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Metagenomic Approaches for Studying Microbial Community of Soil: Review

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Abstract: ***In the era of "omics" there are various techniques have been identified such as genomics, proteomics, transcriptomics, metabolomics, metagenomics etc. amongst all of these the metagenomic plays significant role in the discoveries of microbial world. The discovery of new microbial world in different ecological environment and their gene provides dynamic information about their diversity and function. The results of molecular approaches reveal the microbial based application in human health and some others such as agriculture, food industries, medicine etc. This revies provides the summarized information about molecular techniques and their aspects in the study of microbial community of soil. It also provides information that the metagenomic can combines with other molecular techniques help more detailed in microbial community and explores that application to scientific world.***

Keywords: DNA, Metagenomics, Metabolomics, Metatranscriptomics, Metaproteomic, FISH, RAPD, DGGE, SSCP, PLFA, ARDRA, RISA

1. Introduction

The soil horizon is considered as one of the most diverse environments on earth, currently it estimated that thousands of different microbial species inhabit in 1g of soil (Delmont et al., 2011) such as *Archaea*, *Bacteria*, *Fungi* and *Protist* which plays significant role in various biogeochemical cycles. They also play important role in the soil formation, erosion control, nutrient cycling, plant health and many more. (Gosal & Mehta, 2015) The soil is formed by aggregation of different geological parent matter. It composed of different minerals particles with different shape, size and chemical characteristics along with various organic compounds which are generated during decomposition states. (Adedeji & Babalola, 2020) The soil is highly abundant with prokaryotic organisms which forms the large component of soil biomass. Usually, the microorganism is strongly adhering to the soil particles (e. g., on sand particles, clay or organic matter complex, pores between the aggregates) result into formation of microhabitat which plays crucial role in soil microbiology. The activity of such biomolecules is generally influenced by the secretion of extracellular enzymes by such microhabitat and accumulated in clay minerals. The metabolic activity and survival rates of such microhabitat microorganisms is strongly affected by the availability of nutrient and water content. (Daniel, 2005)

In soil, the major activities are done by the soil microbes. The organic matters are degraded by various enzymatic reaction which are usually involves in the life processes. Moreover, Soil is strong reservoir of organic carbon and abundant in prokaryotic organisms which are strongly involved in decomposition of such organic matters. Although, having high concentration of organic matter but very low concentration of organic carbon is available to the microorganisms. This is due to the majority of organic matter obtained from plants, animals and microorganisms are converted into the humus which is non degraded by the microorganisms present in the soil. The microbial diversity is distinguished as alpha, beta and gamma diversity. (1) α - diversity – diversity within the local habitat, (2) β - diversity - change of species composition along with gradient and (3) γ - diversity – diversity comprising with different habitat.

Now are days increasing anthropogenic activities introduce various contaminants into the environment beyond their limitation which result into increasing the pollution in the atmosphere. These contaminants can be physical or chemical or biological, occurring, e. g., metal pollutants, volcanic eruptions, atmospheric fallouts, etc. Rapid industrialization and exploitation of natural resources has generated various types of pollutants that could be degradable or nondegradable in nature. Such contaminants be heavy metals, hydrocarbons, chlorinated hydrocarbons, organochlorine compounds, nitroaromatic compounds, organophosphates, monochlorinated pesticides and herbicides, radionuclides have resulted serious environmental and health concern. (Panigrahi et al., 2018) However, majority of soil bacteria are involve in biogeochemical process of soil, to assess the microbial community structure and their function, it is necessary to analyze the environmental samples through DNA/RNA based molecular approach. (Giovannoni et al., 1990)

The soil microbial community can be analysis through culturable as well non - culturable aspects. The culturable approach provides minor information about the total bacterial population present in the soil. (Kirk et al., 2004) In nature, bacteria acquire genes from closely or distantly related organisms by horizontal gene transfer which results into increasing the speciation within microbiota. The diversity analysis is important in order to:

- Comparer the distribution of organism and genetic diversity
- To increase the knowledge about functional role of diversity

- Infer the regulation of biodiversity. (Gosal & Mehta, 2015)

As already known, that the cultural dependent approach provides the information about soil bacterial community which are capable to grow under in In vivo conditions. However, direct microscopic count will provide more accurate results in the terms of quantity of organisms. Due to uncultivability, the major portion of bacterial diversity is still difficult to interpret. (Đokić et al., 2010) To overcome with this problem, most recent emergence of metagenomics allows the study of complete microbial community by sequencing DNA directly extracted from the environmental sample. (Nannipieri et al., 2003)

Table 1: Application of traditional metagenomic approaches to study the microbial diversity.

