

# Assessment of Bacterial Diversity of Sandy-Loam Soil Polluted by Hydrocarbons Using 454 Pyrosequencing

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**Abstract** The study was conducted on polluted soil from a refitting oil station at Pointe-Noire in Congo. The aim of the work was to study the composition of the soil microbial community. Microbial diversity was assessed using the 454 pyrosequencing. The results showed that microbial diversity was represented by 1986 OTUs assigned to the Bacteria domain with 97% of similarity. However, only 246 OTUs were affiliated with 12 Phyla, 24 Classes, 56 Orders and 85 Families. The Proteobacteria (73%), Chloroflexi (16.85%), Bacteroidetes (2.68%) and Actinobacteria (2.65%) were the most representative bacterial phyla. The dominant classes were Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Actinobacteria, Sphingobacteria. The most abundant orders are represented by Rhizobiales (22.94%), Sphingomordales (7.07%), Caulobacterales (6.68%) and three unknown orders corresponding to 28.96%. Bradyrhizobiaceae (14.10%), Sphingomonadaceae (7.05%) and Caulobacteraceae (6.68%) were the best distributed families in the microbial community. This soil could serve for isolation of microorganism consortia for bioremediation.

**Keywords:** 454 pyrosequencing, microbial community, diversity, soil, hydrocarbon

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## 1. Introduction

Oil pollution is a serious environmental problem. In fact, oil activities release into the environment large quantities of crude oil and others products derived to petroleum. Because of their toxicity, they cause ecosystem alteration and public health problems [1]. However, there are microorganisms in the soils with ability to degrade hydrocarbons whether the environment is polluted or not [2]. The number of these microorganisms are very high in chronically polluted areas and increase after a supply of hydrocarbons in sites without contamination [3]. These microorganisms (bacteria, fungi, cyanobacteria and algae) are used in biological processes for the treatment of polluted sites or bioremediation because of their implication in several nutrient cycles through their metabolism [4].

Moreover, the need of knowlegment on the microbial community present in polluted sites is one of the strategies used in the choice of the method of bioremediation. Stefani et al. [5] point out that regardless of the approach adopted, effective bioremediation relies on the ability to study microorganisms that are indigenous in polluted sites. Unfortunately, conventional techniques of enumeration can only recover less than 1% of the total microbial population present in a soil sample [6,7]. In addition, the microorganisms recovered by standard methods are not

representative of those that are highly competitive and numerically dominant in the environment [5,7]. Today the focus is on molecular biology methods, valuable tools, for researching the diversity and structure of microbial communities [8]. High throughput sequencing techniques (or next-generation sequencing) allow for greater recovery of DNA sequences. One of these massive sequencing techniques, the Roche life Science's 454 pyrosequencing, is widely used in the study of microbial community composition in different types of soils polluted by hydrocarbon [5,9,10]. In addition, the composition of a microbial community in an ecosystem and its ability to degrade hydrocarbons are influenced by abiotic and biotic factors in the environment [4,11]. The microorganisms colonize specific environments according to their needs so that few microorganisms can adapt and or survive in all environments. This behavior influenced the difference in the composition of the microbial community from one polluted area to another or between the regions [4].

In Congo, the exploitation, storage, transportation and distribution of oil and refined products have generated areas for the dumping of drilling muds. However, there are some companies that process drilling muds produced by different oil companies. However, in the biological remediation techniques used, the inoculum is imported and its composition is known only to importing companies. The use of local microbial strains is preferable since they are more adapted, acclimated and appropriate

for propagation in bioremediation processes [12]. According to these authors, the treatment of sites polluted by imported microbial populations have disadvantages because some populations may not be effective in the application areas. Indeed, these microorganisms can be consumed by predators (eg protozoa), compete with indigenous microorganisms or be inhibited by organic compounds excreted by the roots of nearby plants [13]. In this sense, it was reported that several marketed inoculums produce different results [14], often failing to meet manufacturers' goals. Therefore, it is important to know the microbial communities that colonize Congo's contaminated soils to be able to select indigenous strains that exhibit hydrocarbon-based activity. This in order to constitute consortia of microorganisms adapted to the tropical conditions in order to use them as inoculum in the bioremediation.

