

HOMOLOGY-DEPENDENT METAGENOMICS STUDY OF AGRICULTURAL SOILS IN AKWA IBOM STATE, NIGERIA

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ABSTRACT

The soil is highly complex and constitutes diverse populations of bacteria and archaea responsible for the several soil functions and plants growth. Prokaryotic study of two agricultural soils was carried out. One soil surrounds an aviation fuel-contaminated lentic ecosystem in Inua Eyet Ikot village, Ibeno, and another surrounds an uncontaminated lentic ecosystem in Shelter Afrique, Uyo, both in Akwa Ibom State, Nigeria. Samples of the surface (0-15cm) soils were collected using hand-held auger into well labeled sterile containers. Metagenomic DNA was extracted from both samples using ZYMO soil DNA extraction Kit. The extracted DNA fragments were purified by electrophoresis and amplified by Polymerase Chain Reaction with the aid of 16S rRNA universal primers 785F (GGA TTA GAT ACC CTG GTA) forward and 805R (GAC TAC CAG GGT ATC TAA TC) reverse primers. Sequence homology of the 16S rRNA gene was performed using Nucleotide BLAST program on NCBI software. A large data of bacterial and archaeal sequences were detected in the analysis with bacteria outnumbering archaea in both soils. The bacterial population in the contaminated soil was 0.55% higher than their counterpart in the uncontaminated soil. Sequences of members of Proteobacteria, Actinobacteria, Firmicute, Acidobacteria and those designated 'unknown' showed dominance in both soils with little variations. Euryarchaeotal and Crenarchaeotal sequences were detected and were the only archaeal representatives found in both soils. The most dominant genus in the contaminated soil was *Nitrospira*. *Nitrospira* sp.-Y14643.1 and *Chromobacterium* sp.-AY701878.1 are highly associated with the soil around the aviation fuel-contaminated ecosystem. *Bradyrhizobium* sp.-AJ558030.1 predominates in the uncontaminated soil. Both soils have high composition of Gram-negative cells. The soil around the contaminated ecosystem constitutes a higher community of prokaryotes compared to that around the uncontaminated ecosystem. The group 'unknown' show higher occurrence at all levels of classification as well as the 'uncultured' and 'unidentified' observed at the species level, in both soils. Soil microbial study by sequence analysis reveals invaluable information on the rich microbial diversity of the soil, the kind that would remain hidden despite routine cultivation. Molecular study is undeniably an important approach to discovering new soil microbial ecology.

Keywords: Homology, Metagenomics, Soil, Aviation Fuel, Contamination.

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INTRODUCTION

Quest for wealth has led to activities that hamper the ecosystems. The soil, water, and air become characterized with uncondusive living conditions and only species that are able to bear stress predominate leading to reduced biodiversity. In a report by Menta, natural ecosystems undergo continuous regulation and flows of energy as well as nutrients. The driving force behind this regulation is an undisturbed community of the soil biological diversity (Menta, 2012). Soil microbial biomass mediates soil activities (Hirsch *et al.*, 2010) and is an important agent in biogeochemical cycling and mineralization (Douglas and Green, 2015) that lead to soil fertility and promotion of plants health (Hirsch *et al.*, 2010). Substances, whose presence in the environment affects these microbial activities, also adversely affect plant growth, as well as detoxification of organic pollutants (Douglas and Green, 2015) in the ecosystem.

Majority of soil microorganisms have not been fully characterized, because they have not been readily culturable on standard cultivation media (Lee and Lee, 2013). Molecular study of microorganisms by polymerase chain reaction targeting the 16S rRNA gene has been useful in the investigation and identification of prokaryotes diversity (Attallah, 2014). Microbial genomes hold a vast amount of information and analysis of these genetic resources enables investigation and successful discovery of such information.

