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# The Molecular Study of Microbial and Functional Diversity of Resistant Microbes in Heavy Metal Contaminated Soil

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## ABSTRACT

The operations of biological systems are challenged by various environmental factors that interrupt biological processes. Accurate identification and predictive models can serve as useful tools for distinguishing microbes and understanding key organisms and their roles. This study was aimed at evaluating the microbial abundance/diversities (bacteria and fungi) present in contaminated soil using molecular approaches. The soil samples were artificially contaminated with heavy metal salts of nickel (25, 50, 75 mg/kg); chromium (50, 100, 150 mg/kg); lead (150, 300, 450 mg/kg) and cadmium (1.5, 3.0, 4.5 mg/kg). Pure culture of bacteria and fungi were identified using 16S rRNA gene sequence analysis for bacteria community and the Internal Transcribed Spacer (ITS) gene for fungi. The results show that the physicochemical properties of the soil reduced significantly at the end of the experiment ($p<0.05$). The microbial loads were low at the initial day 1 compared to the day 30 but only significant for Bacterial in Pb (300 and 450 mg/kg) and Cd (3 mg/kg) soils. Gene sequence analysis of the microbe revealed seven species of bacteria and four fungi species. Fungi had higher GC contents than bacteria in the contaminated soil suggesting their higher stability to pollution. Phylogenetic analysis revealed that the contaminated soil harboured a phylogenetically diverse bacterial population compared to fungi which were more clustered together. Predicted genes and protein show that the microbes have remarkable metabolic diversities. Some of the predicted protein sequences obtained in this study represent novel phylotypes indicating the possibility of discovery of bacteria and fungi with important new biomolecules. Metal-utilizing microbial sequences were predicted which imply a possible role for metal oxidation, reduction and remediation potential.

**Keywords**: Bacterial, Fungi, diversity, heavy metal, phylogenetic analysis, functional prediction.

## 1. INTRODUCTION

Heavy metal contamination has become one important constraint to biological processes (Singh *et al*., 2016). The recalcitrant and tenacious nature of heavy metals lead to severe threat to environment. Elevated level of metals in organism may create stress through interference with the function of enzymes or with the information coding units such as deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) in organism (Robert and Ninshata, 2017). Counterbalancing toxicity due to heavy metal requires complex mechanisms at molecular, biochemical, physiological, cellular, tissue and whole organisms level (Singh *et al*., 2016).

Some organisms are less prone to heavy metals toxicity than others which have resulted to some organisms having more tolerance to heavy metals than others. This heavy metal tolerance has become an important example of core biological concepts such as adaptation and evolution (Robert and Ninshata, 2017). Organism can be tolerant to more than one stress, a phenomenon known as co-tolerance and cross-tolerance. Plants and animals may have similar strategy that can lead to metal tolerance. Chelation is a common strategy through metals binding so that they are not able to interact with sensitive cellular process. For instance, Cobbett and Godsbrough (2002) identified two important classes of metal-binding compounds, metallothioneins and phytochelatins in eukaryotic organisms. Another mechanism of tolerance involves sequestration where metals are compartmentalized to remove them from vital tissues or process. Efflux transporters have been identified to play vital roles in regulating metal concentrations in cells such as arsenic and antimony in eukaryote cell (Maciaszczyk-Dziubinsca *et al*., 2012). These mechanisms have been identified in microbes.

Various microbes can tolerate heavy metals through regulatory transport, compartmentalization and production of siderophores (Schalk *et al*., 2011; Long *et al*., 2012). The advent of molecular and genetic tool has contributed to a renewed understanding of the basis for heavy metal tolerance. Roux, *et al* (2011) examined the role that heavy metal tolerant genes play showed that heavy metal tolerance can evolve rapidly and repeatedly in population exposed to heavy metals. Coubot *et al*., (2007) observed that major genes are often responsible for heavy metal tolerance. Therefore, exposure to heavy metals can lead to evolution of new proteins through mutation. Studies on the genetics of heavy metals tolerance contribute greatly in remediation of polluted sites (Maestri and Mamiroli, 2011) by the discovering of potential genes associated with remediation. Microbes may have genes that confer metal tolerance which might be transferred to relatively distantly related species.

Varieties of organisms can be important indicators (bioindicators) of heavy metal pollution because they are non-tolerant to the metals by their absence (Adesuyi *et al*., 2018). Several studies have focused on identification of tolerant organisms using microbial count, bacteria pigmentation and enzymatic activities. This study identifies and enumerates the cultural bacteria and fungi present in heavy metal contaminated soil. This will give insight into the diversity and the underlying genetic basis for tolerance and possible roles of microorganisms present therein with respect to their applications. The knowledge can be used in genetic engineering aimed at solving environmental problems.

## 2. MATERIALS AND METHODS

### 2.1 Soil Sample Treatment and Collection

Soil samples were collected from the Botanical garden, University of Lagos, Akoka, Yaba. The soil samples were air-dried and sieved before they were experimentally contaminated with heavy metal salts of nickel (25, 50, and 75 mg/kg), chromium (50, 100, and 150 mg/kg), lead (150, 300, and 450 mg/kg) and cadmium (1.5, 3.0, and 4.5 mg/kg) in triplicates. The experiment lasted for 30 days while samples were taken at day 1 (immediately after treatment) and at day 30 (end of the experiment). The soils were analysed for their physicochemical characteristics such as organic matter and pH using the method described by Haluschak (2006).

### 2.2 Microbial Isolation and Load

Soil samples were collected at Day 1 and 3, and immediately transported to the laboratory under aseptic condition. The samples were gradient diluted and aliquot were transferred to appropriate media. The media was prepared according to manufacturer's specifications (nutrient agar for bacterial isolation, 28 g/l and Potato Dextrose agar for fungal isolation, 39 g/l). After incubation, the culture plates were observed for colonial characteristics. Then the cultures were subcultured by using a sterile inoculating loop to pick bacterial growth and placed on prepared nutrient agar by streaking method for bacteria and then incubated for 24 hours. Sterile cork borer was used to pick the fungal growth and placed on the newly prepared potato dextrose agar, then incubated for 72 hours. The pure isolates were labeled M1-M7 for bacteria while fungi were labeled M8-M12. All strains were preserved at -80$^0$C in their respective broth (Wang *et al*., 2011).

### 2.3 DNA Extraction and analysis for Fungi and Bacteria

DNA extraction was done using Ctab method as described by Trindade *et al*. (2007). The aliquot DNA was stored at -20ºC for further analysis. The DNA was checked on a Nanodrop spectrophotometer (model 2000 from Thermo Scientific) to quantify the concentration of the extracted DNA and determine purity by measuring at 260/280 nm (Frank *et al.,* 2008).