The objective of this study is to characterize the microbial community contained in a soil polluted by hydrocarbons. The present work aims to: (i) determine the physicochemical composition of the soil; (ii) the total petroleum hydrocarbon content; (iii) evaluate the bacterial density; (iv) to assess by 454 pyrosequencing the composition of the soil microbial community.

## 2. Material and Methods

### 2.1. Soil Sampling

Soil samples used in the study were taken from a refitting oil station at Pointe-Noire in Congo. The soil was polluted by the hydrocarbons. Three samples: Soil No. 1, Soil No. 2 and Soil No. 3 were taken with an auger into 0-20 cm layer in three zones (southern zone, southeastern zone and northern zone). The soil samples were sieved to remove roots, macrofauna, stones and all other unwanted materials, and placed in polyethylene bags. In the laboratory, the samples were mixed to form a composite sample and stored at 4°C prior to microbial analyses.

### 2.2. Soil Physicochemical

Soil texture was analyzed after a pretreatment with hot H<sub>2</sub>O<sub>2</sub> by sieving on a nested sieve column and sedimentation. Some of the samples were air dried and sieved to <2mm for the determination of soil chemical properties. The pH was determined in water (ratio w/v 1:2.5) by using a pHmeter (Stater 3100, OHAUS Corporation) after stirring for 30 minutes with a magnetic stirrer [15]. The total carbon (TC) was measured using the Walkley and Black method. Total nitrogen (TN) was analyzed according to the Kjeldahl method. Total phosphorus (TP) was determined colorimetrically by the molybdenum blue method [16]. Total petroleum hydrocarbons (TPH) from the soil were quantified using the RemediAid Total Petroleum Hydrocarbon kit (Chemetrics, USA) following the manufacturer's instructions. Briefly, the method consists in extracting petroleum hydrocarbons from the soil by dichloromethane. Then the hydrocarbons react with the aluminum chloride to give a colored compound.

The colored product is directly proportional to the concentration of petroleum hydrocarbons contained in the extract.

### 2.3. Soil Microbial Analysis

Microbial enumeration of total aerobic mesophilic flora (TAMF) was performed on PCA medium (Flukachemy, Spain) using the standard decimal dilution technique. Briefly, 10 g soil was stirred into 90 ml of sterile physiological saline in a 250 ml flask. Then, a dilution series of 10<sup>-1</sup> to 10<sup>-5</sup> was prepared from this initial dilution. Then 50µl of each dilution were inoculated in three petri dishes containing the PCA medium.

### 2.4. Extraction and Quantification of DNA

Total microbial community DNA was extracted from 250mg of the composite soil by using the MoBio soil kit (MoBio Laboratories, Inc., Carlsbad, California, USA) following the protocol proposed by the manufacturer. After extraction, the quality of the DNA was determined on 1% agarose gel while the concentration was quantified by using a NanoDropUV-Vis spectrophotometer (NVNR 2031275, France) according to the manufacturer's protocol.

### 2.5. Amplification of RNAr 16 Genes and Pyrosequencing

Bacterial soil diversity was evaluated by the pyrosequencing technique after extraction of total genomic DNA from the soil. The gene encoding the 16s rRNA was amplified by PCR using the Ready-To-Go Taq polymerase (Amersham Pharmacia, USA). The following primer pair 27-Forward (5'-AGAGTTTGGATCCTGGCTCAG-3') and 1492-Reverse (5'-GGTTACCTTGTTACGACTT-3') [17], covering the bacteria was used to amplify the region of interest. The reaction mixture of a total volume of 25 µL contained 0.25 mM of 27F primer, 0.25 mM of 1492R primer and 20 ng of template DNA. The amplification program consisted of initial denaturation at 94 °C for 8 min, followed by 30 denaturation cycles at 94 °C for 30 sec, hybridization at 56 °C for 30 sec, and 72 °C elongation for 1 min. A last elongation step was carried out at 72 °C for 15 min. Finally, the PCR products were cooled to 10 °C. The PCR products were verified by electrophoresis on 1.5% agarose gel (wt / vol) in 0.5 M TAE buffer stained with SYBR Safe stain (Invitrogen, France). A molecular size marker (100bp Ladder-Biolabs) was used as a standard to compare the size of the fragments obtained. Electrophoresis was performed at 80 V for 30 min. The results were visualized with a GelDoc System 2000 transilluminator (BioRad, Richmond, California, USA). The purified DNA extract was sent to the Lubbock Research and Testing Laboratory (TX, USA) for pyrosequencing. A DNA fragment containing the V1-V2 variable regions of the 16S rRNA genes was amplified using primers 28F (5'-GAGTTTGGATCCTGGCTCAG-3') and 388R (5'-TGCTGCCTCCCGTAGGAGT-3'). This fragment was sequenced using the Rock Life GS FLX 454 Pyrosequencer.