Deoxyribonucleic acid (DNA)- or ribonucleic acid (RNA)-based methods allow better characterization of microorganisms. Molecular biological methods which practically involve isolation of the total DNA, amplification of microbial signature rRNA sequences (Sharma *et al.*, 2013) and DNA sequencing of the rRNA sequences (Lee and Lee, 2013) to obtain material(s) for further analysis have revealed an enormous reservoir of unexplored microbes over the years (Sharma *et al.*, 2013). Among these are the metagenomic approaches which involve the extraction of DNA from soil (Delmont *et al.*, 2011) known as the soil metagenome. Metagenomics which is the genomic study of microorganisms involves collective investigation of microbial genomes from a mixed population of microorganisms (Neelakanta and Sultana, 2013). The method increases access to the genetic resources contained in the soil (Ghazanfar *et al.*, 2010) in soil analyses. The recovered gene sequences are used to identify organisms (Fakruddin and Mannan, 2013) and also their functions.

Hirsch *et al.*, (2010), suggested that for proper management and minimization of the negative environmental impacts, there is a need for detailed and predictive understanding of the microbial communities of the soil. From estimate, over 90% of species that constitute the microbial communities in the environment obviously do not form colonies or escape cultivation using conventional techniques (Chikere *et al.*, 2011). The opportunities for

the discovery of new organisms and the development of resources based on microbial diversity are greater (Jurgens, 2002). In this study, a survey was carried out on two soils ecosystems using metagenomics approaches with the objective to identify the communities of prokaryotes present in them. The first soil surrounds an aviation fuel-contaminated lentic ecosystem while the second one surrounds a lentic ecosystem with no history of contamination. Findings from this study will serve as a reference material on the soil microbial diversity.

Materials and methods

Collection of Sample

Samples of soil surrounding an aviation fuel-contaminated lentic system on 04° 32.647' N, 007° 59.951' E, Ibeno and another soil on 04° 58.519' N, 007° 57.908' E behind Graceland High School at Shelter Afrique, Uyo, were collected by removing the weeds on the surface to obtain the top soil (0-5cm). The samples were collected using a hand-held auger into different sterile and well labeled containers. The soil around the contaminated ecosystem was labeled 'contaminated soil'. The second soil was labeled 'uncontaminated soil'. Both were transported to the laboratory on ice.

Community DNA Extraction and Metagenomics Analysis

Total DNA was extracted from the samples using the ZYMO Soil DNA Extraction Kit (Model D 6001, Zymo Research, USA). Individual soil's crude DNA extract was purified by electrophoresis on a 0.7 % low

melting agarose gel at 70V for 3hrs. The purified extracts underwent amplification by Polymerase Chain Reaction with the aid of 16S rRNA 785F (GGA TTA GAT ACC CTG GTA) forward and 805R (GAC TAC CAG GGT ATC TAA TC) reverse primers. The programme of amplification consisted of denaturation at 94 °C for 1 min, and 30 cycles of 94 °C for 20 s, annealing at 53 °C for 25 s, and extension at 68 °C for 45 s, with a final extension at 68 °C for 10 min. PCR products were separated electrophoretically in 1% agarose gel as described by Sambrook *et al.*, (2000) and visualized with the aid of ethidium bromide under ultraviolet illumination. The PCR amplicons of the 16S rRNA genes were sequenced by Next-Generation Sequencing Technologies (NGSTs) using the Miseq Illumina platform. Amplified and sequenced 16S rRNA gene products were analyzed for sequence homology using Nucleotide BLAST program on NCBI software (<http://www.ncbi.nlm.nih.gov/BLAST>).

Results and Discussion

Metagenomics 16S rRNA gene sequence analysis of the two soils metagenome revealed enormous communities of bacteria and archaea. The contaminated soil had 99.82% bacterial community, 0.15% unknown and 0.03% archaeal community while the uncontaminated soil had 99.27% bacteria, 0.59% unknown and 0.14% archaea. Top and representative members of each taxon are presented in figure 2 to figure 6. Table 1 and 2 present members of bacteria and archaea as well as cyanobacteria found in the contaminated

and uncontaminated soil respectively, at species level.