Metagenomic tools	Application	Sample	References
ARDR	To assess the shift in microbial community caused by the copper contaminated soil	Copper polluted soil	(Smit et al., 1997)
	To estimate the soil microbial diversity and comparison of culture bacterial diversity with environmental diversity	Sandy loam and organic soil	(Øvreås & Torsvik, 1998)
	Assessment of microbial diversity with help of 16S rRNA gene amplicon	Heavy metal contaminated sandy soil	(Ellis et al., 2003)
	To investigate the heterotrophic microbial community and characterization of long - term contaminated soil with chromium	Tannery soil contaminated with chromium	(Viti & Giovannetti, 2005)
	Analyze the microbial community and microbial functions in Heavy metal contaminated soil	Heavy meatal contaminated clay soil	(Pérez - De - Mora et al., 2006)
	Comparison of bacterial community of two agriculture soil contaminated with copper	Agriculture soil polluted with copper	(Dell'Amico et al., 2008)
	Assessment of rhizosphere soil bacterial community to tolerate zinc and lead	Rhizosphere soil	(W. hui Zhang et al., 2012)
DGGE	Assessment of bacterial community by combine study of PCR - DGGE analysis	Slit loam soil	(Gelsomino et al., 1999)
	Combine study of FAME and PCR - DGGE to analyze the microbial community of long - term contaminated soil	Heavy metal contaminated soil	(Kozdrój & van Elsas, 2001)
	Analysis of bacterial community of 3 - chlorobenzote contaminated soil to reveals the microbial profile of 3 - CB degrading bacteria.	Forest soil contaminated with 3 - CB	(Morimoto et al., 2005)
	To assessment of protease activity of rhizospheres and bulk soil bacterial community	Rhizosphere soil	(Sakurai et al., 2007)
	To assess the microbial community composition and their enzymatic activity against As and Cd	As and Cd contaminated soil.	(Lorenz et al., 2006)
FISH	Combine use of 16S rRNA to analyze the bacterial community of polychlorinated biphenyl contaminated soil	PCB contaminated soil	(Nogales et al., 2001)
	Microbial community analysis of Siberian tundra soil	Tundra soil	(Kobabe et al., 2004)
	To assessment of microbial community soil by combine study of FISH - MAR	Agricultural soil	(Wagner et al., 2006)
	The estimation of rhizobial bacterial community and their genetic diversity against the As.	Rhizosphere soil contaminated with Arsenic	(Cavalca et al., 2010)
	Combine study of CARD - FISH to assessment of microbial community associated with hydrocarbon contaminated sediments.	Hydrocarbon contaminated sediments	(Tischer et al., 2012)
RAPD	Characterization and functional analysis of microbial diversity	Soil and aquatic sample	(Franklin et al., 1999)
	To assessment of microbial diversity of agriculture soil	Agriculture soil	(Yang et al., 2004)
	to study the effect of Pb, Zn and Cd on microbial community and degradation of isoproturon	Heavy metal contaminated soil	(Suhadolc et al., 2004)
	To investigate the microbial community and their enzymatic activity in the presence of plant species against the Pb and Cd contaminant	Paddy soil	(Gao, Zhou, Mao, Zhi, Zhang, et al., 2010)
	To analyze the change in genetic and microbial diversity by use of lands	Land soil	(Gao, Zhou, Mao, Zhi, & Shi, 2010)
RISA	To analysis of microbial community of fresh water	Lake water	(Fisher & Triplett, 1999a)
	To estimation of microbial community capable to degradation of PAH under low aerobic and nitrate reducing conditions	Arctic site soil sample contaminated with polycyclic aromatic hydrocarbons	(Eriksson et al., 2003)
	Hydrocarbon degrading microbial community in oil spill contaminated site	Water sample contaminated with petroleum oil	(Kostka et al., 2011)
	Combine study of PCR - DGGE with RISA to analyze the microbial community of rhizosphere soil	Rhizosphere soil	(Srivastava et al., 2016)
SCCP	Used to analysis of culture and non - cultured microbial community of rhizosphere soil	Rhizosphere soil	(Schwieger & Tebbe, 1998)

	Combine study of PCR - SSCP with 16S rDNA sequencing to analyze the soil diversity	Slit loam soil	(Stach et al., 2001)
	To estimate the microbial diversity of rhizosphere soil by use of specialized PCR primers	Rhizosphere soil	(Schmalenberger et al., 2001)
	The automated SCCP combined with DGGE to assessment of microbial diversity of acid mine drainage	Acid mine drainages soils	(Hong et al., 2007)

The genome of the organism in soil diversity contains all the information about diversity amongst the species. The use of high throughout put molecular approach for analysis of microbial communities can results into identify the microbial diversity in different species habitat. (Fierer et al., 2012) The metagenomic approaches are mainly carried out it two dimension (e. g., targeted metagenomic and shotgun metagenomics). The targeted metagenomics involve the study of single gene of interest in such way that it reveals compete genetic information such genes present in environment. This method is implying to study how a particular gene is diverse and abundant in the environment, it is also frequently utilized to study how diverse small subunit of rRNA (16S/18S rRNA) are in the sample. The targeted metagenomics deals with the direct extraction of environmental DNA with gene of interest being amplified (e. g., using PCR and primers designing based on targeted gene of interest to be amplified), then NGS (next generation sequencing) is being used to sequence the gene of interest which result in to production of huge data which further analysis by various bioinformatic tools. While shotgun metagenomic involves the extraction of environmental DNA, fragmented by restriction enzyme activities followed by the sequencing libraries preparation which is further used to analyze the complete genome of sample via NGS. The major limitation of metagenomic is, it requires complete sequencing of sample to obtain the good coverage of genome of organism to analyze their functional potential in environment. (Gana et al., 2019).

