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16S metagenomic analysis and taxonomic distribution of enriched microbial consortia capable of simultaneous biodegradation of organochlorines by illumina platform

Saghee Madhu Raju[1,2] and Rajkumar Bidlan[2#]

[1]Research Scholar, Rayalaseema University, Kurnool, India
[2]Dr. Bidlan's Research Institute, Hyderabad, India
[#]Present Address: Department of Biotechnology, Delhi Technological University, Shahbad Daulatpur, Delhi, India

ABSTRACT

Organochlorine pesticides are ubiquitous group of recalcitrant molecules that accumulate in food chains and have inherent toxic effects and adverse health effects. To circumvent the problem, microbial communities are found to be promising candidates for degrading the organochlorine pesticide's and removal of residues. In this study, a novel microbial consortium isolated from Yamuna and Godavari rivers capable of simultaneous biodegradation of organochlorine pesticides (DDT and Lindane) was subjected to metagenomic sequencing. This consortia used was enriched by progressively increasing concentrations of Lindane and DDT (organochlorine pesticides) for months till a stable Lindane and DDT tolerant population was established, and found to be degrading mixture of organochlorine pesticides with concentrations up to 30 ppm of DDT and Lindane. Currently, in the realm of our knowledge very few metagenomic analysis were carried out to characterize the consortia and understand the biodiversity of microbial communities in the riverine ecosystems, that was found to be unique and highly efficient in bio-degradation of organochlorine pesticides. The study concluded biodiversity with a shannon alpha-diversity index of 3.0317 and identified 871 species with *Brevundimonas diminuta* (previously assigned to the genus *Pseudomanas*) having abundance ratio of 17.57 % followed by *Stenotrophomonas acidaminiphila* in the mixed consortium and deciphered the systematic and functional contexts within riverine metagenome.

KEY WORDS: MICROBIAL CONSORTIUM, BIOREMEDIATION, DICHLORODIPHENYLTRICHLOROETHANE, HEXACHLOROCYCLOHEXANE, LINDANE, METAGENOMICS, AMPLICON, ILLUMINA

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INTRODUCTION

Organochlorine pesticides (OCPs) were excessively used globally for pest control and agricultural purposes and public health control (Aktar *et al.,* 2009). OCPs are ubiquitous group of recalcitrant molecules that degrade slowly and accumulate through food chains (Amrita *et al.,* 2007) and produce a significant magnification at each tropic level. One of the major sinks for persistent organic pollutants discharged into environment is the water ecosystem i.e. rivers and lake beds. Organochlorine pesticides were detected in rivers where higher concentrations of Lindane, Endosulfan and DDT were found (Pandey *et al.,* 2011)and the residue presence was even detected in drinking and bottled water (Mutiyar *et al.,* 2011). It is highly essential and vital to remove these pollutants from the environment, from the sinks primarily water and soil ecosystems to finally eliminate their residues. Microorganisms are found to be potential degraders of organochlorine compounds, notably water and soil habitants belonging to genera *Bacillus*, *Pseudomonas*, *Arthrobacter, Klebsiella, Acinetobacter, Alcaligenes, Flavobacterium* and *Micrococcus* were found to be effective bio-degraders (Kafılzadeh *et al.,* 2014 Eric et al 2017).

In this paper, we present the findings of metagenomic analysis leveraging next-generation sequencing (NGS) performed using Hiseq 2500 system (Kumar *et al.,* 2015). The Metagenomics was carried on the defined microbial consortium identified from water ecosystems, Yamuna River (North India) and Godavari River (South India) capable of simultaneous degradation of organochlorine pesticides (Bidlan, 2003). The taxonomic distribution and biodiversity among the microbial consortium was established that comprised of interacting microbial populations (Oulas A *et al.,* 2015 Eric *et al.*, 2017).