### 2.4 Polymerase Chain Reaction (PCR) Amplification

The Internal Transcribed Spacer (ITS) gene for characterization of fungi, ITS universal primer set which flank the ITS1, 5.8S and ITS4 region was used. The primers used are

ITS 1: 5' TCC GTA GGT GAA CCT GCG G 3'

ITS 4: 5' TCC TCC GCT TAT TGA TAT GC 3'

PCR conditions include a cycle of initial denaturation at 94°C for 5 min, followed by 35 cycles of each cycle comprised 30secs denaturation at 94°C, 30secs annealing of primer at 55°C, 1.5 min extension at 72°C and a final extension for 7min at 72°C (Wawrik *et al*., 2005).

For bacteria, PCR reaction cocktail consisted of 10 µl of 5x GoTaq colourless reaction, 3 µl of $MgCl_2$, 1 µl of 10 mM of dNTPs mix, 1 µl of 10 pmol each 27F 5'- AGA GTT TGA TC TGG CTC AG-3' and - 1525R, 5'-AAGGAGGTGATCCAGCC-3' primers and 0.3units of Taq DNA polymerase (Promega, USA) made up to 42 µl with sterile distilled water 8µl DNA template. PCR carried out in a GeneAmp 9700 PCR System Thermalcycler (Applied

Biosystem Inc., USA) PCR profile an initial denaturation, 94°C for 5 minutes; 30 cycles, of 94°C for 30s, 50°C for 60s and 72°C for 1 minute 30 seconds ; and a final extension at 72°C for 10 minutes and chill at 4°C. Then GEL electrophoresis was carried out. Analysis was according to manufacturer's guideline (Wawrik *et al*., 2005). The amplified fragments were ethanol purified in order to remove the PCR reagents according to Trindade *et al*. (2007). The amplified samples were then sequenced .

### 2.5 Sequencing

The amplified fragments were sequenced using a Genetic Analyzer 3130xl sequencer from Applied Biosystems using manufacturers' manual while the sequencing kit used was that of Big Dye terminator v3.1 cycle sequencing kit. Bio- Edit software and MEGA 6 were used for all genetic analysis (Wawrik *et al*., 2005).

### 2.6 Phylogenetic Analysis

Phylogenetic analysis was performed on the sequences of isolated microorganisms using MEGA 6 software (Tamura *et al*., 2013). Both the bacteria and fungi isolate sequences were analysed together in order to determine their relationship. Sequences were prepared in FASTA format and aligned Using ClustaW option of the program (Tamura *et al*., 2013). A phylogenetic tree was constructed based on maximum likelihood with 1000 bootstrap. The resulting tree was analysed and interpreted based on its clustering pattern and topology.

### 2.7 Gene and Protein Prediction Analysis

The sequences were automatically annotated using specialized software (FGENESB, Softberry Inc., Mount Kisco, NY, USA) and the RAST server (http://rast.nmpdr.org). The genes that were identified were compared to databases of protein sequences using the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). FGENESB is a suite of operon and gene-finding programs for automatic annotation of bacterial genomes. The FGENESB gene prediction algorithm is based on Markov chain models of coding regions and translation and termination sites
(http://linux1.softberry.com/berry.phtml?topic=fgenesb&group=help&subgroup=gfindb). The genes that were identified were compared to databases of protein sequences using the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

A second round of annotation was done using BacMet (http://bacmet.biomedicine.gu.se)—a manually curated database of antibacterial biocide- and metal-resistance genes. Finally UNIPROT data was assessed to determine their other metabolic activities.

### 2.8 Statistical Analysis

The significance of experiments was tested using one-way Analysis of Variance (ANOVA) ($p<0.01$, 0.05). Also, Least Significant Difference (LSD) test at $P < 0.05$ confidence interval was also done. All data were processed using GraphPad (version 7.0).

## 3. RESULT

### 3.1 Physicochemical characteristic of the soil

The soil pH and organic matter of the control and heavy metals experimentally contaminated soils is presented in Table 1. The pH values decreased at the final day of the experiment for

each of the metal treatment. However, the reduction was not significant (P>0.05) except in the control and 300 mg/kg treat of pb (p<0.05). The highest decrease in soil pH was observed in soil treated with 300 mg/kg contamination of pb (6.50%) while the least was in 75 mg/kg Ni contamination (0.14%).

On the other hand, the soil organic matter decreased significantly (p<0.05) (p<0.01) at the end of the study. However, the reduction in the control treatment was not significant. The soil organic matter reduction was highest in 4.50 mg/kg Cd contaminated soil (30.55%) followed by 3.00 mg/kg Cd contaminated soil (29.05%) while the least was observed in the control soil (8.40%).

**Table 1: Physicochemical characteristics of the soil**

| Metals | Treatments (mg/kg) | pH | | | Organic matter | | |
|---|---|---|---|---|---|---|---|
| | | Initial (Day 1) | Final (Day 30) | Percentage change (%) | Initial (Day 1) | Final (Day 30) | Percentage change (%) |
| Control | 0.00 | 7.53 | 7.08* | 5.98 | 79.02 | 72.38 | 8.40 |
| Pb | 150.00 | 7.21 | 6.89 | 4.44 | 77.90 | 58.05** | 25.48 |
| | 300.00 | 7.08 | 6.62* | 6.50 | 77.32 | 56.45** | 26.99 |
| | 450.00 | 6.89 | 6.57 | 4.64 | 77.00 | 56.45** | 26.69 |
| Cr | 50.00 | 7.29 | 6.97 | 4.39 | 78.12 | 58.39** | 25.26 |
| | 100.00 | 7.15 | 6.88 | 3.78 | 75.12 | 57.27* | 23.76 |
| | 150.00 | 7.11 | 6.73 | 5.34 | 73.03 | 56.18* | 23.07 |
| Ni | 25.00 | 7.30 | 6.98 | 4.38 | 78.46 | 59.07** | 24.71 |
| | 50.00 | 7.17 | 6.85 | 4.46 | 78.25 | 57.61** | 26.38 |
| | 75.00 | 7.09 | 7.08 | 0.14 | 78.21 | 56.31** | 28.00 |
| Cd | 1.50 | 7.32 | 7.00 | 4.37 | 78.84 | 57.99** | 26.45 |
| | 3.00 | 7.21 | 6.89 | 4.44 | 78.45 | 55.66** | 29.05 |
| | 4.50 | 7.10 | 6.78 | 4.51 | 78.07 | 54.22** | 30.55 |

Values with asterisks shows significant difference between the initial day of treatment and the final day. * = p< 0.05, **= p<0.001.

