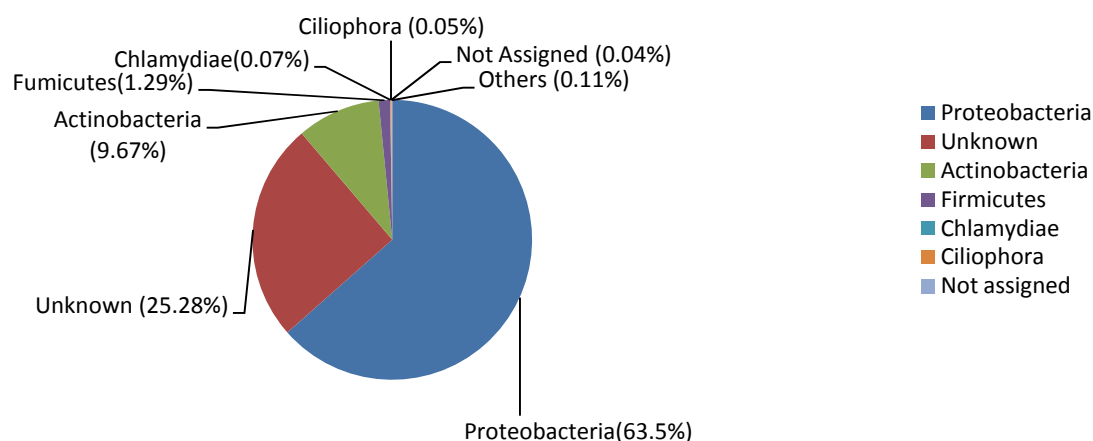250           from the soil metagenomics is shown in Figs. 5a and b.
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254    Fig. 1: Top phylum classification of bacterial isolates obtained from the crude oil contaminated soil.
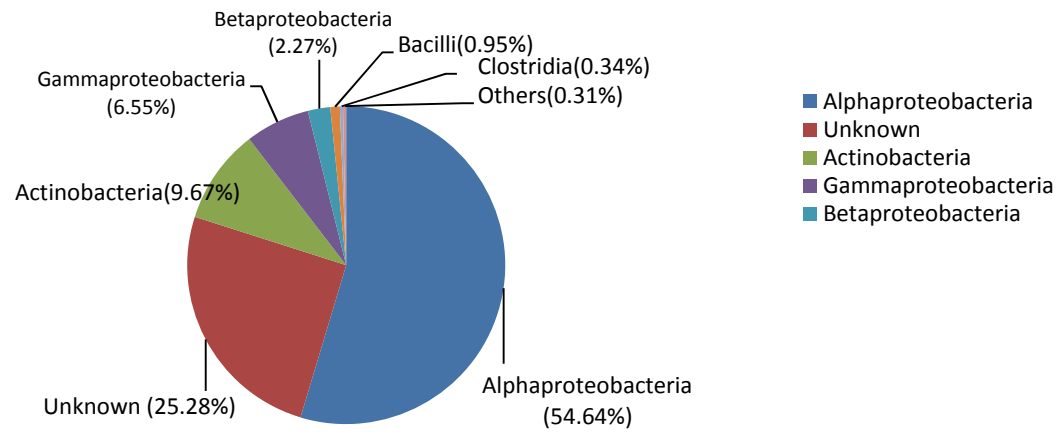
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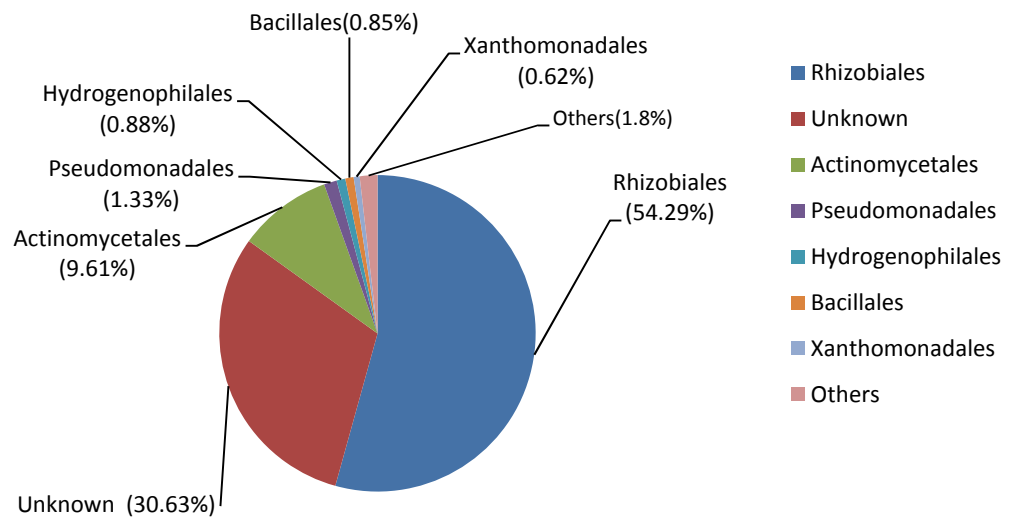257    Fig. 2: Top class classification of bacterial isolates obtained from the crude oil contaminated soil