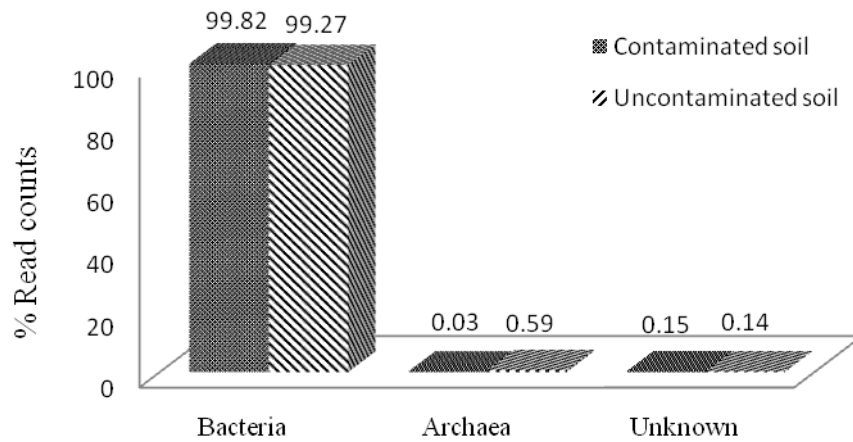


Figure 1: Kingdom classification of gene sequences detected in the contaminated and uncontaminated soils.

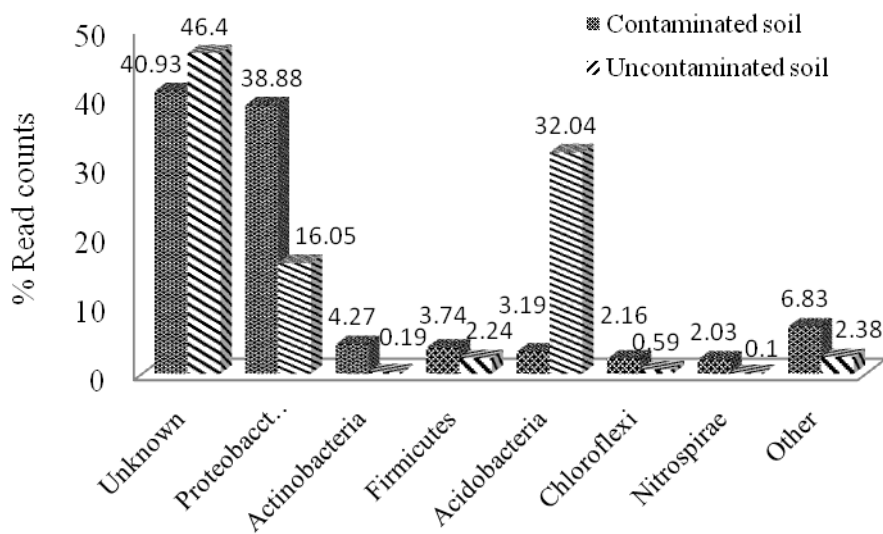


Figure 2: Relative abundance of top 7 and 5 out of 23 and 21 prokaryotic phyla detected in the contaminated and uncontaminated soils respectively. Other is the sum total of all classifications with percentage read of <1%.