The assessment of bacterial diversity can be achieved by three different approaches (Fig.1) such as

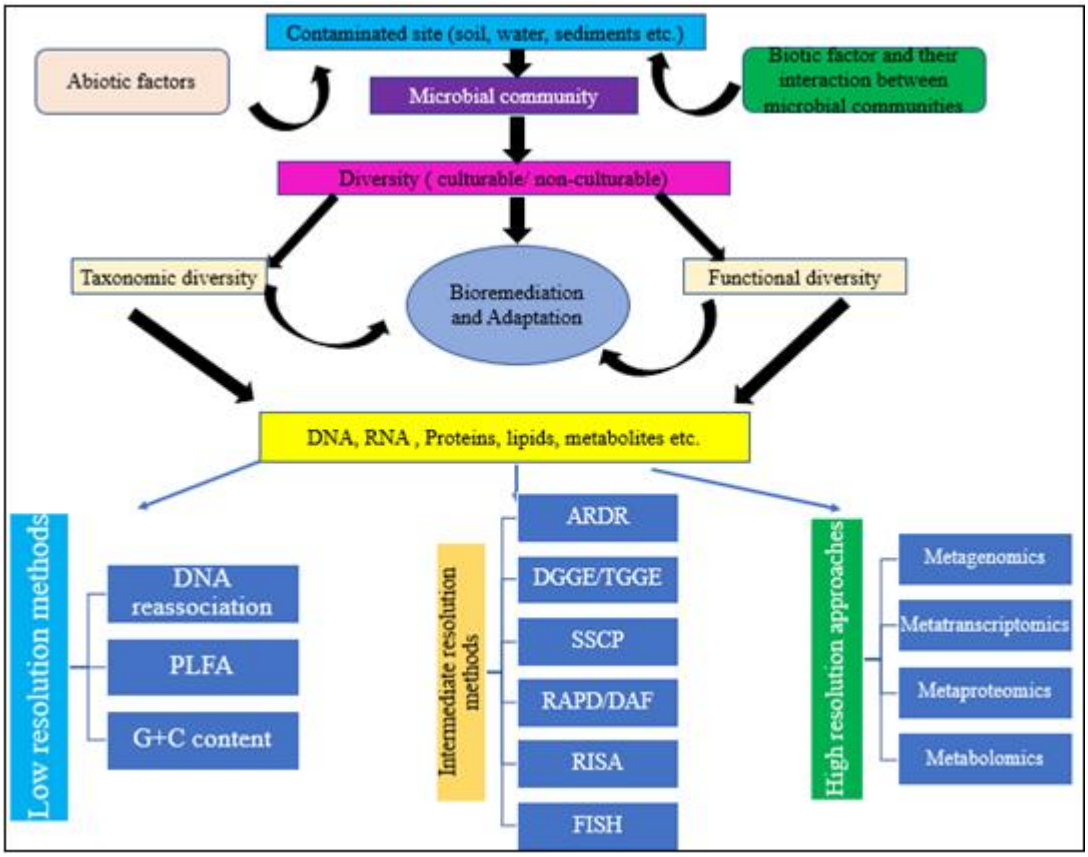

Figure 1: Methods for assessment of microbial diversity 1) Low resolution methods 2) Intermediate resolution methods 3) High resolution methods.

Low resolution approach

Low resolution methods are traditional molecular approaches based on nucleic acids or some molecular marker to study the microbial diversity and their functions and characterization in different environmental conditions

DNA reassociation

DNA reassociation is simple molecular approach which deals with the extraction of DNA from environmental sample followed by purification, denaturation and reannealing. The direct extraction of environmental DNA from microbial community contains the mixture of different DNA obtained from different microbiota. The proportion of DNA concentration also varies due to distribution of microorganism in environment also varies. This method is based on hybridization or reassociation of DNA molecules which is also depends on the sequence similarities. As, the complexity of DNA increases the rate of hybridization or

reassociation will decreases. (Gosal & Mehta, 2015; Torsvik, Goksoyr, et al., 1990) Moreover, the rate of DNA hybridization provides the information about variety of sequence present in the environment which directly reflect the microbial and genetic diversity of organism. (Torsvik, Salte, et al., 1990) The DNA reassociation can be used for the analysis of genome size and complexity. There are various factors which need to monitor during the DNA reassociation such as, the concentration of DNA, time of incubation. (Øvreås & Torsvik, 1998) the microbial community diversity is depends on the amount of genetic information present in the community and their distribution amongst the environment (evenness). (Torsvik, Salte, et al., 1990) use the approach to study the genetic diversity of bacterial population against the phenotypic approach.