MATERIALS AND METHODS

Lindane γ-HCH (insecticidal isomer) was of 97% purity and obtained from Sigma- Aldrich, USA. DDT, 99.4% pure, was donated by Hindustan Insecticides Ltd, India. All other chemicals and reagents used in the study were of analytical grade and were purchased from standard manufacturers. The microbial consortium subjected to Metagenomic analysis was isolated from Yamuna (North India) and Godavari rivers (South India) and enriched by progressively increasing concentrations of Lindane and DDT (organochlorine pesticides) for months till a stable Lindane and DDT tolerant population was established in the flask (Bidlan 2003). DNA was isolated using Xcelgen Bacterial gDNA kit and quality of gDNA was checked on 0.8 % agarose gel (loaded 5 µl) for the single intact band. The gel was run at 110 V for 30 min. 1 µl of each sample was loaded in Nanodrop 8000 for determining A260/280 ratio. The DNA was quantified using QubitdsDNA HS Assay kit (Life Tech). 1 µl of each sample was used for determining concentration using Qubit® 2.0 Fluorometer (Ogata *et al.*, 1990).

The amplicon library was prepared using Nextera XT Index Kit (Illuminainc) as per the 16S Metagenomic Sequencing Library preparation (Eric J. *et al.,* 2017). Primers for the amplification of the V3-V4 hyper-variable region of 16S rDNA gene of bacteria and Archaea are used for this study (Table-1).

The amplicons with the Illumina adaptors were amplified by using i5 and i7 primers that add multiplexing index sequences as well as common adapters required for cluster generation (P5 and P7) as per the standard Illumina requirements (Esling *et al.,* 2015). The amplicon libraries were purified by 1X AMpureXP beads and checked on Agilent High Sensitivity (HS) chip on Bioanalyzer 2100 and quantified on fluorometer by QubitdsDNA HS Assay kit (Life Technologies).

After obtaining the Qubit concentration for the library and the mean peak size from Bioanalyser profile, library was loaded onto HiSeq 2500 at appropriate concentration (10-20 pM) for cluster generation and sequencing (Sharpton, 2014). Paired-End sequencing allows the template fragments to be sequenced in both the forward and reverse directions. Kit reagents were used in binding of samples to complementary adapter oligos on flow cell. The adapters were designed to allow selective cleavage of the forward strands after re-synthesis of the reverse strand during sequencing. The copied reverse strand was then used to sequence from the opposite end of the fragments (Blomquist *et al.,* 2013).

The libraries were prepared from sample after amplifying the V3-V4 region of the 16S segment. Size of library was 644 bp and the library was sequenced using the Illumina sequencing chemistry to generate ~150 Mb of data per sample. The next generation sequencing (NGS) for the sample was performed on the Illumina platform, HiSeq 2500 (Kumar *et al.*, 2015).

Paired end sequence stitching was carried out for sample using FLASH (Fast Length Adjustment of Short reads) with parameter minimum overlap of 10 bases to merge paired-end reads from next-generation sequencing experiments (Tanja *et al.*, 2011). QIIME (Quantitative Insight into Microbial Ecology) was used for analyzing 16S metagenome data from NGS platforms and, is implemented in python language (Kuczynski *et al.,* 2011). Chimeras composed of DNA from two or more microbial species which are artifacts made during the PCR process. They were filtered first, using usearch61 algorithm (de novo, abundance-based), from the Flashed/stitched data then taken for analysis. A total of 2,44,283 non chimeric sequences from sample were used for OTU pick. In the next step, the similar sequences were clustered,

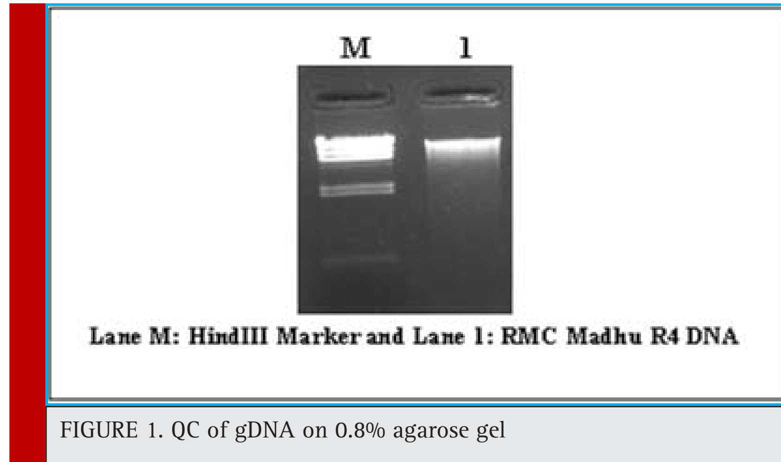


FIGURE 1. QC of gDNA on 0.8% agarose gel

Table 1. Primers used in the Study

Oligo Name	Oligo Sequence (5' to 3')	Length of primer	Product size (Approx.)
Prokaryote V3-Forward	CCTACGGGNBGCASCAG	17	~ 460 bps
Prokaryote V4-Reverse	GACTACNVGGGTATCTAATCC	21	