### 3.2 Microbial Colony Count

The bacterial and fungal colony count from soil treated with different concentrations of heavy metals is shown in Table 2. There was a decrease in microbial count in all the treatments in the initial day after contamination. Comparing the control with treatments (final), significant reduction (p<0.05) (p<0.01) was observed in bacterial count for 3.0 and 4.5 mg/kg of cadmium as well as 300 and 450 mg/kg of lead. However, there was stimulation of growth of the organisms in soil treated with heavy metals on the final day (day 30). The bacteria growth of Ni-tolerant strains at 75 mg/kg, Cd-tolerant strains at 1.5 and 3.0 mg/kg, and Pb- tolerant strains at 300 and 450 mg/kg from the soil were significantly higher (p<0.05) (p<0.001) when compared with the treated soil samples of initial day. Bacterial growth was highest in the last day (day 30) of soil treated with 150 mg/kg of chromium ($204x10^8$ CFU/g) while lowest in the initial day (day 1) of treatment with 450 mg/kg of lead ($46x10^8$ CFU/g). Fungi growth was highest ($33x10^8$ CFU/g) in the controlled soil of the final day (day 30) and lowest in the initial soil sample treated with 50 mg/kg of chromium. The microbial growth were both time and dose dependent. Analyses of the microbial groups show that bacteria were significantly more predominant than fungi.

**Table 2: Microbial Colony Count for the Different Heavy Metal Concentration (CFU/g)**

| Metals | Treatments (mg/kg) | Bacteria ($10^{8}$) | | Fungi ($10^{8}$) | |
|---|---|---|---|---|---|
| | | Initial (Day 1) | Final (Day 30) | Initial (Day 1) | Final (Day 30) |
| Control | 0.00 | 182.00 | 122.00 | 21.00 | 33.00 |
| Pb | 150.00 | 76.00 | 140.00 | 22.00 | 28.00 |
| | 300.00 | 52.00* | 168.00** | 13.00 | 19.00 |
| | 450.00 | 46.00* | 154.00* | 16.00 | 22.00 |
| Cr | 50.00 | 84.00 | 148.00 | 9.00 | 16.00 |
| | 100.00 | 118.00 | 180.00 | 15.00 | 23.00 |
| | 150.00 | 130.00 | 204.00 | 14.00 | 26.00 |
| Ni | 25.00 | 92.00 | 158.00 | 10.00 | 12.00 |
| | 50.00 | 88.00 | 142.00 | 18.00 | 23.00 |
| | 75.00 | 76.00 | 178.00* | 13.00 | 17.00 |
| Cd | 1.50 | 68.00 | 162.00* | 19.00 | 22.00 |
| | 3.00 | 46.00* | 138.00* | 10.00 | 15.00 |
| | 4.50 | 28.00** | 86.00 | 13.00 | 18.00 |

* = Significant difference between control and treatment at 0.05 down the vertical axis. ** = Significant between control and treatment at 0.01 down the vertical axis.

### 3.3 DNA Quality and Quantity

Result of the DNA analysis from the microbial clones is shown in Table 3, figure 1 and 2 (bacterial and fungi respectively). Sample M8 had the highest DNA yield (2720.9 ng/µl) which was significantly higher than other samples ($P<0.05$). The other samples were not significantly different ($P<0.05$) from each other. The DNA quality in all the samples were not significantly different from one another ($P<0.05$). The amplified purified PCR products examined in gel electrophoresis revealed a band with molecular size ~1500. More so, fungi isolates examined showed bands with molecular sizes ranged between 480bp - 700bp.

Table 3: Result of DNA quality and quantity in concentration as measured using Nanodrop spectrophotometer (Bacteria: M1-M7; Fungi: M8-M12)

| S/n | Sample ID | DNA quantity (ng/µl) | DNA quality | |
|---|---|---|---|---|
| | | | 260/280 | 260/230 |
| 1 | M1 | 403.5 | 2.13 | 2.22 |
| 2 | M2 | 216.7 | 1.87 | 2.08 |
| 3 | M3 | 329.9 | 1.89 | 1.79 |
| 4 | M4 | 333 | 1.95 | 1.53 |
| 5 | M5 | 288 | 1.51 | 0.79 |
| 6 | M6 | 161.2 | 1.81 | 1.12 |
| 7 | M7 | 197.7 | 1.94 | 1.95 |
| 8 | M8 | 2720.9* | 1.94 | 1.25 |
| 9 | M9 | 304.1 | 1.33 | 0.62 |
| 10 | M 10 | 337.4 | 1.91 | 1.15 |
| 11 | M 11 | 1113.9 | 1.92 | 1.25 |

| | | | | |
|---|---|---|---|---|
| 12 | M 12 | 375.8 | 1.62 | 1.01 |

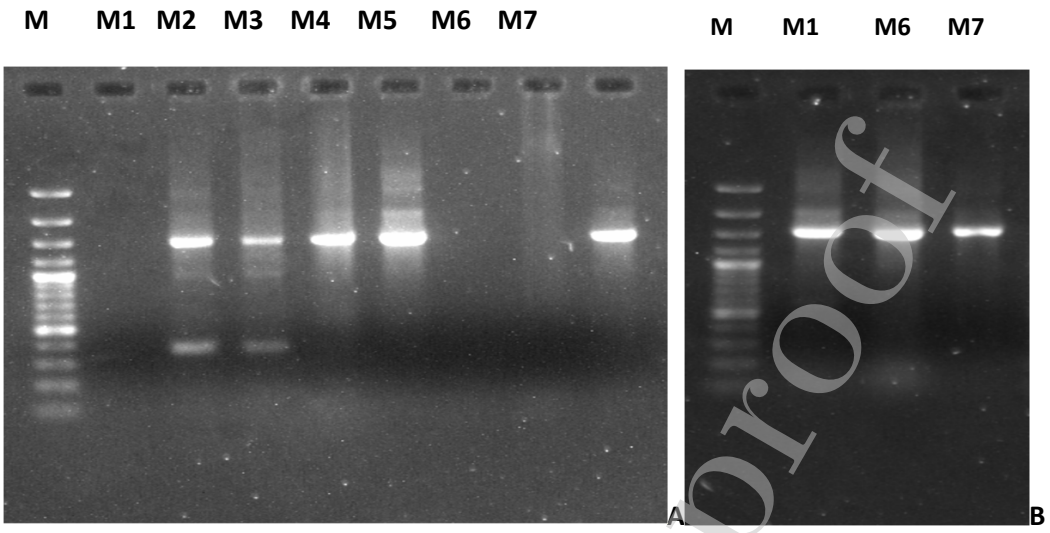


Figure 1: Nucleotide sequences of 16S genes amplified from bacterial samples using 27F and 1525R primers**.** A**.** positive amplification at first run of PCR B. Amplifications in samples 1, 6 and 7 in repeated PCR assay which did not test positive at first testing