PLFA (Phospholipid Fatty Acid Analysis)

Phospholipid fatty acid analysis is culture independent approach which use for analysis of microbial community structure. The sample for PLFA analysis is driven from whole viable microbial community, and each species provides the significant biomass. (Singh et al., 2006) PLFA are crucial component of cell membrane and it can be used as potential biomarkers to study the microbial diversity as they are available in significant proportion under the natural environment. (Gosal & Mehta, 2015; Kozdrój & van Elsas, 2001) The phospholipids are abundantly present in microbial cell, cell membrane and not in other part of cell as storage component. Usually, the cell membrane is composed of multilayer lipid membrane and degradation of membrane cause death of microbial cells. Moreover, the phospholipids can act as important bioindicator of living microbial biomass. (Fakruddin, 2015) The fatty acid molecules plays important role in determination of microbial community as their specificity towards the microbial species. The changes in fatty acid patterns under different environmental conditions could be used as specific biomarkers to describe the microbial community and their physiological state under microbial taxa. (Gosal & Mehta, 2015; Misko & Germida, 2002) The phospholipids of microbial cells are easily extracted from the soil samples, which allows the study of large proportion of microbial community resent in soil. (Nannipieri et al., 2003).

There some limitation of this approaches like (1) this method is based on the specific fatty acid signature molecules. Therefore, a specific signature molecule is not known for all kind of microorganism and many times the specific fatty acid is not linked with specific group or microorganisms. (2) As method depends on the fatty acid molecules, if any changes in signature molecules occurs due to various environmental factors will results into generation of false community data and (3) Bacteria capable to produce wide range of PLFA and fatty acids depends on the growth and environmental conditions. It is not necessary that signature molecule produce by specific group of bacteria will be specific to the same group of species in all condition.

Intermediate resolution methods

The community fingerprinting approaches are based on conserved genes like rRNA gene to be known as Intermediate resolution methods. This method involves the amplification of 16S rRNA genes by extraction of environmental DNA from soil, followed by cloning and sequencing to determine the genetic diversity of microbial community present natural environment. These methods can be differed from each other on the basis of length, restriction patterns and denaturation.

Amplified ribosomal DNA restriction analysis (ARDRA)

Amplified ribosomal DNA restriction analysis is one of the most commonly used tools to study the microbial diversity which depends on the DNA polymorphism. (Deng et al., 2008) Here, the PCR amplified 16s rRNA fragments are cut or digested by specific restriction enzymes at specific site and then the small fragments are separated via gel electrophoresis. Based on restriction site different DNA will cut at different location which results in formation of unique profile of the community which being to be analyzed. (Gich et al., 2000) Banding patterns in ARDRA can be used to screen clones or be used to measure bacterial community structure (Kirk et al., 2004)

The ARDRA techniques provide information about the genetic diversity among the cultivable bacteria in natural population. Most research data reveals that the organic soil isolates having higher biodiversity than the other soil. (Øvreås & Torsvik, 1998) they found that sandy loam soil having higher biodiversity than the organic soil, but when ARDRA was combined with REP - PCR it was found that the higher biodiversity among the cultivable bacteria. This was due to the ARDRA provides information about the organisms belonging to one specific phylogenetic group. They also conclude that even if bacteria belong to different phylogenetic groups, can functionally related. However, when this technique applied to the complex community it is not useful. On comparative study of high chronic chromium contaminated and uncontaminated soil it was observed that the heterotrophic cultivable bacteria were higher in contaminated site as compare to the uncontaminated soil, this is due to the negative effect of chromium on bacterial community leads to shifting of microbial load due to reduction in decomposition of nutrient cycle and organic matter. (Viti & Giovannetti, 2005) They found that the cultivable heterotrophic bacterial community from Chromium contaminated soil having high Cr (IV) tolerance were belong to the same genus represent in the unpolluted soil. The ARDRA approach for high Pb contaminated soil by (W. hui Zhang et al., 2012) is one of the most appropriate way to demonstrate the bacterial community of rhizosphere soil. In this study they found fifteen different ARDRA restriction pattern of Amplified 16S rDNA amongst 47 isolates from which majorly belongs to the Pb resistant. This leads to finding of almost four major groups and 8 different genera from which *Pseudomonas* was commonly found in all samples. The ratio of Pb resistant bacteria in rhizosphere soil was different in the high, moderate and low Pb contaminated soil.

The ARDRA approaches provide significant data with regards to bacterial diversity and changes amongst the community. This method is more effective, especially when combining with other molecular methods. Molecular techniques provide wide range to study the microbial diversity. However, much more study and optimization are requiring for understating soil microbial ecosystem.

DGGE (Denaturing Gradient Gel Electrophoresis)

DGGE is advanced form of gel electrophoresis which is used to separation of PCR amplified DNA fragments on electrophoresis contain polyacrylamide gel containing linear gradient of denatured DNA. (Panigrahi et al., 2018) (Fig.2) the separation of DNA fragments is based on their electrophoretic mobility in gel. The drawback of DGGE/TGGE is high exposure of chemical denaturant or high temperature can lead to complete dissociation of DNA strands. To overcome with this, during PCR step 5'GC clamped (30 - 50kb) forward primers are used. The amplified DNA fragments ranges from 400 - 500bp which are separated on gel. (Chakraborty & Das, 2014).