## 2.6. Statistical Analysis and Bioinformatics

Bioinformatic treatment was performed according to [18]. Briefly the sequences were analyzed and processed using the Mothur v.1.33.3 program (<http://www.mothur.org>). Sequencing errors were reduced using an implementation of the Amplicon Noise algorithm and low-quality sequences were eliminated. Then the sequences were sorted keeping only the highest quality readings (Q>35). Chimeras were eliminated using Mothur's chimera.uchime command. The sequences were aligned and classified according to the reference database SILVA v.102. Then they were grouped into Operational Taxa Units (OTUs) using the nearest neighbor algorithm with 97% sequence similarity. The Greengene database has been used for taxonomic assignments of OTUs. The determination of the relative abundances of each taxonomic group was carried out by the Microsoft Excel 2016 software. The OTUs were used to assess the community diversity and evenness with respectively Shannon diversity indice

$$(H' = -\sum_{i=1}^{S_{obs}} \frac{n_i}{N} \ln\left(\frac{n_i}{N}\right), \text{ with } S_{obs} \text{ the number of observed OTUs, the number of individuals OTU, and } N \text{ the total number of individuals in the community) and Shannon equitability index. In addition, the Bray-Curtis matrix was calculated [19] to assess the dissimilarity between the taxa using Primer software version 5.}$$

## 3. Results

### 3.1. Characterization of the Soil

The results show that the soil had a slightly alkaline pH around 7.7 (Table 1). These results reveal also that soil texture was sandy-loam. It had high sand 75.31%, low clay and silt respectively 13.77% and 10.92%. The total carbon and total nitrogen contents were respectively 2.5 % and 0.07%, with a higher C/N ratio (35.7). The total phosphorus content was 0.04%. The degree of pollution evaluated by the TPH content was 1441.66 mg/kg of soil. The soil had a large cultivable TAMF  $1.53 \cdot 10^7$  CFU/g of soil.

Table 1. Soil parameters (mean values and standard deviation)

Parameters	Values
Clay (%)	13.77 ± 2.00
Silt (%)	10.92 ± 3.93
Sand (%)	75.35 ± 2.21
pH	7.7 ± 0.32
TC (g/kg)	2.5 ± 0.05
TN (g/kg)	0.07 ± 0.004
TP (g/kg)	0.04 ± 0.01
C/N	35.7
TPH (mg/kg)	1441.66 ± 244
TAMF (CFU/g)	$1.53 \cdot 10^7 \pm 6.4 \cdot 10^6$

TPH: Total petroleum hydrocarbons; TAMF: Total aerobic mesophilic flora

### 3.2. Amount and Amplification of DNA

The DNA concentration of the entire polluted soil microbial community obtained after extraction was 1.28 ng/μl. The control of PCR products on 1% agarose gel shows a single band at 1200 bp for the soil tested (Figure 1).

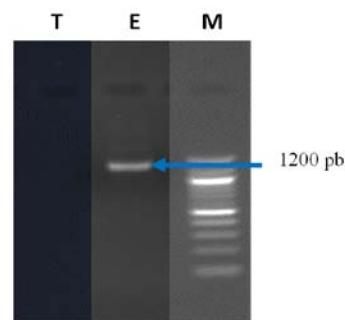


Figure 1. 1.5% agarose gel electrophoresis of PCR amplicons (TAE buffer wt/v; M: 100bp Ladder - Biolabs ; T: H<sub>2</sub>O ; E: PCR Amplicons of soil microbial community)