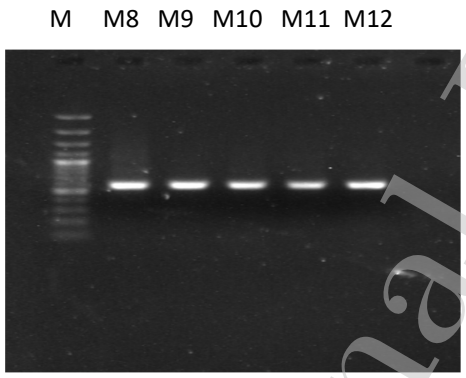


Figure 2: The gel electrophoresis of purified amplified PCR products with band sizes run on the gel for fungi

### 3.4 Molecular Identification of Microbial Isolate

BLAST analysis revealed that all the sequences were bacterial and fungal in nature. They could be classified into eleven species (Table 4). M1 was identified as *Ochrobactrum anthropi* with identity 91%. *Ochrobactrum pseudogrignonense* had 95% identity, *Pseudomonas aeruginosa* had 97% identity and the identity of *Achromobacter xylosoxidans* was 97%. Samples M5, M6 and M7 sequences analysis were *Diphyllobothrium latum, Novosphingobium pentaromativorans* and *Kaistia adipata* respectively with their various respective similarities of 100%, 80% and 98%. The fungal family sequence analysis showed *Aspergillus niger* having similarity of 98%, *Penicillium verruculosum* with identity of 94% while sample M11 and M12

respective identity of 97% and 86% belonged to *Penicillium citrinum* and *Aspergillus flavus* family. The overall guanosine plus cytosine nucleotide (G+C) content of bacteria ranged from 40.2% to 59% while fungi ranged between 57.20% to 61.00%. GC contents of *D. latum* differed significantly ($P<0.05$) from those of other species while the rest of the species were not significantly different from each other.

**Table 4: Identity of the Microbial Isolate from Contaminated Heavy Metal Soil**

| ORGANISM TYPE | SAMPLE ID | ORGANISM | THYMINE | ADENINE | CYTOCINE | GUANINE | G+C | IDENT |
|---|---|---|---|---|---|---|---|---|
| Bacteria | M 1 | *O. antropi* | 22.8 | 22.7 | 27.8 | 26.6 | 54.4 | 91% |
| | M 2 | *O. pseudogrinense* | 24.9 | 21.8 | 25.4 | 27.9 | 53.3 | 95% |
| | M 3 | *P. aeruginosa* | 20.3 | 25.7 | 23.1 | 30.9 | 54.0 | 97% |
| | M 4 | *A. xylosoxidans* | 21.7 | 23.7 | 24.4 | 30.2 | 54.6 | 97% |
| | M 5 | *D. latum* | 29.4 | 30.4 | 19.7 | 20.5 | 40.2* | 100% |
| | M 6 | *N. pentaromativorans* | 21.7 | 19.3 | 29.5 | 29.5 | 59.0 | 80% |
| | M 7 | *K. adipata* | 20.6 | 24.4 | 23.3 | 31.7 | 55.0 | 98% |
| Fungi | M 8 | *A. niger* | 21.0 | 18.1 | 30.2 | 30.8 | 61.0 | 98% |
| | M 10 | *P. verruculosum* | 22.1 | 18.8 | 32.0 | 27.1 | 59.1 | 94% |
| | M 11 | *P. citrinum* | 20.0 | 20.6 | 31.6 | 27.8 | 59.4 | 97% |
| | M 12 | *A. flavus* | 23.0 | 19.8 | 28.0 | 29.2 | 57.2 | 86% |

### 3.5 Phylogenetic Analysis

Phylogenetic constructions based on nucleotide sequence of the organisms were compared with each other. The Neighbour-Joining distance analysis with sequence difference and topology showed distinct lineages (Figure 3). Two major clusters (bacteria and fungi) were observed from the tree topology. Fungi were more clustered than bacteria. *O. antropi, O. pseudogrinense,* D*. latum* and *N. pentaromativorans* are out group.

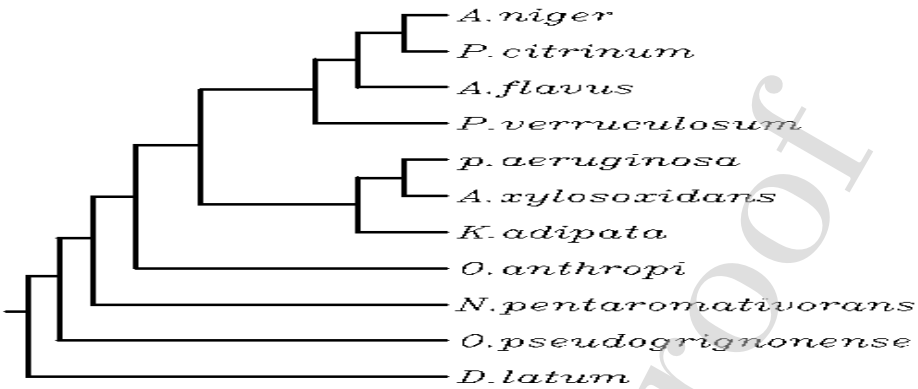


Figure 3: Phylogenetic relationship among all microbial isolates observed in the study.