The DGGE pattern has been utilized to understand the Bacterial community of Cd and as contaminate soil. The study reveals that the major microorganisms including bacteria (e. g., *Firmicutes, Cytophaga - Flavobacterium group, Actinobacteria, Proteobacteria*) and fungi. These microbes show strong enzyme activity against the menaquinones as increasing soil contamination with Cadmium which results into the shifting of microbial community. (Lorenz et al., 2006) In another approach DGGE methods applied to study the microbial community structure of long - term contamination with heavy metals. The study indicates that, there is no change in the number of heterotrophic bacterial community in all Silesian soils. (Kozdrój & van Elsas, 2001) . The higher organic carbon in high polluted sites indicates that, the accumulation of heavy metals by bacterial community. A study has demonstrated for identification and characterization for *benA* gene coding for 3 - chlorobenzoate degradation. *ben A* bands phylogenetically analyzed by NJ tree construction using the nucleotide sequence of DGGE bands and indicates that the diverse group of bacteria mostly related to *Burkholderia spp*. (Morimoto et al., 2005)

A comparative study has been carried out to investigate the effect of organic and inorganic fertilizer on soil protease activity of rhizosphere and bulk soil. A study reveals that the alkaline metalloprotease (apr) and neutral metalloprotease (npr) genes activity affected by the Organic and inorganic fertilizers treatment resulting into both bacterial community plays different roles in different soil. TheDGGE and Phylogenetic analyses indicates that most of *apr* are closely belongs to *Pseudomonas fluorescens* and *npr to Bacillus magaterium.* (Sakurai et al., 2007)

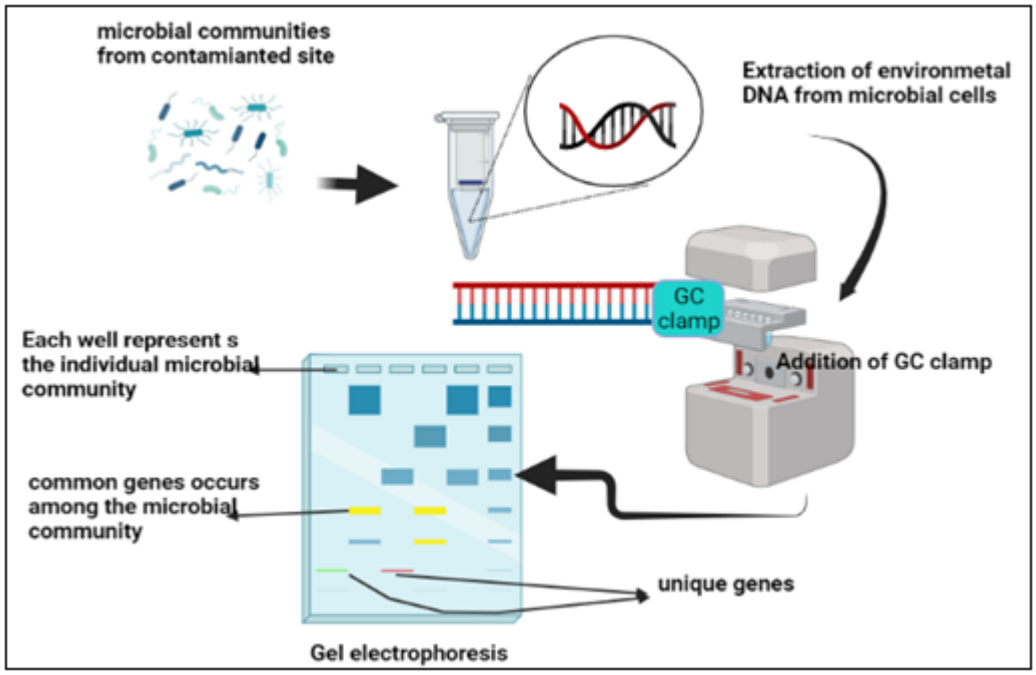

Figure 2: Schematic representation of denaturing gradient gel electrophoresis. Created with BioRender. com

SSCP (Single - Strad - Conformation Polymorphism)

SSCP is another technique which is based on electrophoresis approach similar to the DGGE. (Fig.3) Here the amplified environmental PCR products are subjected under the nondenaturing gel electrophoresis for separation on single stranded DNA. (Schwieger & Tebbe, 1998) initially this method was developed for detection of human genetic variation. SSCP involves, under nondenaturing gel electrophoresis condition, the denatured PCR amplified single strand DNA will fold to form a secondary and tertiary confirmation based on their nucleotide sequence and their physiochemical conditions.