i.e., sequences coming from the same genus, together into one representative taxonomic unit called as Operational Taxonomic Unit (OTU). The basis of this sequence clustering is 97% sequence similarity and implemented through UCLUST algorithm. OTU-picking identified highly similar sequences across the samples and provided a platform for comparisons of community structure. All the sequences from all the samples were clustered into Operational Taxonomic Units (OTUs) based on their sequence similarity.

A representative sequence was selected for each of these OTU's picked. As these OTU's made up of a group of sequences, they were represented through one sequence to assign a taxonomic name to the group. Thus repre-

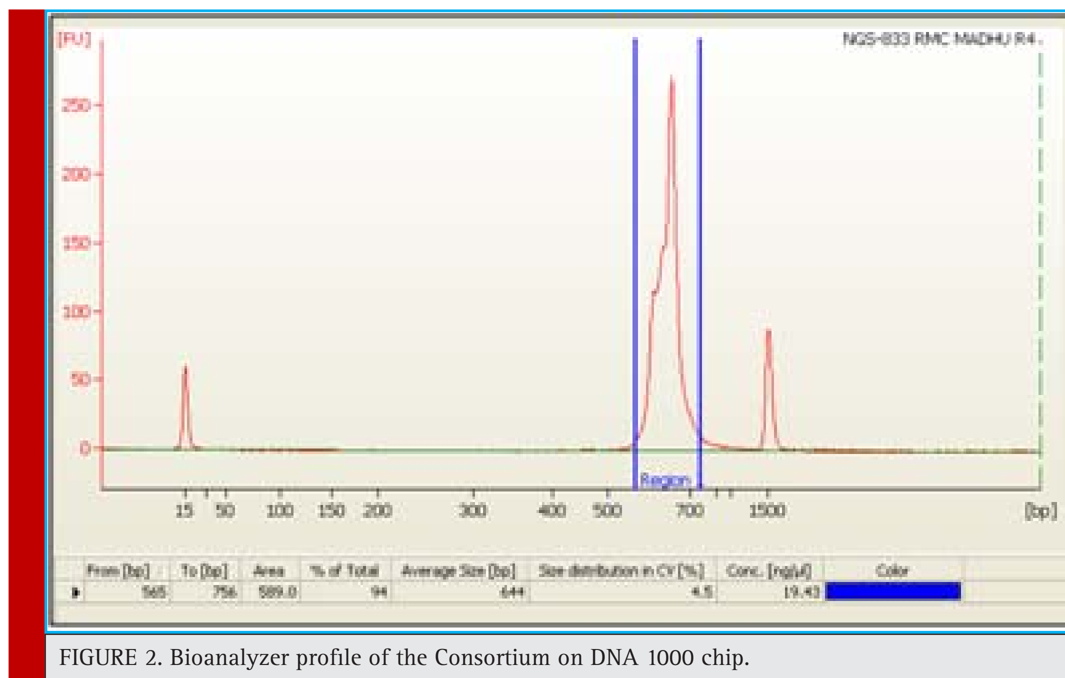


FIGURE 2. Bioanalyzer profile of the Consortium on DNA 1000 chip.