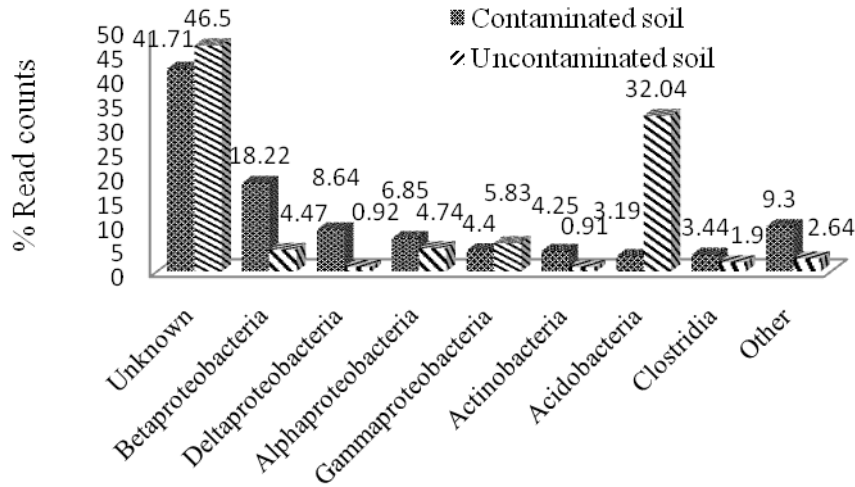


Figure 3: Relative abundance of top 8 out of 32 and 34 classes of prokaryotes detected in the contaminated and uncontaminated soils respectively. Other is the sum total of all classifications with percentage read of <1%.

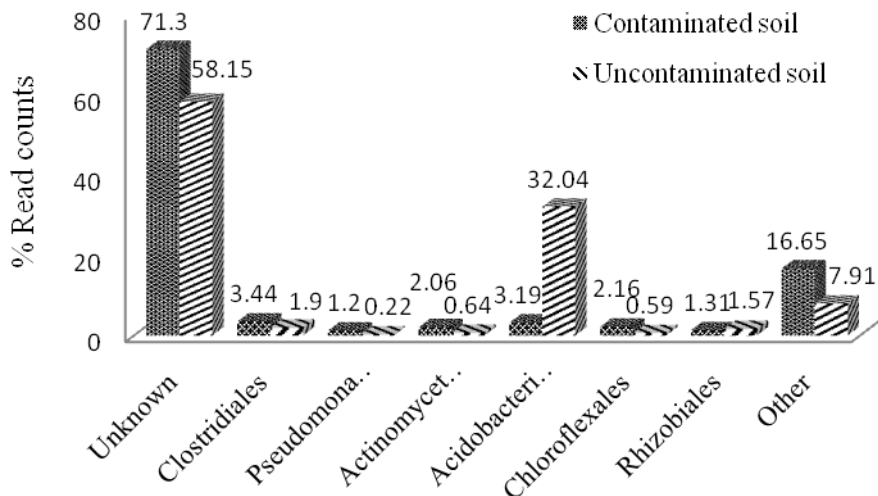


Figure 4: Relative abundance of top 7 and 4 out of 59 and 65 orders of prokaryotes detected in the contaminated and uncontaminated soils respectively. Other is the sum total of all classifications with percentage read of <1%.