SSCP is simple and more straightforward technique as compare to the DGGE/TGGE, as it not requires a GC clamps, gradient gel, or specific equipment and well suited for separation of small fragments up to 500bp. (Schmalenberger et al., 2001) Capillary electrophoresis SSCP is an advanced version of SSCP in which the fluorescently labeled PCR fragment are analyze using automated DNA sequencer. (Panigrahi et al., 2018) The major advantage of the CE - SSCP is, it allows the processing of multiple samples parallelly. Similar approach was done by (Hong et al., 2007) to monitoring and analyze sulfate reducing microbial community of mine drainage. Another approach based on PCR - SSCP combining with

16S rDNA sequencing to study the diversity in soil. (Stach et al., 2001)

SSCP is highly applicable for study community analysis, the combination with automated DNA sequencer help to distinguish 16S - 23S rRNA interspacer regions of selected bacterial strains. (Schwieger & Tebbe, 1998; Stach et al., 2001) The limitation of SSCP technique is, after initial denaturation during electrophoresis, it requires high rates of reannealing DNA strands especially when the high concentration of DNA requires for estimation of high diversity community analysis. Another limitation is, after electrophoresis of double stranded PCR products it appears more than one bands on gel. Which be resolve via using phosphorylated primers during the PCR amplification and followed by specific exonuclease digestion of phosphorylated strands. (Rastogi & Sani, 2011)

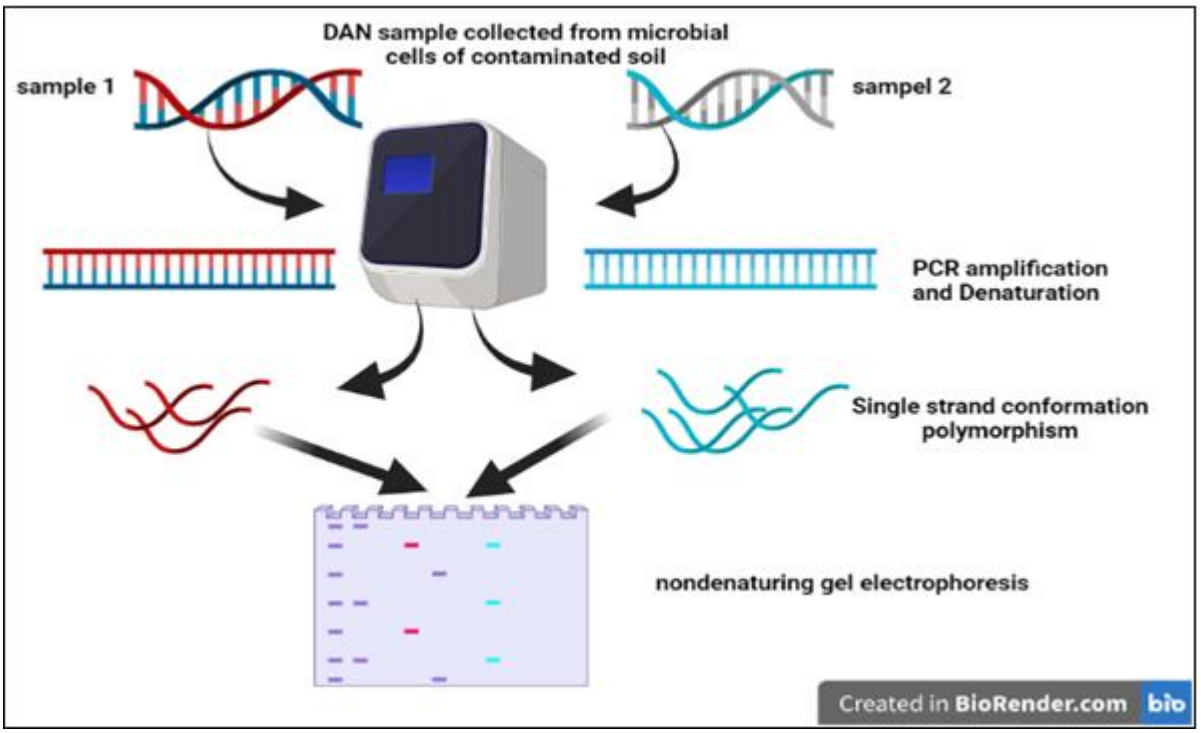

Figure 3: Systematic representation of single strand conformation polymorphism analysis

Random Amplified Polymorphic DNA and DNA Amplification Fingerprinting (RAPD/DAF)

It is simple and rapid method which is based on PCR amplification helps to identification of significant genetic markers and determination of microbial genetic diversity within different microbial habitat. In this method the random primers are selected and amplified by PCR and anneal randomly at the multiple regions of genomic DNA under low annealing temperature. (Franklin et al., 1999) this amplified products are further analysis by electrophoresis, where the gel is stained with fluorescent dye; which is further analyze under the imaging system.