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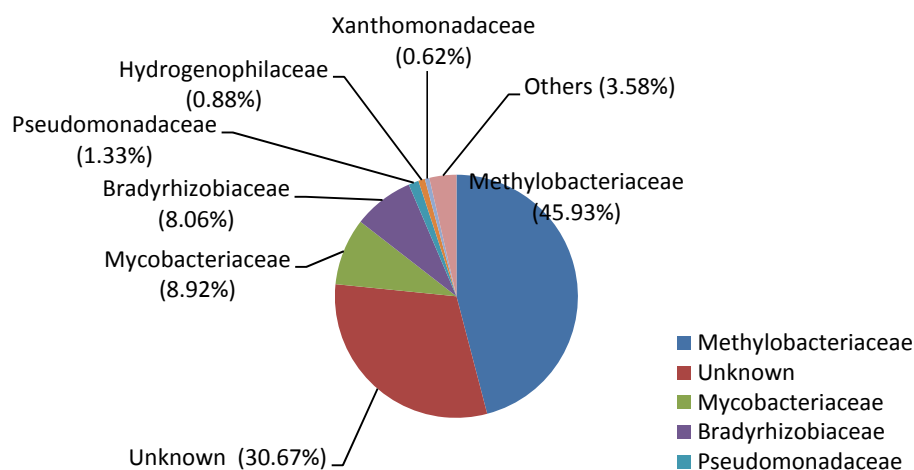260    Fig. 3: Top order classification of bacterial isolates obtained from the crude oil contaminated soil.

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Xanthomonadaceae
(0.62%)

Hydrogenophilaceae
(0.88%)

Pseudomonadaceae
(1.33%)

Bradyrhizobiaceae
(8.06%)

Mycobacteriaceae
(8.92%)

Others (3.58%)

Methylobacteriaceae
(45.93%)

Unknown (30.67%)

■ Methylobacteriaceae
■ Unknown
■ Mycobacteriaceae
■ Bradyrhizobiaceae
■ Pseudomonadaceae

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264     Fig. 4: Top family classification of bacterial isolates obtained from the crude oil contaminated soil.