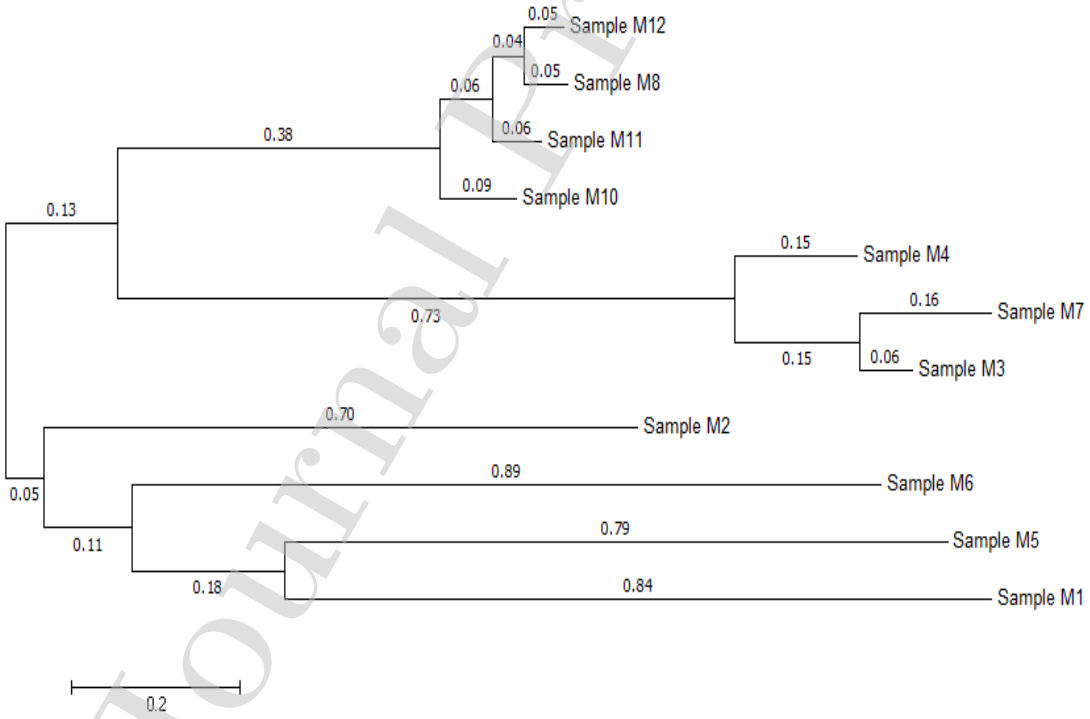


Figure 4: The Neighbour-Joining distance analysis

### 3.6 Remarkable Metabolic Diversities of The Microbial Group

The different biochemical functions (enzyme/proteins) expressed in the different microorganisms are shown in Table 5. *D. latum* contains the highest number of biochemical

functions (31 proteins) followed by K. *adipata* (28 proteins). *A. niger* had the lowest number of proteins (14 proteins). Methyltransferase is present in all the microbes. Peptide binding proteins, metallothionine family and ATPase P are present in 10 out of the 11 microbes. Cop D genes product, coppertranslocation P-type ATPase, ATPase G, copper binding protein and tonB dependent sidersphore are only present in *D. latum*. Molybdate ABC transporter substrate-bin is present in *K. Adipata.* Cation transporter ATPase, E1-E2 family protein is present in *p. Veruoleulosum.* Zinc/cadmium/mecury/lead-translocation proteins only present in *O. antropi. Tran-2-enoyl-CoA-reductase* is only present in *P. citrinum.* Oxidases were present in *O. atropi, D. latum, P. veruculous, P. citrinum.* Efflux protein was present in all except *P. verruculos, K. adipata* and *A. niger.*

Table 5: Remarkable Predicted Enzymes/Proteins in the Microbial Group using BACMET and UNIPROT database

| **Enzymes/proteins** | *O. antropi,* | *O. pseudogrin* | *P. aeruginosa* | *A. xylosoxida* | *.D. Latum* | *N. pentaroma tiegma* | *K.. adipata* | *A. niger* | *P. verruculos* | *P. citrinum* | *A. flavus* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| methyltransferase | + | + | + | + | + | + | + | + | + | + | + |
| UbiE methyl tranferase | - | - | - | - | - | + | + | - | - | + | + |
| copper-transporting P-type ATPase | - | - | - | - | + | + | + | - | - | - | + |
| arsenite S-adenosylmethyltransferase | - | - | + | - | + | + | + | + | + | + | + |
| copper resistance protein CopC | + | + | + | + | + | - | + | - | - | - | - |
| cation transporting P-type ATPase | + | - | + | + | + | + | + | + | - | + | + |
| ATPase P | - | + | + | + | + | + | + | + | + | + | + |
| putative nickel resistance protein | - | - | - | - | + | - | + | - | + | + | - |
| Twin-arginine translocation pathway sign... | + | + | + | + | + | + | + | + | - | - | + |
| zinc/cadmium/mercury/lead-transporting | + | - | + | - | + | - | + | - | + | - | + |
| cation transport ATPase, E1-E2 family protein | - | - | - | - | - | - | - | - | + | - | - |
| molybdate ABC transporter substrate-bin.. | - | - | - | - | - | - | + | - | - | - | - |
| molecular chaperone DNA K | + | + | + | + | + | - | + | + | + | - | - |
| metallothionein, family 14 | + | + | + | + | - | + | + | + | + | + | + |
| copper oxidase | + | - | - | + | - | - | + | + | + | + | + |
| ABC transporter family protein | + | + | - | - | + | - | + | + | - | + | - |
| zinc/cadmium/mercury/lead-translocation | + | - | - | - | - | - | - | - | - | - | - |
| Mercuric reductase | - | - | + | + | + | - | + | - | + | + | - |
| MerA gene | - | - | - | - | - | - | + | - | - | - | + |
| Nickel resistance protein | - | - | - | + | + | - | + | - | - | + | - |
| Copper resistance protein | + | - | - | + | - | - | + | + | + | - | - |
| ArsM gene product | - | - | - | - | - | - | + | + | - | - | - |
| Ferroxidase | - | - | - | + | + | - | + | + | - | + | + |
| Extracellular solute binding protein | - | - | - | - | + | - | + | - | - | | - |
| Major facilitator super family protein | - | - | + | + | + | + | + | - | - | - | - |
| Sortase sorted copper resistance surface | - | - | - | - | + | - | + | - | | - | - |
| Oxidases | + | - | - | - | + | - | - | - | + | + | - |
| $Fe^{2+}/Zn^{2+}$ uptake regulation protein | + | - | - | - | - | - | - | - | - | - | - |
| Efflux protein | + | + | + | + | + | + | - | - | - | + | + |
| Transporter | - | - | + | - | - | - | - | - | - | - | - |
| CopD gene product | - | - | - | - | + | - | - | - | - | - | - |
| Acid shock protein precursor | - | - | - | - | + | - | - | - | - | - | - |
| Copper translocation P-type ATPase | - | - | - | - | + | - | - | - | - | - | - |
| ATPase G | - | - | - | - | + | - | - | | - | - | - |
| Copper binding protein | - | - | - | - | + | - | - | - | - | - | - |
| YegM gene product | - | - | - | - | + | - | - | - | - | - | - |
| tonB dependent siderosphore | + | - | - | - | + | - | - | | - | + | - |
| TOBE domain containing protein | - | - | - | - | + | - | - | - | - | - | - |
| Solute binding protein | - | - | - | - | + | - | - | - | - | - | - |
| Trans-2-enoyl-CoA-reductase | - | - | - | - | - | - | - | - | - | + | - |
| Molybdenum-pterin binding domain protein | - | - | - | - | - | - | - | - | - | - | + |
| Perisplasmic solute binding protein | - | - | - | - | - | - | - | - | - | - | + |
| NADH cytochrome B5 reductase | - | - | - | - | - | - | - | + | + | + | - |
| Genomic island protein protein | + | + | + | + | + | - | + | - | - | - | - |
| Virulence associated protein E | - | + | + | - | - | + | - | - | + | - | - |
| Collagen | - | + | + | - | - | + | + | - | - | - | - |
| Peptide binding protein | + | + | + | + | + | + | + | + | + | + | - |
| Bacterial SH3 domain | + | + | + | + | + | + | + | - | - | - | - |
| FecR | + | + | - | - | - | - | - | - | - | - | - |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| H/ACA ribonuclease | | | | | | | | | | | |

+ = present. - = absent.