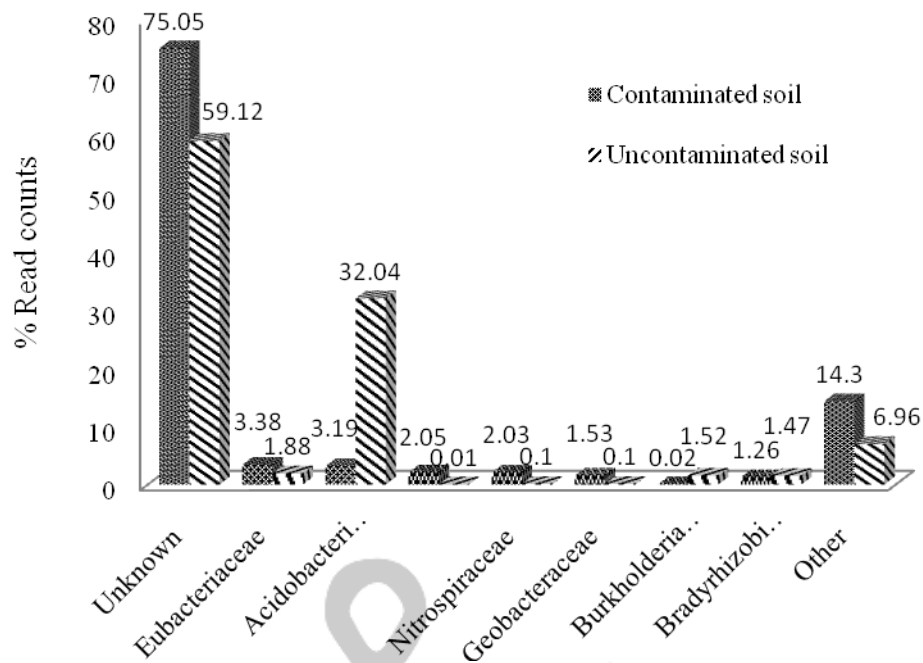


Figure 5: Relative abundance of top 7 and 5 out of 95 and 105 families of prokaryotes detected in the contaminated and uncontaminated soils respectively. Other is the sum total of all classifications with percentage read of <1%.

Table 1: Top 25 of prokaryotic species detected in the soil around the contaminated lentic ecosystem.

Species Diversity	%Read Count	Percentage nucleotide identity match	Accession number
Uncultured bacterium	26.94	96	AJ548899.1
Uncultured acidobacteria	12.77	96	AM884631.1
Unidentified bacterium	3.80	96	AJ518257.1
<i>Nitrospira</i> sp.	1.68	93	Y14643.1
<i>Chromobacterium</i> sp.	1.15	97	AY701878.1
Unidentified eubacterium	1.63	97	AJ232828.1
Uncultured Rhodospirillaceae	1.43	96	AM159320.1
Uncultured <i>Geobacter</i> sp.	1.37	84	AM159295.1
<i>Acinetobacter</i> sp.	0.74	92	AJ410290.1
<i>Achromobacter xylosoxidans</i>	0.50	91	AY189752.1
<i>Bdellovibrio bacteriovorus</i>	0.44	91	AF148941.1
<i>Gemmata obscuriglobus</i>	0.41	98	X85248.1
<i>Defluviicoccus vanus</i>	0.37	96	NR_041771.1
<i>Delftia</i> sp.	0.34	92	AB164685.1

<i>Frankia</i> sp.	0.28	93	U60287.1
<i>Enterobacter cloacae</i>	0.25	93	AF030416.1
<i>Kouleoithrix aurantiaca</i>	0.11	91	AB079638.1
<i>Klebsiella oxytoca</i>	0.22	90	U78183.1
<i>Pseudomonas putida</i>	0.20	89	AE015451.1
<i>Methylobacterium</i> sp.	0.20	85	AY904733.1
<i>Polyangium cellulorum</i>	0.13	90	AF387629.1
<i>Bacillus</i> sp.	0.12	93	AY159884.1
<i>Dermatophilus</i> sp.	0.12	94	AJ244775.2
<i>Streptomyces</i> sp.	0.11	90	AB123037.1

Table 2: Top 26 of prokaryotes detected in the soil around the uncontaminated ecosystem.

Species Diversity	%Read Count	Percentage nucleotide identity match	Accession number
Uncultured bacterium	29.89	91	AJ534633.1
Uncultured acidobacterium	25.65	98	KF225943.1
<i>Bradyrhizobium</i> sp.	1.20	95	AJ558030.1
Uncultured eubacterium	1.15	97	AJ292907.1
<i>Burkholderia cepacia</i>	0.91	91	AB114607.1
Uncultured <i>verrucomicrobia</i>	0.41	90	AY694604.1
<i>Delftia</i> sp.	0.31	90	AB164685.1
Uncultured <i>Legionella</i> sp.	0.30	87	AY924076.1
Uncultured <i>Holophaga</i> sp.	0.09	94	AJ519371.1
<i>Planctomyces</i> sp.	0.05	92	Y14640.1
<i>Nevskia</i> sp.	0.05	78	DQ242479.1
<i>Brochothrix thermosphacta</i>	0.05	97	M58798.1
<i>Achromobacter xylosoxidans</i>	0.04	91	AY189752.1
<i>Bacillus gelatini</i>	0.04	83	AJ586347.1
<i>Streptomyces coelicolor</i>	0.03	93	AL939130.1
Uncultured <i>Chlorobi bacterium</i>	0.03	90	AJ519647.1
<i>Enterococcus raffinosus</i>	0.01	91	AJ301838.1
<i>Dechlorosoma</i> sp.	0.01	90	AY171616.1
<i>Roseateles depolymerans</i>	0.01	96	AB003626.1
<i>Rhizobium</i> sp.	0.01	98	AY500261.1
<i>Synechococcus elongates</i>	0.01	89	CP000100.1
<i>Hahella chejuensis</i>	0.01	85	CP000155.1
<i>Flavobacterium gelidilacus</i>	0.01	90	AJ871226.1
<i>Rubrivivax gelatinosus</i>	0.01	90	AJ871464.1
<i>Sphingomonas</i> sp.	0.01	90	AY694604.1