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266            Table 3: BLAST output results
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| BLAST HIT | Read | Count % |
|---|---|---|
| 1. *Methylobacterium* sp. | 5857 | 45.90 |
| 2. uncultured bacterium | 3143 | 24.63 |
| 3. *Mycobacterium* sp. | 1130 | 8.86 |
| 4. *Bradyrhizobium* sp. | 1020 | 7.99 |
| 5. uncultured gamma | 505 | 3.96 |
| 6. *Pseudomonas* aeruginosa | 165 | 1.29 |
| 7. beta *proteobacterium* | 134 | 1.05 |
| 8. *Thiobacillus prosperus* | 106 | 0.83 |
| 9. *Stenotrophomonas maltophilia* | 79 | 0.62 |
| 10. *Enterobacter* sp. | 69 | 0.54 |
| 11. *Brachybacterium paraconglomeratum* | 54 | 0.42 |
| 12. bacterium btn | 46 | 0.36 |
| 13. *Alicyclobacillus* hesperidum | 35 | 0.27 |
| 14. *Ochrobactrum* anthropi | 32 | 0.25 |
| 15. unidentified eubacterium | 26 | 0.20 |
| 16. *Bacillus* sp. | 24 | 0.19 |
| 17. uncultured alpha | 23 | 0.18 |
| 18. *Staphylococcus* aureus | 22 | 0.17 |
| 19. *Achromobacter* sp. | 11 | 0.09 |
| 20. uncultured beta | 11 | 0.09 |
| 21. No hits | 10 | 0.08 |
| 22. uncultured chlamydia | 9 | 0.07 |
| 23. *Geobacillus thermoleovorans* | 9 | 0.07 |
| 24. *Anaerobic bacterium* | 9 | 0.07 |
| 25. *Spirulina subsalsa* | 9 | 0.07 |
| 26. *Bradyrhizobium japonicum* | 8 | 0.06 |
| 27. *Dietzia* sp. | 8 | 0.06 |
| 28. *Mycobacterium* heidelbergense | 8 | 0.06 |
| 29. uncultured streptococcus | 7 | 0.05 |
| 30. *Alicyclobacillus acidiphilus* | 7 | 0.05 |

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|---|---|---|---|
| 299 | 31. uncultured eubacterium | 7 | 0.05 |
| 300 | 32. *Clostridium* sp. | 7 | 0.05 |
| 301 | 33. alpha proteobacterium | 7 | 0.05 |
| 302 | 34. *Providencia vermicola* | 6 | 0.05 |
| 303 | 35. *Thiobacillus* sp. | 6 | 0.05 |
| 304 | 36. *Chroococcidiopsis* sp. | 6 | 0.05 |
| 305 | 37. uncultured rothia | 6 | 0.05 |
| 306 | 38. *Acidovorax delafieldii* | 6 | 0.05 |
| 307 | 39. *Bacterium 'smarlab* | 5 | 0.04 |
| 308 | 40. *Ralstonia pickettii* | 5 | 0.04 |
| 309 | 41. *Grimontella senegalensis* | 5 | 0.04 |
| 310 | 42. *Staphylococcus epidermidis* | 5 | 0.04 |
| 311 | 43. *Leuconostoc mesenteroides* | 5 | 0.04 |
| 312 | 44. *Sphaerobacter thermophilus* | 4 | 0.03 |
| 313 | 45. *Streptomyces* sp. | 4 | 0.03 |
| 314 | 46. *Actinomycete* species | 4 | 0.03 |
| 315 | 47. *Comamonas testosteroni* | 4 | 0.03 |
| 316 | 48. *Paracoccus* sp. | 4 | 0.03 |
| 317 | 49. *Sphingomonas* sp. | 3 | 0.02 |
| 318 | 50. *Micrococcus lylae* | 3 | 0.02 |
| 319 | 51. *Microcoleus vaginatus* | 3 | 0.02 |
| 320 | 52. *Bacillaceae bacterium* | 3 | 0.02 |
| 321 | 53. uncultured brevundimonas | 3 | 0.02 |
| 322 | 54. *Aeromonas* sp. | 3 | 0.02 |
| 323 | 55. *Pseudonocardia yunnanensis* | 3 | 0.02 |
| 324 | 56. uncultured hyphomicrobium | 3 | 0.02 |
| 325 | 57. *Sulfuricurvum kujiense* | 3 | 0.02 |
| 326 | 58. *Bacillus soli* | 3 | 0.02 |
| 327 | 59. *Micrococcus luteus* | 3 | 0.02 |
| 328 | 60. uncultured chloroflexi | 2 | 0.02 |
| 329 | 61. *Acidovorax* sp. | 2 | 0.02 |
| 330 | 62. uncultured soil | 2 | 0.02 |
| 331 | 63. *Oscillatoria amphigranulata* | 2 | 0.02 |
| 332 | 64. *Bacteroidetes bacterium* | 2 | 0.02 |
| 333 | 65. uncultured fusobacterium | 2 | 0.02 |
| 334 | 66. *Kocuria* sp. | 2 | 0.02 |
| 335 | 67. *Alcaligenes* sp. | 2 | 0.02 |
| 336 | 68. *Clostridium indolis* | 2 | 0.02 |
| 337 | 69. *Acidocella* sp. | 2 | 0.02 |
| 338 | 70. uncultured pseudomonas | 2 | 0.02 |
| 339 | 71. uncultured actinobacterium | 1 | 0.01 |
| 340 | 72. *Shewanella putrefaciens* | 1 | 0.01 |
| 341 | 73. *Bacillus sphaericus* | 1 | 0.01 |
| 342 | 74. *Morganella* sp. | 1 | 0.01 |
| 343 | 75. uncultured scenedesmus | 1 | 0.01 |
| 344 | 76. agricultural soil | 1 | 0.01 |
| 345 | 77. uncultured chloroflexus | 1 | 0.01 |
| 346 | 78. *Pseudomonas stutzeri* | 1 | 0.01 |
| 347 | 79. *Rhodopila globiformis* | 1 | 0.01 |
| 348 | 80. unidentified bacterium | 1 | 0.01 |
| 349 | 81. uncultured archaeon | 1 | 0.01 |
| 350 | 82. *Bifidobacterium* sp. | 1 | 0.01 |
| 351 | 83. uncultured rubrobacteridae | 1 | 0.01 |
| 352 | 84. *Dysgonomonas* sp. | 1 | 0.01 |
| 353 | 85. *Chondromyces crocatus* | 1 | 0.01 |
| 354 | 86. *Pseudomonas pertucinogena* | 1 | 0.01 |
| 355 | 87. *Agrobacterium tumefaciens* | 1 | 0.01 |
| 356 | 88. *Saprospira* sp. | 1 | 0.01 |
| 357 | 89. *Delftia* sp. | 1 | 0.01 |
| 358 | 90. uncultured candidate | 1 | 0.01 |