### 3.3. Relative Abundance of Phyla

After elimination of the unique sequences due to sequencing errors and deletion of the chimeric sequences, 28805 sequences were retained. These sequences were grouped, with a similarity of 97% of sequences, in 1896 operational taxonomic units (OTUs). All these OTUs have been classified as belonging to the Bacteria domain. However, only 246 OTUs were affiliated with a phylum. Twelve bacterial phyla were identified including four phyla with a relative abundance ranging from 2.65% to 73% and an unknown phylum with an abundance of 1.27%. The most representative bacterial phyla were: Proteobacteria (73%), Chloroflexi (16.85%), Bacteroidetes (2.68%), Actinobacteria (2.65%) (Figure 2). The Shannon index indicates low phyla diversity ( $H' = 1.18$ ) due to unequal distribution of the number of OTUs. In fact, equitability index (E) was 0.34 showing that the majority of individuals (OTU) were distributed in a small number of phyla. The similarity dendrogram between phyla shows two groups (Figure 3). The first group was comprised by the phyla with abundances ranging from 0.25 to 75.12%. This group counted three clusters within there were Proteobacteria and Chloroflexi with 37% similarity, Firmicutes and Acidobacteria 85% of similarity, Actinobacteria, bacteroidetes and unknown phylum. The second group included the phyla with abundances <0.25. These phyla covered a cluster that was apart all other clusters with 99.5% of similarity.

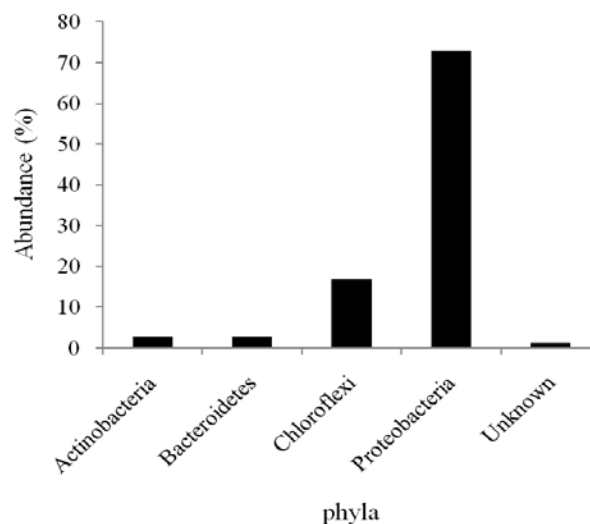


Figure 2. Relative abundance of major bacterial phyla

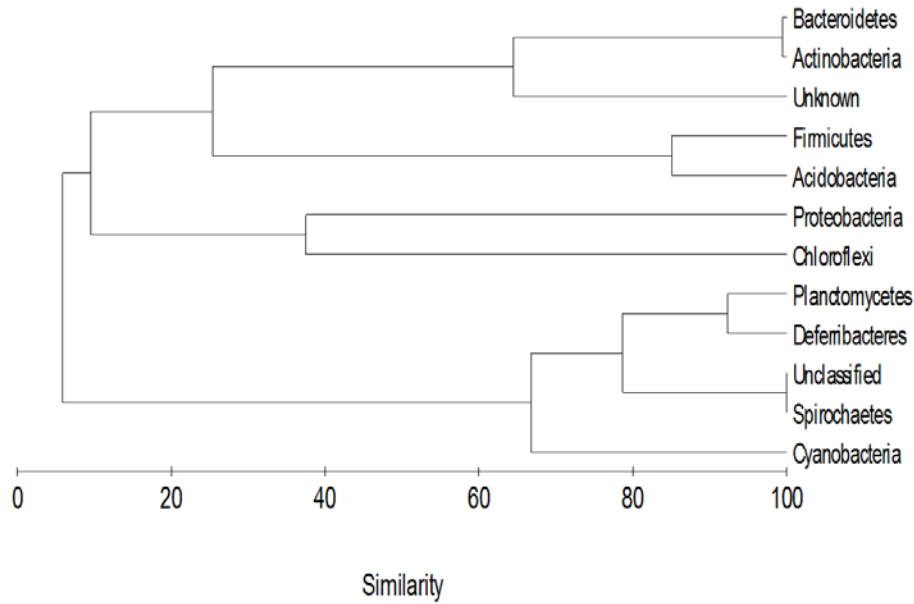


Figure 3. Similarity between phyla obtained by the Bray-Curtis matrix of the relative abundance of OTUs