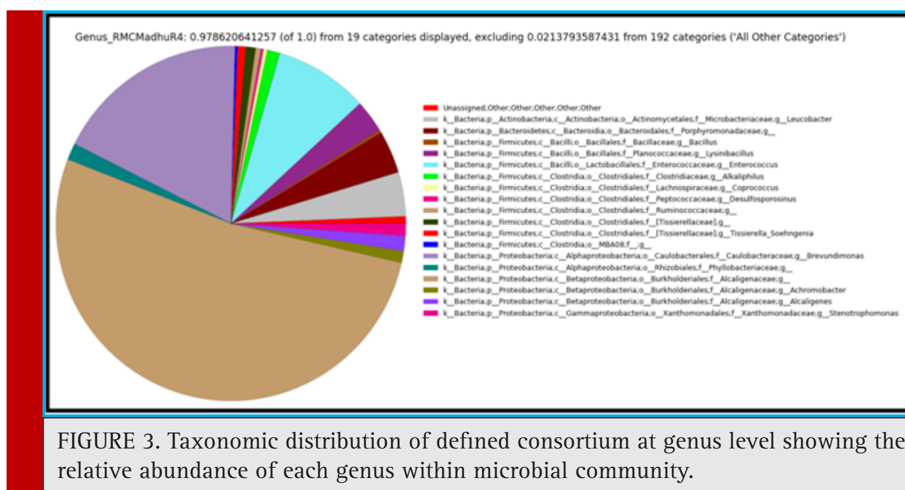


FIGURE 3. Taxonomic distribution of defined consortium at genus level showing the relative abundance of each genus within microbial community.

Table 2. Relative Abundance of the genus in the consortium

Taxonomy (Genus)	Abundance
Brevundimonas	17.60%
Enterococcus	8.50%
Leucobacter	3.90%
Lysinibacillus	2.90%
Alcaligenes	1.40%

Table 3. Alpha-diversity

Sample	shannon	Observed species	chao1
Consortiaenriched with pesticides	3.0317	871	871

sentative set of OTUs were prepared which consist of 2,911 sequences. With representative sequence in hand, the taxonomic names to these sequences were assigned at 90% sequence similarity. This is done using UCLUST algorithm, where query is representative sequences and subjects that are curated sequences at greengenes database.

RESULTS AND DISCUSSION

Diversity calculation for each sample was performed and compared the types of communities, using the taxonomic assignments.

α-DIVERSITY

α-Diversity or within-sample diversity is calculated using an OTU table which gives idea about species richness. Alpha diversity summarizes the diversity of organisms in a sample using different metrics in a habi-

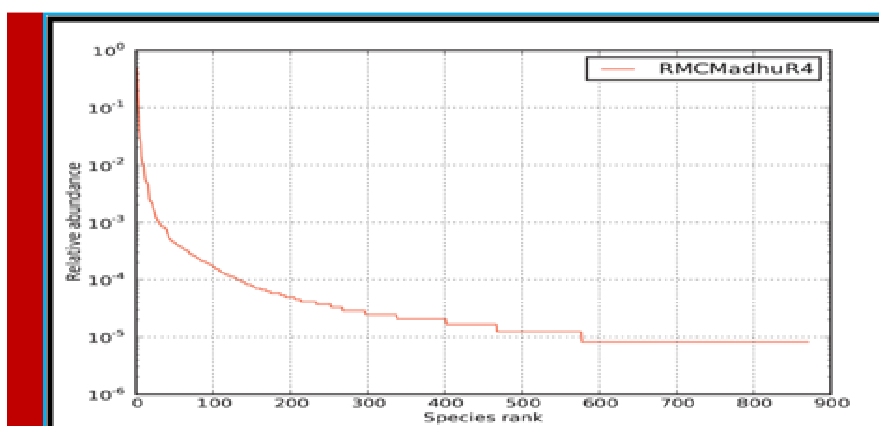
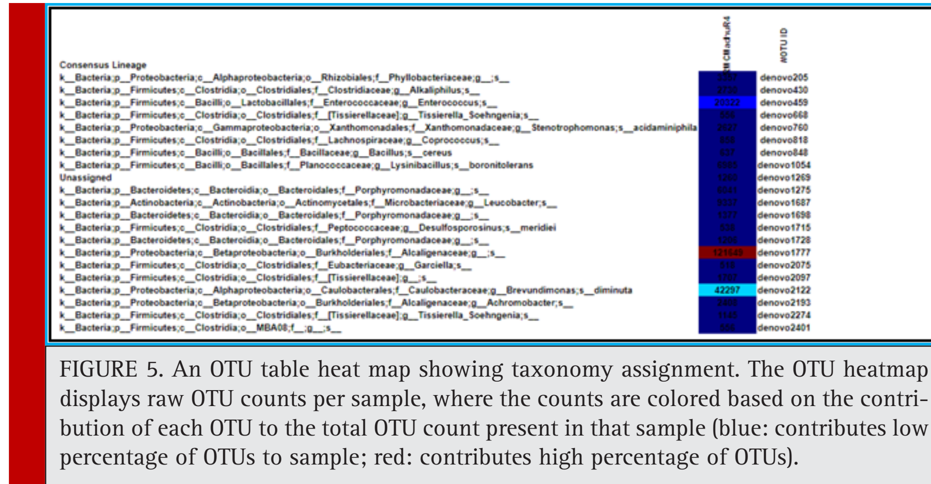