| 359 | 91.  *Rhizobium* sp. | 1 | 0.01 |
| 360 | 92.  uncultured gloeothece | 1 | 0.01 |
| 361 | 93.  uncultured delta | 1 | 0.01 |
| 362 | 94.  *Marinospirillum alkaliphilum* | 1 | 0.01 |
| 363 | 95.  uncultured rhodospirillaceae | 1 | 0.01 |
| 364 | 96.  *microbulbifer* sp. | 1 | 0.01 |
| 365 | 97.  uncultured sphingomonas | 1 | 0.01 |
| 366 | 98.  *Clostridium subterminale* | 1 | 0.01 |
| 367 | 99.  *Cetobacterium somerae* | 1 | 0.01 |
| 368 | 100.*Mobiluncus curtisii* | 1 | 0.01 |
| 369 | 101.uncultured methanogenic | 1 | 0.01 |
| 370 | 102.uncultured syntrophorhabdaceae | 1 | 0.01 |
| 371 | 103.*Aquaspirillum peregrinum* | 1 | 0.01 |
| 372 | 104.*Dellovibrio bacteriovorus* | 1 | 0.01 |

373

374

marinospirillum_alkaliphilum
bacterium_bhn
providencia_vermicola
grimontella_senegalensis
microbulbifer_sp.
aeromonas_sp.
aquaspirillum_peregrinum
pseudomonas_aeruginosa
uncultured_pseudomonas
uncultured_bacterium
uncultured_chlamydia
alpha_proteobacterium
rhizobium_sp.
uncultured_methanogenic
ralstonia_pickettii
acidovorax_delafieldii
comamonas_testosteroni
beta_proteobacterium
uncultured_beta
uncultured_brevundimonas
uncultured_hyphomicrobium
uncultured_sphingomonas
bradyrhizobium_japonicum
paracoccus_sp.
sphingomonas_sp.