Prokaryotic Diversity:

Analysis of the rRNA genes revealed a large data of bacterial, archaeal and also cyanobacterial sequences. Sequences affiliated with bacteria predominated in both soils as seen in Figure 1. The bacterial population in the contaminated soil was 0.55% higher than their counterpart in the uncontaminated soil. The archaeal members were less by 0.11% in the contaminated soil than the uncontaminated soil. This predominance nature of bacteria has been observed previously (Udotong *et al.*, 2015; 2017). Taxonomic classification of the sequences detected from the contaminated soil revealed prokaryotic representatives from 23 phyla, 32 classes, 59 orders, and 95 families (Figure 2 through 5). Species from 151 genera were detected. As observed in the figures, the group "Unknown" led in the compositions. Similar observation was recorded in other ecosystems (Udotong *et al.*, 2018). The percentage occurrence of members in each taxon was higher in the contaminated soil than the uncontaminated soil. The prokaryotic diversity of the contaminated soil based on taxonomic analysis was classified into 2 known kingdoms (Fig. 1), 23 phyla (Fig. 2), 32 classes (Fig. 3), 59 orders (Fig. 4), and 95 families (Fig. 5). These numbers were quite higher than observed in the uncontaminated soil. Actinobacteria (4.27%) followed next in abundance after the Unknown (40.93%) and Proteobacteria (38.88%) before Firmicute (3.74%) and Acidobacteria (3.19%) in the contaminated sample (figure 2). However, Acidobacteria (figure 2) followed in

abundance before Proteobacteria (16.05%) in the uncontaminated sample and represented 22214 of the reads at 32.04%. Similar compositions at phyla level have been recorded by Nair *et al.*, (2013) in the study of mangrove soil. Lee and Lee (2013) and Delmont (2011) through culture-independent analysis reported that majority of the soil microbial diversity also belongs to these phyla. Actinobacteria are Gram positive bacteria. They are dominant in terrestrial and aquatic ecosystems and are highly involved in the decomposition of organic matter and making nutrients available for plants uptake (Servin *et al.*, 2008). In a report by Jurgens (2002), the application of new molecular approaches has led to the discovery of high numbers of novel and unexpected "non-extreme" archaeal phenotypes. The presence of Euryarchaeotal and Crenarchaeotal sequences were detected in both soils in this study and a similar finding has been documented by Jurgens, (2002). The low relative abundance of archaea in this study validates report by Fierer *et al.*, (2012). Betaproteobacteria was relatively higher in the class (Figure 3) lineage in the contaminated soil while members of Alphaproteobacteria were the highest in the uncontaminated soil. Both have a common ancestor, Proteobacteria and are active in fixing nitrogen in plants where the Betaproteobacteria, especially, oxidize ammonium to nitrate. Acidobacteria occurred most in the uncontaminated soil (22,214 reads at 32.04%). These groups of bacteria are physiologically diverse in the soil environment (Eichorst *et al.*, 2007). Clostridiales (3.44%) occurred most in the contaminated soil at the class level while

Acidobacteriales (32.04%) was the highest in the uncontaminated soil (Figure 3). Most representatives of Clostridiales are known to be saprophytic in the environment. Aside members of the Uncultured and Unidentified, the genus *Nitrospira* (1.69%) and *Chromobacterium* (1.15%) as seen on Table 1 predominate as they contain high numbers of species from the contaminated soil, and this gives evidence that these species are probably the most active members in this environment. The uncontaminated soil had most species to belonging to the genus *Bradyrhizobium* (1.20%) (Table 2). All phyla, class, order, family and genus members with relative abundance less than 1% were grouped and designated 'other' in the figures.