FIGURE 4. Rank abundance plot of Microbial Consortium



Consensus Lineage	CMadhuR4	#OTU ID
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Phyllobacteriaceae;g__;s__	3357	denovo205
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Alkaliphilus;s__	2730	denovo430
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Enterococcaceae;g__Enterococcus;s__	20322	denovo459
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Tissierellaceae];g__Tissierella_Soehngenia;s__	556	denovo668
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae;g__Stenotrophomonas;s__acidaminiphila	2627	denovo760
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Coprococcus;s__	858	denovo818
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae;g__Bacillus;s__cereus	637	denovo848
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Planococcaceae;g__Lysinibacillus;s__boronitolerans	6985	denovo1054
Unassigned	1260	denovo1269
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;g__;s__	6041	denovo1275
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Microbacteriaceae;g__Leucobacter;s__	9337	denovo1687
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;g__;s__	1377	denovo1698
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptococcaceae;g__Desulfosporosinus;s__meridiei	538	denovo1715
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;g__;s__	1206	denovo1728
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Alcaligenaceae;g__;s__	121649	denovo1777
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Eubacteriaceae;g__Garciella;s__	518	denovo2075
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Tissierellaceae];g__;s__	1707	denovo2097
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Caulobacterales;f__Caulobacteraceae;g__Brevundimonas;s__diminuta	42297	denovo2122
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Alcaligenaceae;g__Achromobacter;s__	2408	denovo2193
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Tissierellaceae];g__Tissierella_Soehngenia;s__	1145	denovo2274
k__Bacteria;p__Firmicutes;c__Clostridia;o__MBA08;f__;g__;s__	556	denovo2401

FIGURE 5. An OTU table heat map showing taxonomy assignment. The OTU heatmap displays raw OTU counts per sample, where the counts are colored based on the contribution of each OTU to the total OTU count present in that sample (blue: contributes low percentage of OTUs to sample; red: contributes high percentage of OTUs).

tat/sample. The below table summarizes the α-Diversity, where the columns correspond to alpha diversity metrics and the rows correspond to samples and their calculated diversity measurements (Lozupone, Catherine *et al.,* 2007).

The rank abundance curve representing species richness and species evenness is shown in Figure 4. Species richness can be viewed as the number of different species on the chart and species evenness is derived from the slope of the line that fits the graph.

The OTU table was developed to visualise as a heatmap where each row corresponds to an OTU and each column corresponds to a sample. The higher the relative abundance of an OTU in a sample, the more intense the color at the corresponding position in the heatmap.