375

376

377   Fig. 5a: Phylogenetic tree of 16S rRNA gene sequences of bacterial isolates obtained from crude oil
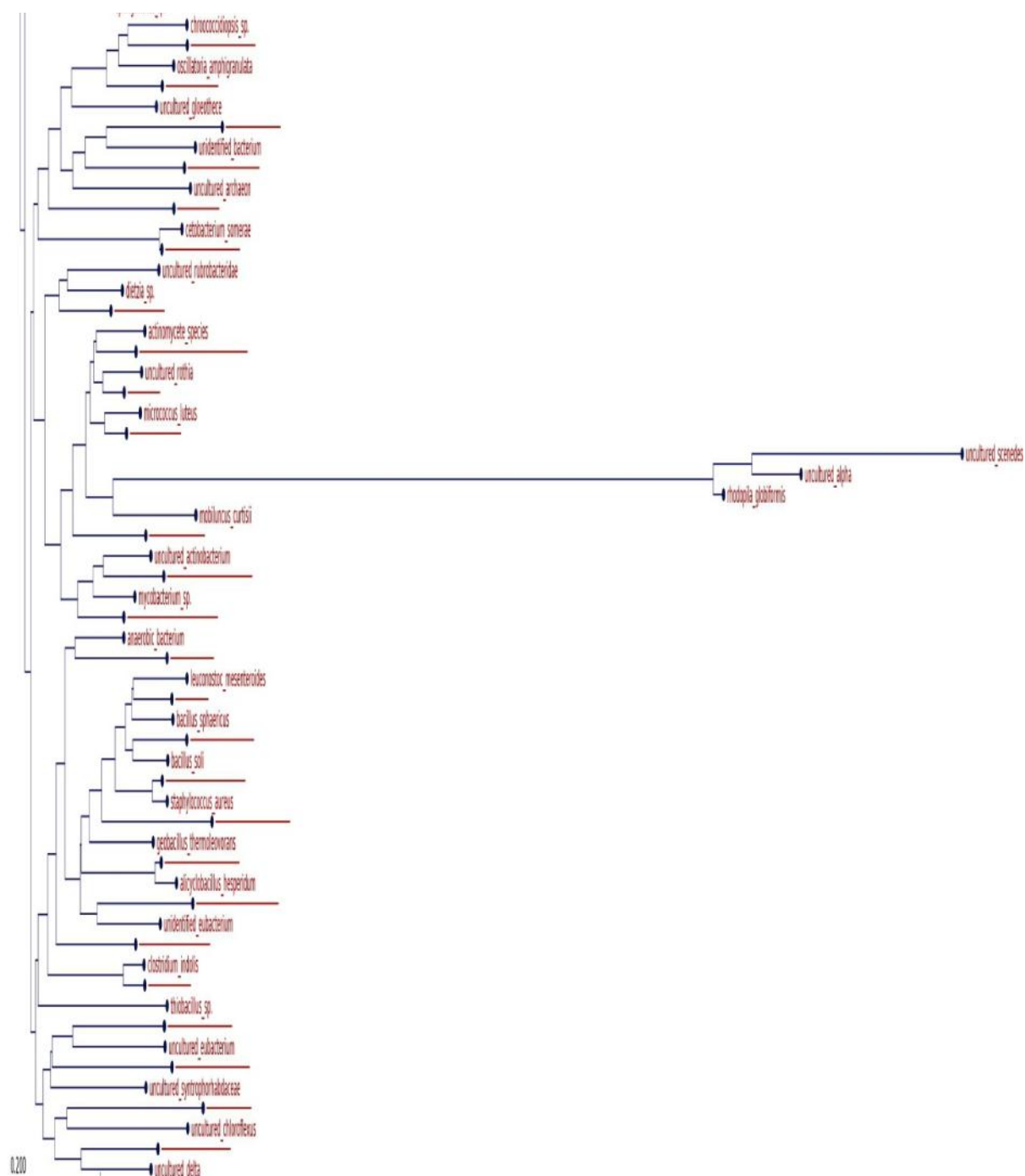378   contaminated soil sample.

379

380

381    Fig. 5b: Phylogenetic tree of 16S rRNA gene sequences of bacterial isolates obtained from crude oil
382    contaminated soil.