Species Diversity:

A large percentage of sequences affiliated to sequences of microorganisms denoted 'uncultured' and 'unidentified' were detected in the soils. These include Uncultured bacterium with the accession number AJ548899.1, Uncultured acidobacterium-KF225943.1, Uncultured *Geobacter* sp.-AM159295.1, Uncultured *Sterolibacterium* sp.-DQ279355.1, etc. (Table 1) and Uncultured bacterium-AJ534633.1, Uncultured eubacterium-AJ292907.1, Uncultured *Legionella* sp.-AY924076.1, Uncultured *Holophaga* sp.-AJ519371.1, etc. (Table 2). The relatively high abundance of these groups of prokaryotes suggests that a vast number of soil microorganisms have affiliation with organisms of no scientifically specified genus. The soil around the contaminated ecosystem showed the presence of

Nitrospira sp.-Y14643.1 and *Chromobacterium* sp.-AY701878.1 in higher percentage than *Acinetobacter* sp.-AJ410290.1, *Achromobacter xylosoxidans*-AY189752.1, *Bdellovibrio bacteriovorus*-AF148941.1, *Gemmata obscuriglobus*-X85248.1, *Defluviicoccus vanus*-NR_041771.1, *Delftia* sp.-AB164685.1, *Frankia* sp.-U60287.1, and many more on Table 1. The uncontaminated soil showed lower abundances of *Acinetobacter* sp.-AJ410290.1, *Nevskia* sp.-DQ242479.1, *Brochothrix thermosphacta*-M58798.1, *Enterococcus raffinosus*-AJ301838.1, *Delftia tsuruhatensis*-AJ606337.1, *Achromobacter xylosoxidans*-AY189752.1, *Bacillus gelatini*-AJ586347.1, *Streptomyces coelicolor*-AL939130.1, *Dechlorosoma* sp.-AY171616.1, *Synechococcus elongates*-CP000100.1, etc. A large number of these species are Gram negative bacteria. This population demonstrates the position of Gram negative bacteria as the dominant and most active bacterial communities in the soil ecology. *Nitrospira* species are nitrite-oxidizers. *Chromobacterium* is the soil's normal flora and antibiotics producers. The species *C. violaceum* produces the violacein antibiotics (Kodach et al., 2006). *Bradyrhizobium* species form symbiotic relationships with leguminous plants in the soil where they fix nitrogen in exchange for carbohydrates from the plants. The detection of sequences affiliated with *Synechococcus elongates* in the uncontaminated soil confirms the present of the group, cyanobacteria as an important component of the prokaryotic community. *S. elongates* is a photosynthetic bacteria and therefore responsible for primary production

(Scanlan and Nyree, 2002) in the environment.

Conclusion

Soil study using sequence analysis was carried out for the first time on these two agricultural soils. BLAST results showed sequences affiliated with species of bacteria, archaea and cyanobacteria. Sequences affiliated with bacterial community predominated in both soils. The contaminated soil displayed higher number of prokaryotes than observed in the uncontaminated soil. The presence of Euryarchaeotal and Crenarchaeotal sequences were detected in both soils. Cyanobacterial sequences were also found in the uncontaminated soil. The contaminated soil revealed the presence of more than 75.37% of species designated 'uncultured' and 'unidentified'. This included Uncultured bacterium (26.94%) and Unidentified bacterium (3.80%). Gram negative bacteria dominate both soils. Molecular analysis of the soil ecology is an important tool to discovering new microorganisms.

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