### 3.7 Predicted Protein Sequences of the Isolated Microbes and their Energy Metabolism

The Predicted proteins were distributed into different functional group (table 6). When compared with the closest group (*Geobacter sulfurreducen*), various protein sequences were predicated from the genome of the organisms. In *O. antropi,* 5 protein sequences were predicted and out of these, 4 were distributed into Cr, Mn, Fe, Co, Zn, Ni, Cu and Cd energy metabolism while one protein sequence was found to be a novel phylotype. *O. pseudogrinonense* contained 5 protein sequences which were distributed into Cr, Ni, Co, Cr, $H_2O_2$, $NaNo_2$ metabolism.

Three proteins sequences were predicted from *P. aeruginosa* while 4 were predicted from *A. xylosoxidans*. *A. xylosoxidans,* 3 protein sequences were predicted to perform metabolism of Cr, Mn, Fe, Co, Zn, Ni, Cu, Cd, Ga, etc while one was predicted to be of the novel phylotype. *D. latum* latum was predicted to harbour three novel proteins while the remain one was predicted to perform metabolic activity. *N. pentaromativorans* was predicted to has 4 proteins with energy metabolism of Cr and Cu. In *K. adipata,* 3 protein sequences were predicted to metabolise Cr, As and Sb. In *A. niger*, 3 protein sequences were predicted and were distributed into Cr, Zn, Cr metabolism. *P. verruculosum*, harboured 5 protein sequences distributed in Cr, Zn, W, As, n-hexane metabolism. Two protein sequences were predicted from *P. citrinum* for Cr, Zn and W metabolism. Two out of 3 proteins sequences in *A. flavus* were predicted to metabolize Cr and Cu while the remain one is a novel phylotype.

Table 6: The Predicted Protein Sequences and their Energy Metabolism using FGENESB and BLAST

| Organism | Closest group | Predicted protein sequence | Predicted energy metabolism |
|---|---|---|---|
| *O. antropi* | Geobacter | MASQESVVAGASQPHHPLLLDPIIGEGNSVCDTSTPVVVDPPGGATKTNTPLIEVPQSVS VVSRKQIEMQNAQSVTEMLRYVPGVSIETYGPDPKGYDWIFMRGFNAQSTSSYQNGLRQL SNSYTFFRTDPYELDSIEVLRGPVSSLYGQSDAGGLVNRVTKKPQAEASHEASVEYGS | Cr, Mn, Fe, Co, Zn, Ni, Cu, Cd. |
| | | VTQIADNHANSYEDRQILQFVGWPKHIRQRSGTVGGQLFFVATIVRGQNSAIGQASLTRI NALALGQYRCKHYF | |
| | | MRVDMKSRTQTGRGQGSQALLKTGLSAHLLAASALSFDHGIHVRYGW | Cr, SDS, SDC |
| | | MPLLLAGFSCYFMAATQPLRGLDTSETRSNDR | New protein |
| | | SANGGTITALGTAFDVKLIDRAVTVSVVEHAVSVAFGQSDPVRLDEGWQITYSGDEAALP QRADQQTVEAWRNDRIIFEDVPLSRVLSELERYRRGRIFLTDTEIGNMPVTAIFDTRDAE AALATIAETLPVRVLNGSGWVTVVTRR | Cr, Mo |
| *O. pseudogrinonense* | Geobacter | MVHNGVAVDGRGAPVRLWGRASHLVRVTSAGLTAQHLVIAVLVVALAVAVCILGTCVPCM PDGSNNHL | Cr, Ni, Co. |
| | | LQNDCGPLEGGVSSEPRIRPSGGEDNDLISIDLQRFQLRMYRWNRCNCLFLRIIRCKTAS HFCWKCYK | |
| | | MTWSNRTIQGRSLIVLALAVQFASMTVSPALAFEIFGV | |
| | | LASEFDHGSVRRLVTQSSLIPSLIQPTSRLQEDRQMLTFVDADVKAHIGSASGHRWDEAI FVATGPNSAIGQAADTD | |
| | | RPRLPDALPSGSYLNGQPDRVQSQLLPATALRRCFSQSVPHRWPNTGGGRRKHYHRDHWK CDSWGDEGYMACAETSSMATTIDLDGLAQLNNACTGYCPESMRH | Cr, $H_2O_2$, CCCP, BAC, $NaNo_2$ |
| *P. aeruginosa* | Geobacter | LRPYSPGGRLIALAAPTKISRIPTASRHRLRRGLPGYLILFAPPRFRTSVSVSVQVVAFA DWWFLPYIYAFHRLHKEMSTTRLPYC | Cr, Triclosan |
| | | MWSTSKQRGEILPGLDMLRTFQRWIGAFGNSDTGAAWLSSARVVRCWVKSRNERNPCP | |
| | | GPSGVTYSYSDDHYVLTVPLAVRLATSGATHSHGVTGGVYKARERIHRDILIHDY | ‘’ |
| *A. Xylosoxidans* | Geobacter | VAGRPLKPATDRRLGEPLPHQLANPISAAPIVQGLAIPTFPRRAYAVLATLSGSYPPLPG RFRYITHPSATGHQTELRAAARLAL | Cr, Cu |
| | | VGIARPCTIGAADIGLASWWGNGSPRRRSVAGLRGRPATLGLRHGPDSYGRAAVGNFGQW GETLIQPSPRVLMKAFGIVKHFWLERKRQWVNTPAKLTVPARISTG | Cr, Mn, Fe, Co, Zn, Ni, Cu, Cd, |
| | | MQFQELSSGDFTSFFPNQPTRDFTPSNSD | |
| | | MREEPPIGEAAYLGLTTDRFDASEAWGGKQD | New protein |
| *D. latum* | Geobacter | MDALRKKWTIYVLPTNYNTSRFVGAWYLLYLKTSGYALIICRQLSCFPLNVVKIIV | |
| | | MPPRNAMCYNLWAAHRWSIFFLTRPFCLHNMPLGNFFEGKYYIGIQRCLIFPGDFGKVRT NPNHI | New protein |
| | | LPPPRGLVFVSRKSRRSSCKGVSARSCYFTRKKGNNHAHRQYSVLMHPNFMAICK | |
| | | MFYCIHLIENGMVTIVFAFRVSIGQAAHFARMAIKQDFQKAAYSDH | New protein. |
| | | LHLRSVRPWGSLFEGQTEWCSNVYFTSLQISMSRSVLKTGSLPFIRYIYIF | New protein |
| *N. pentaromativorans* | Geobacter | MPVETHDAAKEIVMRRDIRSFSADMPAMVSGSSQKLSALRLATSSRCSKHNRRARAIVHH RHTHPEKRQNLYHRLVRAAGKAMNKNCALSECDA | Cr, Cu. |
| | | VSVCSCTCMRPGARLRPQVLFHRTDNQTVVRTDQDQYIMPVVAHSSHARLVTG | |
| | | MHVHEQTDTPSVCFRRSLKPHANSAFAVEDPHEVQGLASAMSPAAARLSADQDICSGHCE CRHGRLRGGLFFQATPLDPVTYPMPLTQLDWRRCSHWRGHLCYHPPSS | |
| | | MLSPAVFLSCADEAITRLEGLVPTQSNRSDEQIEWQQFATPPRLAWMAAKACAISAAE | Cr, Cu. |
| *K. adipata* | Geobacter | VLYNPKAVITHAAWLDQACAHCPIFPTAASRRSLGRVSVPVWLVILSDQLRIVALVSHYL TN | Cr, As, Sb. |
| | | VFFLISTNFTSTLGVPLTSPGLKIASMKGSSEVEPRDFTPHLTIRLRTLYAQ | |
| | | MWFNSKQRAEPYQLLTCLVWVPETVSFKFGWREHRCCIGCQSARVVRCWVKSTQRAATPR LLVCHHSVGLL | |
| *A. niger* | Geobacter | MPEPRDPLLKVLTDCIQSTQTARFQTVFVLGSPAGTGPGGRGALPGGRQCGGPAEATGYR | Cr, Zn |
| | | MDREVGPKGPALGIPLAYFDMLKFSGYPYLIRGQPGKNGWKTSAGAGQSYRACDRSPIRS RIGRGAAAAFRARPPGEGDGDPTHKPGLRAAMTLGQACPPEYQGAQCAFKDSMIH | Cr, SDC |
| *P. verruculosum* | Geobacter | MLYVASSHTHRSKMWHPRALREKKTPLVGTAALFFGGAAPPPPRWGNPPPPPRGHGYLPP LFFLLLPPEKQIYFLFFFF | Cr, Zn, W, SDC |
| | | MVISPPFFFFSSPQKNKYIFYFFFFKTPLLPVPSFWGAGRKPPPPATRWPRWAPKPTGV | |
| | | MILPRGNLRKGSIPGGRGLRGPNLPTPCLYTPVAFGRAHRGHLVAGGTSSPGPAPRRSAP | Cr, As |
| | | MPGGAMCVQRFDDSRNSAIHITYRISLRSSSMPEPRDPLLKVLTIFIVLRQPIFIRVHGA LRRGAGPGDDVPPATRWPRWARPKATGV | Cr, n-hexane |
| | | MPVRASFLPSSTACVLGAVPPGTCPKGSGDVRLVLERMGLCHSLGTGLRGFVTSIVSHGD LGSRDSHP | |
| *P. citrinum* | Geobacter | IITECGPLGAQPPTRVARTYVASAGPAPADGPPERCLKLQSETYNEI | |
| | | LGVGWRRPGLLERVTKPHTLEDRTRCRRCLSGPSPRRGGRGPTHKPGLRAAMTLGQACPP EYQRAQCAFKNSMIH | Cr, Zn, W, SDC. |