383
384    Metagenomic approach was used to identify the autochthonous bacterial population in the polluted
385    soil sample used in the investigation and results show that bacteria were predominant in the crude oil
386    impacted soil. Proteobacteria were the predominant bacterial population and this shows that the
387    proteobacteria are major players in a crude oil polluted soil ecosystem. Proteobacteria are gram
388    negative group of bacteria. This probably accounts for the predominance of gram negative rods

389 identified via culture-dependent approach described above. The data also showed that the soil was

390 mainly dominated by the Alphaproteobacteria (54.64%), followed by the Actinobacteria (9.67%),

391 Gammaproteobacteria (6.55%), Betaproteobacteria (2.27%) and Bacilli (0.95%) as well as Clostridia

392 (0.34%); as the most dominant classes of bacteria.

393

394 The order taxonomical classification showed that Rhizobiales (54.29%) were the most dominant group

395 followed by the Actinomycetales, Pseudomonadales, Bacillales, Xanthomonadales and

396 Hydrogenophilales.

397

398 The BLAST data indicated the dominant bacteria to be *Methylobacterium* sp. making up 45.90 % of

399 the population. The uncultured bacterium was 24.63% and *Mycobacterium* sp., 8.86%. Also,

400 *Bradyrhizobium* sp. was 7.99% and uncultured gamma, *Pseudomonas aeruginosa* and beta

401 proteobacterium accounted for 3.96, 1.29 and 1.05% of the bacterial population respectively. These

402 organisms have been identified by other researchers to be associated with biodegradation. Molecular

403 studies on microbial diversity have interestingly been related to degradation potentials of these

404 organisms. Kanaly *et al*. (2000) [17] examined the degradation of benzo[α]pyrene by a group of

405 bacteria by using the DGGE technique, in order to determine their dynamics during the degradation

406 process, and identified *Sphingomonas paucimobilis EPA505*, *Mycobacterium str.PYR-1*, and

407 *Alcaligenes denitrificans WW1* to be present in the consortium. These organisms are well known to be

408 associated with the degradation of aromatic compounds, as *Mycobacterium* and *Pseudomonas*

409 species have been also reported to be able to degrade PAH [18]. In crude oil, carbazole is often

410 present with its alkylated derivatives which often have monomethyl, dimethyl, trimethyl, and

411 tetramethyl side chains on different carbon positions of carbazole (called C1-, C2-, and C3-, C4-

412 carbazoles, respectively). Researchers have isolated strains of *Pseudomonas* species, which could

413 not only degrade carbazole efficiently, but also showed nitrification and denitrification ability [19].

414

415 In the overall, one hundred and four (104) diverse bacterial species were identified as shown in table

416 3. The five (5) different bacterial genera (*Acetobacter* sp*., Pseudomonas* sp., *Arthrobacter* sp.,

417 *Bacillus* sp. and *Micrococcus* sp.) isolated via cultural approach were similar to the bacterial species

418 identified using metagenomics. However, a comparison of the number of isolates obtained using both

419 methods clearly indicates that molecular techniques such as metagenomics increases the

420 understanding of bacterial diversity as well as functionality during bioremediation. Cultural techniques

421 only help to culture and identify minute population of microorganisms [20], representing about 1% of

422 the total microorganism found in a crude oil contaminated site [10].

423

424

425 The phylogenetic tree showed the predominant bacterial community in the hydrocarbon polluted soil

426 in relation to each other, using a scale of 0.2 and uncultured delta (0.01) at the root of the tree. The

427 phylogenetic sequence relationship shows that the most distant groups/species are the uncultured

428 scenedesmus (0.01%), uncultured alpha (0.18%) and *Rhodpila globiformis* (0.01%). These groups

429 however, belong to the same clade with *Mobiluncus curtisii* (0.01%). The *no hits* (0.08%) were also

430    seen to be distantly related to the uncultured *Clamydia* (0.07%) which is related to the alpha
431    proteobacteria (0.05%). The tree shows numerous clades for the 104 BLAST output result. This
432    shows the crude oil contaminated soil is composed of diverse groups of microorganisms and thus,
433    implies the hydrocarbon polluted soil is a reservoir for diverse bacterial groups.