RAPD uses single primer pairs or mixture of primers which targets multiple location of genome to generate specific DNA fragments via polymerase chain reaction. The primary structure of such primers can be design randomly or target specific. (Malik et al., 2014) RAPD and DAP are highly susceptible to the annealing temperature, quality and quantity of template DNA, $MgCl_2$ concentration and Primers as well, hence it is necessary to carefully developed the laboratory protocols to reproducibility. Moreover, usually all RAPD markers are dominant, so it is difficult to differentiate that whether, a DNA fragment is amplified from homo or heterozygous locus. (Panigrahi et al., 2018) Therefore, the determination of codominant RAPD markers which are different size from DNA segments is rarely occurs. However, such mismatch amongst primer and template leads to complete absence or decrease in the amount of PCR products which results into difficulties in finalize the results. (Yang et al., 2000) RAPD analysis is useful for examination of epidemiological relatedness amongst two isolates of the same species (e. g., *E. amylovora*). (Cooper & Rao, 2006) The combine study of RAPD with PLFA (Phospholipid fatty acid analysis) shows that the bioavailable heavy metals can reduce the degradation rates of herbicide isoproturon (IPU) in indigenous microbial community of agriculture soil. (Suhadolc et al., 2004) Similar approach was done by the (Yang et al., 2004) to study microbial community diversity of agriculture soil polluted by pesticides and chemical fertilizers. (Gao, Zhou, Mao, Zhi, Zhang, et al., 2010) Uses the PCR - RAPD method to understand the effect of two plants species existence in soil microbial community and their enzymatic activity in the presence of Cd and Pb contaminants. The detailed study indicates that existence of plant species increases the microbial population under heavy metal stress conditions, it also shows that planted soil enhance the soil genetic polymorphisms. Another study also indicates that heavy metals produce negative effect on microbial enzyme activity and their community. (Gao, Zhou, Mao, Zhi, & Shi, 2010)

RISA (rRNA intergenic spacer analysis)

rRNA intergenic spacer analysis is PCR based microbial community analysis approach which involves the amplification of a specific region in rRNA gene operon

between 16S and 23S subunit (Fisher & Triplett, 1999b; Panigrahi et al., 2018) known as intergenic spacer region (ISR) (Fig.4). RISA uses the oligonucleotide primers which are specific to the conserved region in 16S and 23S genes. Usually, the RISA fragments can be obtained from the most dominant bacteria in the environment sample. Generally, the rRNA operon plays structural functional role i. e., region encodes for tRNAs which is depends on the bacterial species.

However, the taxonomic value of the intergenic spacer region based on the heterogenicity of length and nucleotide sequence. Significantly the ISR were ranges between 120 to 1200bp with majority of 120 to 500bp. The automated version of the RISA is known as ARISA which involves the fluorescent labeled primers and ISR fragments are automatically detected by the laser detector. (Fisher & Triplett, 1999b)

ARISA (Automated ribosomal intergenic spacer analysis) is frequently used for analysis of variety of habitats such as soil, water, human gut (Kovacs et al., 2010) and it is very effective approach for comparative study of microbial community of different profile pattern. (Fomina. M. O., 2015) used RISA to analyze the effect of polycyclic aromatic hydrocarbons (Pyrene) and copper, either alone or in combination to fungal community in

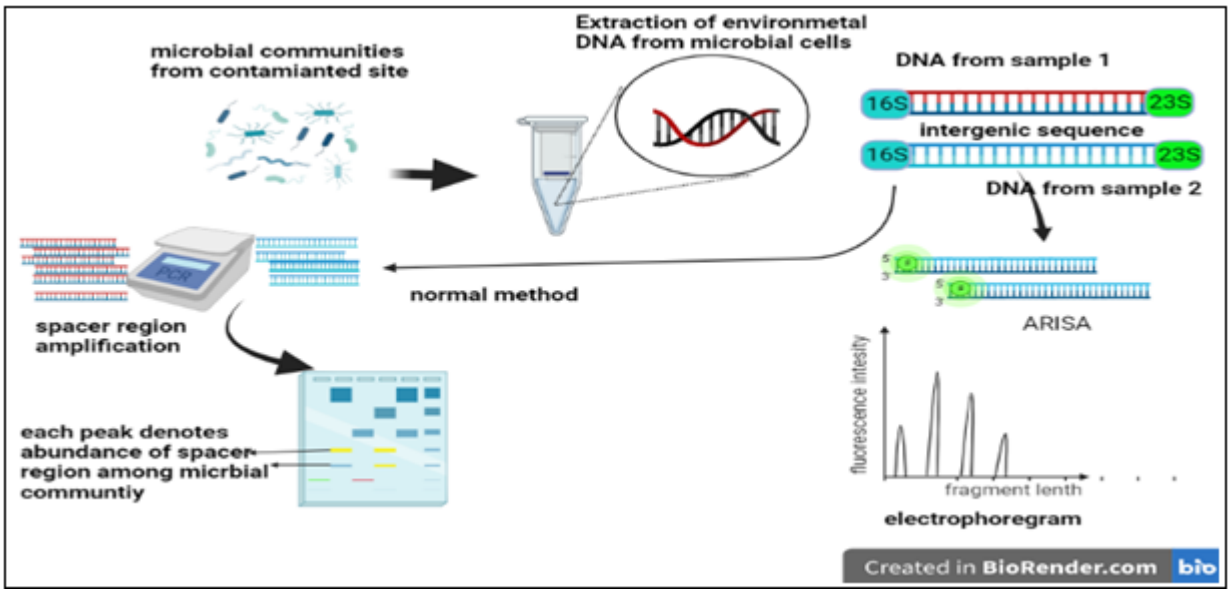

Figure 4: A schematic review of RISA

soil microcosms. The detailed study with coupling metagenomic sequence and bioinformatic analysis, it is observed that the Cu contamination reduce the metabolic activity of indigenous microorganisms against PHA utilization. However, the RISA analysis identified the copper sensitive fungal community belongs to species *Cryptococcus pseudolongus.*