| | | | |
|---|---|---|---|
| *A. flavus* | Geobacter | MVLQRTHRYRDIHNFSPHFSPFGTYFGTRRSIVESF | New protein |
| | | MAGPPKQLRYSKHGWEVGLARNPTLGNGPSVGEPAEGSLPSVGFLASPTSHPCLLYLSCF GGPAIHGRRGLSAPGPRPPETPRTLSDLVKSELIVSQSVKTFNNGSLGSGIDEERSEMR | Cr, Cu |
| | | MPVRASLLPIKHGLCVGSSSPLRGGRAPKAAAAPRPILERMGLCHRSVGPAGACRTQINL FQVDLRIQLGIEP | Cr, Cu |

## 4. DISCUSSION

The environmental microbial community composition and diversity are sensitive key indicators to the impact of pollutant on the microbial ecology system over time (Zhao *et al.,* 2014). The study was aimed at evaluating the microbial abundance/diversities (bacteria and fungi) present in heavy metal contaminated soil. Research has found out that metabolic activities of microorganisms in an environment can often be connected to the pH of the system that is under examination. Our study has found out that the metals slightly reduced the soil pH. pH reduction below 7.0 may have negative impact on the metabolic activities of the microbes. Adeleye *et al*., (2018) reported that pH range of 7 to 8 can support optimum microbial activities. However, it was found out that the soil pH was suitable for the survival of the microbes. Moreso, the organic matter reduced at the end of the study which suggest energy metabolism. Thus, the microbes utilize this organic matter for energy.

The study noticed reduction of microbial load. The reduction in microbial load at the initial day (day 1) of the treatment can be attributed to destructive effect of the heavy metals to susceptible microorganisms. This is because heavy metals produce stress condition which eliminates most of the non-tolerant organisms (Ekpo and Ebeagwu, 2009). Lima e Silva *et al.* (2012) observed that metals have an inhibitory effect on gelatinase production which negatively impacted on organism's survival. From the result obtained, cadmium and lead had more impact on bacteria compared to fungi which suggests fungi to be more resistant. This is similar to the findings of Gupta *et al.,* (2016) that showed that cadmium and lead are highly toxic to organisms exposed to them. The increase in microbial counts observed at the end of this study suggests recovery of microbes from stress imposed by heavy metal contamination.

This study found out that *A. niger* yielded significantly higher DNA quantity than others. This may suggest the possibility of DNA quantity to influence the GC contents (Khare *et al.*, 2014). *A. niger* having higher DNA yield may have better physiological functions. 16S rRNA gene sequence analysis of contaminated microbial community revealed a bacteria diversity represented by seven species of bacteria including Pseudomonas species. Sequence analysis of rRNA gene library clones derived from bacterial consortia in cadmium contaminated soil by Gupta *et al.,* (2016) revealed two cadmium resistant strains of pseudomonas species. Out of the four fungi species identified in our study, two belonged to Aspergilus species. Fazli *et al*. (2015) reported cadmium tolerance and bioremediation capacity of seven isolates including *Aspergillus* species. Iram *et al*., (2009; 2012) also observed most common fungal strains such as *Aspergillus niger*, *Aspergillus sp, Fusarium sp*., and *Penicillium sp*. have tolerance against the zinc, lead, nickel and cadmium. The dominance of aspergilus suggests their possibility towards precipitation of multi metals.