### 3.4. Relative Abundance of Classes

In this study twenty-two classes were obtained but the Figure 4 shows only the relative abundances of the 8 most representative classes in the soil. Alphaproteobacteria represented the main class with a relative abundance of 45.80%, followed by a class not known in the phloroflexi phylum with 16.39%, then the class of Betaproteobacteria with a relative abundance of 10.42%. The relative abundance of the last five classes: an unknown class in the phylum of Proteobacteria, Actinobacteria, Gammaproteobacteria, Sphingobacteria and an unknown class in the field of Bacteria was respectively 2.82%, 2.60%, 2.36%, 1.98% and 1.27%. Taxonomic diversity evaluated by the Shannon index was low 2.3. It could mean that the OTUs have been distributed only in a few taxonomic classes. The In other hand, the result shows two groups of classes. A group composed of the most representative classes with Alphaproteobacteria, Betaproteobacteria, Chloroflexi and Gammaproteobacteria. The second group includes all the least abundant classes.

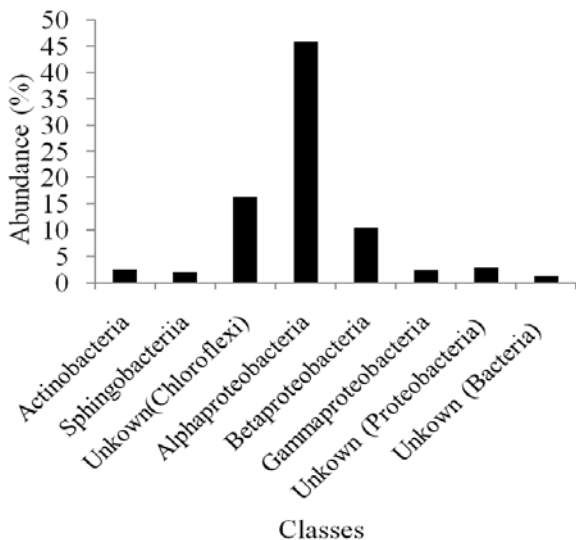


Figure 4. Relative abundance of the major bacterial classes

### 3.5. Relative Abundance of Orders

The 246 OTUs were grouped into 55 taxonomic orders. However, only 16 orders have relative abundances ranging from 1.27% to 22.94%. The Figure 5 shows that the rhizobiales represent the dominant order with an abundance of 22.94%, followed by an unknown order in the phylum Chloroflexi with an abundance of 16.39%, then come the sphingomonadales (7.07%), an unknown order (7.01%) in the class of betaproteobacteria, caulobacterales (6.68%) and an unclassified order (5.56%) of gammaproteobacteria. The left over orders, Actinomycetales, Sphingobacteriales, an unknown order of the class Alphaproteobacteria, Burkholderiales, Rhodospirillales, Methylococcales, Xanthomonadales and an unknown order of the class of Gammaproteobacteria have relative abundances less than 5%.

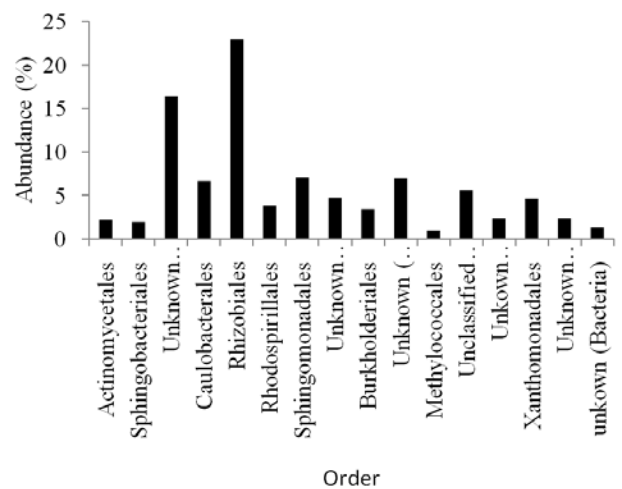


Figure 5. Relative abundance of the most representative orders

### 3.6. Relative Abundance of Families

A total of 84 families were obtained in the soil tested. However, only 18 taxonomic families have a relative abundance of between 1.27% and 16.32% (Figure 6).