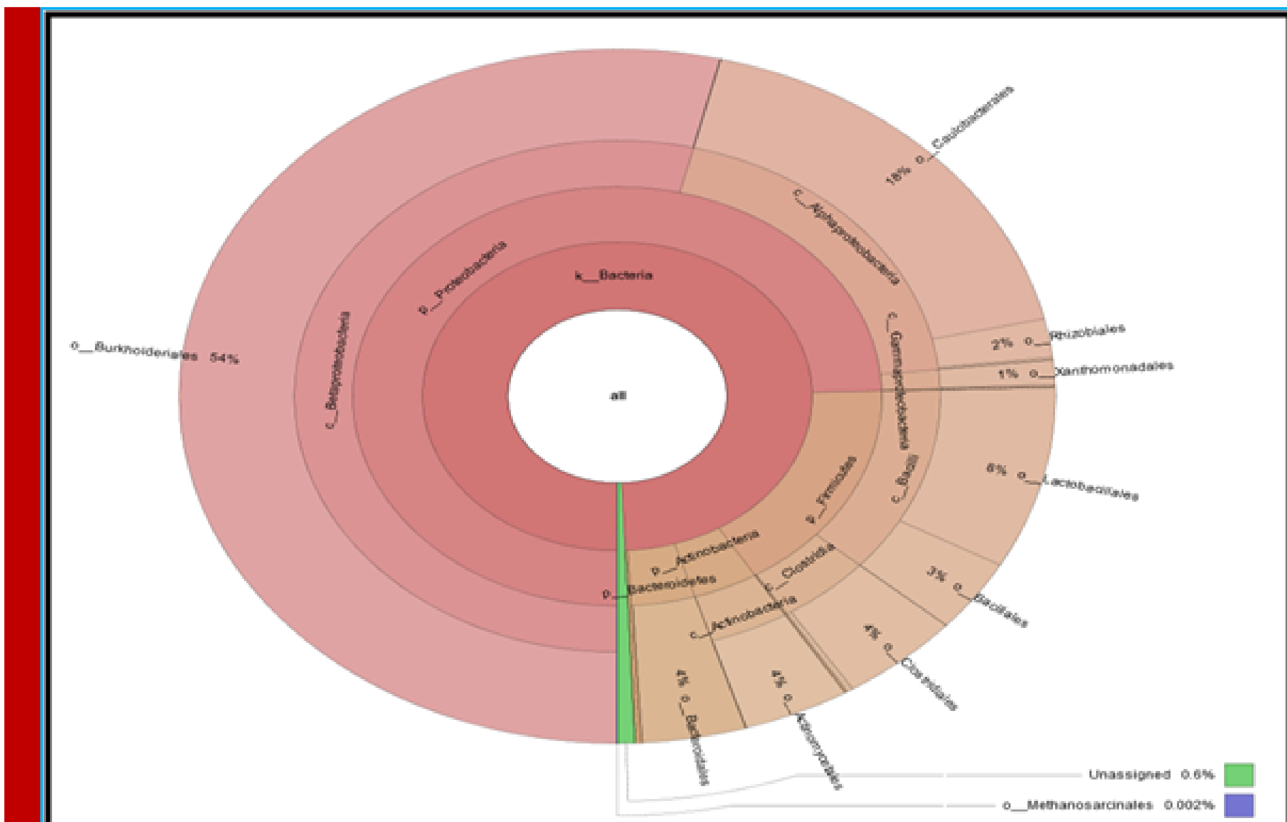


FIGURE 6. Krona graph for taxonomy assignment for Microbial Consortium at order level.

Table 4. Organisms Identified through Metagenomic Characterization by Hiseq2500, Illumina Platform, NGS	
Total Reads	**5,88,408**
Total number of stitched reads	2,81,957
Number of OTUs	2,911
Abundant phylum	Proteobacteria
Abundant class	Betaproteobacteria
Abundant order	Burkholderiales
Abundant family	Alcaligenaceae
Abundant genus	Brevundimonas
Abundant species	dimunita
shannon alpha-diversity	3.0317
Observed species	871

Krona graph tool was used to display abundance and hierarchy simultaneously using a radial space-filling display. The Krona chart features a red-green colour gradient signifying average value within each taxon (Ondov *et al.,* 2011)

CONCLUSION

The metagenomic sequencing comprehensively sampled all genes in all organisms present in microbial consortia and evaluated bacterial diversity and abundance of microbes (Table-4). This study also identified at genotypic level any unculturable microorganisms that are otherwise difficult or impossible to analyze (Handelsman J. *et al.,* 2004). The study concluded biodiversity with a shannon alpha-diversity of 3.0317 and identified 871 species genotypically, with *Brevundimonas diminuta* having abundance ratio of 17.57 % followed by *Stenotrophomonas acidaminiphila* in the mixed consortium. This consortia characterized was found to be degrading mixture of organochlorine pesticides with concentrations up to 30 ppm of DDT and Lindane confirmed by GC-MS/MS. Although research has been carried out using on single strain and single compound of organochlorines, the current study data provides an insight on how bacterial communities in mixed consortia are taxonomically distributed and their biodiversity. The metagenomic characterization identified the consortia in a definitive manner which acts as promising solution for bioremediation of organochlorine mixtures.

NCBI Sequence Accession Number: DNA sequences obtained have been deposited at National Center for Biotechnology Information (NCBI) Sequence Read Archive under the bioproject ID PRJNA420925 and accession codeSRX348847.

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