The identified bacteria strains in this study have high GC contents. This could have helped them towards tolerance to heavy metals. One important feature of the GC base pair is its higher

thermal stability compared with the AT base pair. In bacteria, an increase in GC content correlates with a broader tolerance range for a species (Smarda *et al*., 2014). The GC content of the bacteria was slightly less than observed GC by Wu *et al*., (2012) in soil. Wu *et al*. (2012) reported that GC content of the microbial communities can be globally and actively influenced by the environment. Sometimes, bacteria are able to survive under both harsh and favourable environments. The sequence signatures of their genomes' compositional changes may not always be directly related to their characteristics.

Fungal resistance against metals can be dependent on strain's biological function (Gupta *et al.,* 2016). Indeed, the range of GC contents in fungi was greater than that encountered in bacteria and broadly similar to the values reported for soil samples by Smarda *et al* (2014) which is characteristic of microbe from soil. *A. niger* can be a very important bioindicator for use in biotechnology because it yielded high DNA quantity and has a GC content similar to established GC in soil for active physiological functions. The findings of Smarda *et al* (2014) showed that GC-rich genes facilitate response to environmental stress. It can facilitate complex gene regulation. Therefore, an improved response to environmental conditions might be facilitated by GC-rich genes. Generally, fungi having a higher GC content gives it more advantage to be utilized in a wide range of environmental applications such as remediation.

Phylogenetic analysis revealed that the polluted soil harboured a phylogenetically diverse bacterial population belonging to five clusters of orthologous groups while fungi were more clustered together in one group. Reason may be the combination of selective factors, proximity and functional capacity of microbes (Ning and Beiko, 2015). From a selective point of view, similar environmental conditions such as site pH, oxygen availability, or adhesion potential may support the growth of taxonomically similar sets (Ding and Schloss, 2014, Simón-Soro *et al.,* 2013). Our study reveals that the contaminated soils have diverse bacterial populations. Functionally, phylogenetically distant lineages can share common functional features and functions. Ning and Beiko (2015) also opined that functional similarities exist between operational taxonomic units (OTUs) that belong to different high-level taxonomic groups. Most of microbial sequences analysed in different taxonomic divisions could be related to representatives with known metabolic traits.

The different metabolic functions were predicted in the different microorganisms. These data suggest that these microbes have diverse metabolic potential that could be exploited for environmental and other applications. For instance, the presence of methyltransferase can catalyze the transfer of various chemical groups from one compound to another. Also, arsenite S-adenosylmethyltransferase methylates arsenite to form methylarsonate, (Me-$AsO_3H_2$), which is reduced by methylarsonate reductase to methylarsonite. Methylarsonite is converted into the much less toxic compound dimethylarsinate. Such catalysis can lead to the formation of several methylated intermediates which result to loss of arsenic, from both the medium and the cells (Qin *et al.,* 2006)**.** The twin-arginine translocation (Tat) pathway exports proteins across the cytoplasmic membrane and is responsible for the proper extracytoplasmic localization of proteins involved in a variety of cellular functions (McDonough *et al* 2005). ABC (ATP-binding cassette) proteins serve as active pumps to shuttle substrates and xenobiotic conjugates across a variety of biological membranes (Kretzschmar *et al.,* 2011).

From our present study, *D. latum* can perform highest metabolic activities followed by *K. adipata* while *A. niger* may perform lowest metabolic activities. The presence of reductases suggest that some organisms might have the capability to reduce nickel, cadmium, lead and Cr (VI) while the presence of metallothionine and efflux transporters indicate their remediation potentials towards heavy metals. *MerA* gene, a key enzyme in the process of methanogenesis predicted in *K. adipata* and *A. flavus,* shows these organisms can utilize methane as enormous carbon and energy source. Bidle *et al* (1999) found out that methanogens and methanotrophs can possess this gene and bacterial species related to those found in other hydrocarbon environments.

With sequence analysis, it is possible to predict the nature of energy metabolism of microorganisms present in a specific microbial community (Hugenholtz *et al*., 1998). This study found out that most of the sequences in different taxonomic divisions could be related to representatives with known metabolic traits. Organisms with such physiological properties could, therefore, be expected to reside in heavy metal contaminated site. *O. antropi and A. xylosoxidans* has been predicted to metabolize Cr, Mn, Fe, Co, Zn, Ni, Cu, Cd. Interestingly, such attribute gives it much potential to reside in an environment that is contaminated with such heavy metals. It is evident from this study that the organisms are chromium reducers since the chromium metabolism is valid in all their pathways. They can as well be used in bioremediation of heavy metals due to the presence of metallothionine.

Moreover, most of the predicted protein sequences obtained in this study represented novel phylotypes indicating the possibility of discovery of bacteria and fungi with biotechnologically important new biomolecules. Organisms with the novel protein are *A. antropi, A. xylosoxidans, D. latum* and *A. flavus.* These data suggest that the microbes have diverse metabolic potential that could be exploited for additional environmental or biotechnological applications.

**Conclusion**

The results from microbial data are noteworthy in several regards. Enumeration of microbial counts shows initial suppression of microbial counts and subsequent growth. Metal-utilizing microbial sequences were found in these soils suggests a possible role for metal oxidation and reduction. This can be exploited for the remediation of metals polluted sites. Active chromium energy pathways were predicted to be occurring in all the microbes examined, attributed to their high tolerance to chromium. Lead utilizers did not appear to be highly abundant suggesting lead toxicity. These data show that most of the microbes have a diverse metabolic potential that could be exploited for additional environmental or biotechnological applications. It will now be possible to design probes for *in situ* quantitation of the abundance of particular phylotypes in the soil for possible remediation of metal polluted soil.

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**The Molecular Study of Microbial and Functional Diversity of Resistant Microbes in Heavy Metal Contaminated Soil**

**Highlights:**

- Heavy metal contamination has become one important constraint to biological processes.
- Phylogenetic analysis revealed that the contaminated soil harboured a phylogenetically diverse bacterial population compared to fungi.
- Genes and protein show that the microbes have remarkable metabolic diversities.
- Some of the predicted protein sequences obtained in this study represent novel phylotypes indicating the possibility of discovery of bacteria and fungi with important new biomolecules.
- The knowledge can be used in genetic engineering aimed at solving environmental problems.

**Declaration of interests**

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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