An unknown family in the Chloroflexi phylum has the highest relative abundance (16.32%). It is followed by Bradyrhizobiaceae (14.10%), Sphingomonadaceae (7.05%), Caulobacteraceae (6.68%), an unclassified family of gammaproteobacteria with an abundance of 5.56% and an unknown family. Rhizobiales with an abundance of 5.04%. The remaining twelve families all have relative abundances of less than 5%.

### 3.7. Relative Abundance of Genera

In this study 110 taxonomic genera have been founded. Of these genera only 18 genera have relative abundances greater than 1% (Figure 7). It is an unknown genus in the phylum Chloroflexi (16.39%), Bradirrhizobium (14.08%), of a genus unknown in the phylum Betaproteobacteria (7.02%), Sphingomonas ( 5.95%), an unclassified genus in the phylum Gammaproteobacteria (5.57%), an unknown genus in the order Rhizobiales (5.04%), and an unknown genus of the family Caulobacteraceae (5.03%). The

rest of the genera have a relative abundance of between 5 and 1%. Taxonomic diversity is average with a Shannon index of 4.5. Regarding the distribution of OTUs, the value of equitability index (0.66) shows that the sequences are unequally distributed in the taxonomic classes. Despite the low resolution of pyrosequencing at the species level, a few bacterial genera have been identified including: Bradyrhizobium, Caulobacter, Azorhizobium, Steroidobacter, Phaeospirillum, Pseudoxanthomonas, Vibrio, Acinetobacter, Methylocaldum, Legionella, Coxiella, Providencia, Geobacter, Azospira, Massilia, Comamonas, Parvibaculum, Rhizobium, Hyphomicrobium, Mesorhizobium, Phenyllobacterium, Pelotomaculum, Desulfosporosinus, Paenibacillus, Brevibacillus, Ammoniphilus, Prochloron, Dehalococcoides, Levilinea, Candidatus, Amoebophilus, Prevotella, Streptomyces, Prauserella, Propionibacterium, Nocardioides, Microbacterium, Agromyces, Burkholderia, Pigmentiphaga, Achromobacter, Sphingopyxis, Sphingomonas, Sphingobium, Novosphingobium, Roseomonas, Komagataeibacter, Rhodobacter, Paracoccus, Xanthobacter.