434

435    Similar study involving the excision, reamplification and sequencing of dominant DGGE bands in
436    biostimulated soils revealed the presence of distinct hydrocarbon degraders like *Corynebacterium*
437    spp., *Dietzia* spp., low G+C Gram positive bacteria and some uncultured bacterial clones.
438    Phylogenetic analysis of the 16S rRNA gene sequences of these dominant bacterial communities was
439    conducted using the neighbour joining method of PHYLIP. The researcher observed two distinct
440    clades appearing in the tree with members of the *Actinobacteria* and *Firmicutes* clustering separately
441    [21]. In another study, the analysis of 16S rRNA of the isolated isolates from a crude oil impacted soil
442    in the Niger Delta region of Nigeria showed the species belonged to eight bacterial genera namely:
443    Achromobacter, Alcaligenes, Azospirillus, Bacillus, Lysinibacillus, Ochrobactrum, Proteus, and
444    Pusillimonas, with Alcaligenes as the dominant genus [22].

445
446    The variations in the number of hits of bacterial species observed in this study conform to the fact that
447    the polluted soil has diverse bacterial groups with different levels of adaptation. The ability of these
448    bacteria to metabolize or adapt to crude oil hydrocarbons is relative to the possession of degradative
449    genes or resistance genes [23]. These studies have revealed the phylogeny of the polluted soil used
450    in this study, which shows the diverse species of bacteria. These bacterial species differ in their
451    structure and function. For example *Thiobacillus* sp. is known to be associated with iron oxidation and
452    inorganic sulphur reduction (*Thiobacillus ferroxidans*). Some others are associated with heavy metal
453    reduction. This implies the polluted soil ecosystem harbours diverse bacterial population having
454    different structures and function which aid the process of bioremediation. While some of these
455    organisms are hydrocarbonoclastic, others (like *Pseudomonas* sp.) are known for biosurfactant
456    production. Biodegradation of petroleum hydrocarbon pollutants is therefore feasible in such
457    ecosystem. However, the fate of these crude oil pollutants will partly depend on the ecological/site
458    characteristics of the impacted soil. Gbarain kingdom is an oil rich region in the Niger Delta area of
459    Nigeria. This area is replete with crude oil pipe lines traversing the land and water body. This may be
460    partly responsible for *Thiobacillus* sp*.* ranking among the top 8 in theBLAST output report. This
461    revelation does not only make a case for bioremediation but also buttresses the critical need for
462    pipeline coating and biomonitoring.

463

464    4.0 CONCLUSION
465    The bacterial community composition of a crude oil polluted soil obtained from Gbarain Kingdom of
466    Bayelsa State, Nigeria suggests the presence of a rich and versatile group of hydrocarbon utilizing
467    bacteria adapted for crude oil degradation. These hydrocarbonoclastic bacterial species are involved
468    in the utilization of the petroleum hydrocarbons spilled at the site sampled and they have the
469    potentials to facilitate the bioremediation of the contaminated soil through natural attenuation or
470    enhanced bio-treatment protocols. Data obtained in this study make it obvious that only a very small

471 proportion of the bacterial population in the soil can be isolated and identified through cultural
472 techniques. Therefore, a detailed insight into the agents and dynamics of crude oil degradation in soil
473 thus, requires in-depth metagenomics which provide the structure and function of the diverse
474 microbial genera for the purpose of exploring and harnessing the potentials of these autochthonous
475 bacterial populations. The emergence of next generation gene sequencing (NGS) technique has
476 enabled the identification of a wide range of bacteria which were hitherto, unidentifiable via culture-
477 based methods. The NGS method is performed independent of PCR products and boycotts the
478 limitations associated with such techniques and is suitable for the study of the bacterial community
479 structure of a crude oil impacted media. However, cultural methods are not to be sacrificed for
480 identification using molecular characterization as both methods are important in bioremediation
481 monitoring.

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