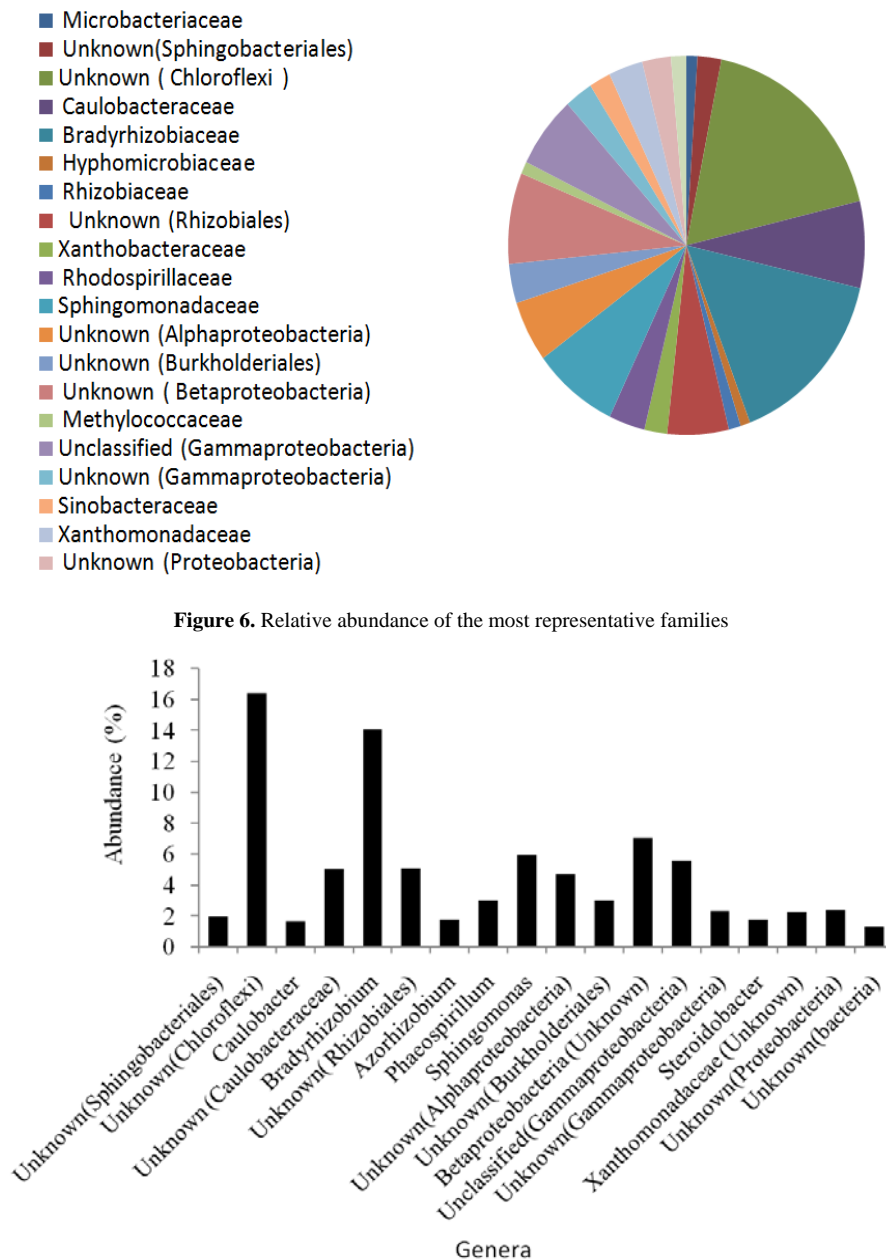


Figure 7. Relative abundance of the most representative genera

## 4. Discussion

Restoring polluted ecosystems requires a deep understanding of the microbial response to contaminants, both in terms of function and composition of the microbial community [20]. Next-generation sequencing techniques allow for greater recovery of DNA sequences, hence a deeper knowledge of the structure of the microbial community into the environment [4,5]. The present study was conducted to evaluate the composition of the microbial community in hydrocarbon-polluted soil using the Roche 454 Pyrosequencing technique. The results showed that Proteobacteria, Chloroflexi, Actinobacteria and Bacteroidetes are the dominant phyla. These results are in accordance with those of several authors [4,9,13,15,21] who found that dominant phyla in 16S rRNA and rDNA gene libraries 16S obtained from soils are Proteobacteria, Acidobacteria, Actinobacteria, Verrucomicrobia, Bacteroidetes, Chloroflexi, Planctomycetes, Gemmatimonadetes, and Firmicutes. The differences between our study and the studies of these authors could be explained by the physicochemical properties of soil matrix (pH, total carbon and nitrogen and phosphorus contents), the location zone and TPH concentration. This study was conducted in a tropical country on a sandy loam with a low alkaline pH, total carbon and nitrogen contents, phosphorus content and a relatively high TPH concentration. Whereas in the study of others authors [16,22,23,24] the soils studied were saline-alkaline with pH ranging from pH 7.36 to 9.35 and high nitrogen, phosphorus and organic carbon contents. Our study showed also that of the four dominant phyla, Proteobacteria were the most representative phylum with a relative abundance of 73%. Similar results have been obtained by several authors [11,12,13,22,25,26]. The predominance of Proteobacteria in all analyzed soils might be to their role in biogeochemical processes in soils. In addition, bacteria belonging to the phylum Proteobacteria are known to be able to use aliphatic and aromatic hydrocarbons [13,27]. In accordance with Torsvik and Ovreas [6] the number of bacteria degrading hydrocarbon increases in contaminated soils reaching 10 to 100% of the whole microbial community. However, in others works Acidobacteria followed by Actinobacteria were the most representative phyla in all soil samples studied [11,28]. In this study a proportion 1.27% of OTUs belonging to the Bacteria domain were not affiliated with a phylum. In other works it was also founded unclassified sequences affiliated to the Bacteria domain. These sequences might belong to group of bacteria not yet cultivated or not determined, or belonging to a new bacteria [5,29,30]. In accordance with Sutton and al [10] only three of the five classes of Proteobacteria (Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria) were representative (abundance >1%) . On the other hand, in their study [30] showed that relative abundances of the class Epsilonproteobacteria were very low (<1%) in all soils studied. As in several studies the most representative OTUs attached to Chloroflexi could not be affiliated with a class [5,9,31]. Members of phylum Chloroflexi appear to play an important role in the ecosystem function mainly in degradation organic matter [9,31]. As in the present study Actinomycetales were the most representative in the taxonomic class of Actinobacteria and have been often

isolated from polluted environments in many studies [5,32].

The orders identified in this study have already been isolated from oil-polluted environments in previous studies [5,32]. The Rhizobiales were the most dominant taxonomic order (22.94%). This order includes atmospheric nitrogen-fixing bacteria living in symbiosis with legumes that have an important role in functioning of soil ecosystems [33,34]. The phototropic and chemoorganotrophic bacteria were represented by Sphingomonadales (7.07%), Caulobacterales (6.68%) and Rhodospirillales (3.78%) including the photosynthetic and chemoorganotrophic species involved in oxidation of carbohydrates and alcohols [33]. In the present study Betaproteobacteria were represented by the Burkholderiales (3.40%). Bacteria affiliated with this taxonomic order are known as degraders of aliphatic and aromatic compounds (polychlorobiphenyls, naphthalene and phenanthrene) and are often used in bioremediation processes [33,35]. Xanthomonadales (4.62%) were the only order of Gammaproteobacteria identified. The members of this group degrade polyaromatic hydrocarbons and are also active in polluted soil treatment processes [32].

Despite the difference in relative abundances, most of the bacterial genera identified by 454 pyrosequencing in this study were isolated from oil-polluted areas in previously studies [5,28,27]. These authors have founded in all soils studied high occurrence for the following genera: Agromyces, Mycobacterium, Mesorhizobium, Bradyrhizobium, Rhizobium, Sphingomonas, Streptomyces, Microbacterium and Nocardioideis, Acinetobacter, Legionella, Pseudoxanthomonas, Caulobacter, Sphingopyxis, Hyphomicrobium, Propionibacterium, Phaeospirillum, Massilia, Hyphomicrobium, Geobacter, Sphingobium and Steroidobacter. In addition, in our study a large number of OTUs could not be affiliated with a genre or were unknown. Several authors [27] indicated that these kinds of sequences may represent new microbial species or species not described in the databases. In the studies on soil using pyrosequencing high diversity was founded with shannon index ranging from 5.11 to 9.98. In this interval the contaminated soils had relatively higher diversity than uncontaminated soils [1,5,9,10,11,26]. The phylum diversity in our study was low compared to the finding of these authors. This difference can be explained by the fact that the soil explored in this study has very low levels of total nitrogen, total phosphorus and organic matter. It is known that pollution exerts a selection pressure to propagate hydrocarbon degrading bacteria. However, these selected bacteria need to find in the environment the nutrients necessary for their growth. Low nutrient content (organic matter) would result in low diversity by selecting only the most suitable bacteria. It has been also shown that polluted soils compared to unpolluted soils support a more diverse bacterial community [2,4,7,14]. The variation of bacterial community is controlled mainly by the organic matter and the exposure time [37]. This work showed that diversity in all taxonomic levels was low, although it increased slightly from phylum to genus. The majority of sequences affiliated to Bacteria domain were grouped into a few number of taxa at each taxonomic group. In addition, similarity between phyla and between different taxonomic classes,

based on sequence abundances, confirm this distribution by grouping the most abundant taxa in the same clade.

Although this study did not focus on the biodegradation of hydrocarbons, members of Proteobacteria, Chloroflexi and Actinobacteria phyla isolated from polluted environments are known to be associated with hydrocarbon degradation [6,7,23]. Despite its limitation, the present study might provide a better understanding of bacterial diversity of sandy-loam soil polluted by hydrocarbons. It might be a potential source for isolation of consortia of microorganisms for bioremediation. Nevertheless, further work should be done to study the genes involved in hydrocarbon degradation as well as biodegradation in the laboratory and in the field.

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