Another combining approach of PCR - DGGE and RISA was carried out by (Srivastava et al., 2016) to analyze the physio - chemical properties and microbial diversity of rhizosphere bacterial community of different paddy soil. The further analysis with DCA and PCA analysis of RISA fingerprints demonstrate that the proteobacteria, actinobacteria, and cyanobacteria spp. plays significant role in bacterial community of paddy soil.

RISA has been used to analyze the potential biodegrading microbial population of polycyclic aromatic hydrocarbon at lower temperature under aerobic and nitrite reducing condition. (Eriksson et al., 2003b) study indicates that the dominant microbial population belongs to *Acidovorax, Pseudomonas, Sphingomonas and Variovarax.* In another study of RISA used to investigate the hydrocarbon degrading bacteria and bacterial community response in oil spill contaminated beach sand. (Kostka et al., 2011b; Panigrahi et al., 2018).

FISH (Fluorescence in situ hybridization) 4′, 6 - diamidino - 2 - phenylindole

Fluorescence in situ hybridization is a modern approached where fluorescently labelled DNA probes are used to detect the targeted genes or group of organisms from environmental samples. The environmental sample is initially denatured and then fluorescently labeled probes added in to the denature mixture where they are hybridized with targeted site. Which is further detected by the florescence microscopy. (Cooper & Rao, 2006) The two cloned libraries of rDNA and rRNA was prepare from polychlorinated biphenyl (PCB) polluted soil to analyze the bacterial community of Burkhoderia *spp.* and *Pseudomonas spp.* Study indicates that both sequence clone libraries were closely similar to each other and about 60% of Burkhoderia and Pseudomonas spp were about 98% similar in both clones. (Nogales et al., 2001)

FISH analysis was used to estimate the bacterial community of high arsenic contaminated soil. *Cirsium arvense* is rhizosphere associated bacterial community showing high resistance capacity against Arsenic. (Cavalca et al., 2010) around 64 isolates having the As resistance from which 46 isolates were As (III) (moderate to high) and 18 isolates having As (V) (high) resistant capability. They used Fluorochrome Cy3 labeled 16S rRNA targeted

oligonucleotide probes. This study indicates that isolates having *ArsC* gene which helps in efflux of toxic As and isolates were capable to produce indole acetic acid. (Tischer et al., 2012) used horseradish peroxidise labeled probes to detect archaea and bacteria in petroleum hydrocarbon contaminated sediments samples. They observed that, in source zone and upper part of core 3 and 5 having high abundance of *Bacteria andArchaea.* FISH study indicates that majority of them were belongs to methanogenic group and shows syntrophic relationship amongst contaminant degraders and archaeal methanogens. FISH technique also used to analyze the microbial community of Siberian tundra soil were oligonucleotide probes labeled with cyanine Cy3 or Cy5 which are specific for specific phylogenetic groups. (Kobabe et al., 2004) they used universal protein stain 5 - (4, 6 - dichlorotriazin - 2 - yl) aminofluo - rescein (DTAF) to analyze the bacterial community from top soil horizon to bottom layer. However, study indicates that various parameters such as availability of carbon, nitrogen sources, oxygen, temperature, moisture contents cause the large variation in bacterial count in different soil horizons. As depth increases the cell count decreases.

There are different advanced molecular techniques available which are combined with FISH which helps to study various metabolic and functional properties of microbial community. FISH can be combined with MAR. (Panigrahi et al., 2018) FISH - MAR is one of the advanced and sensitive technique as compare to DNA or RNA stable isotope probing (SIP) (Wagner et al., 2006) FISH - MAR can be helpful in different ways such as to understand the Eco physiological properties of microorganisms or physiological process of microorganisms in the environment. Furthermore, FISH - MAR can be combined with more significantly with microelectrodes which help to estimate the concentration of soluble components in the microenvironment of targeted microorganisms. This advanced technique helps to study the uncultured *Chloroflexacea which* is photoheterotrophic microorganisms which can tolerate high Oxygen concentration and utilize organic carbon which is provided by autotrophic cyanobacteria.

High resolution or advance modern molecular approaches

The method with the highest level of resolution is based on sequencing of the entire soil metagenome followed by careful analysis of the functional genes by various bioinformatic tools. Theses advanced molecular approaches are combined with intermediate resolution methods to study the complete metagenomic diversity of microbial community present in the soil.

Metagenomics

Metagenomics is the one of the most advanced molecular techniques which used to analyze the microbial community directly in their natural environment. The metagenomic involves the analysis of collective genome which is obtained directly from the environmental site, bypassing the isolation or cultivation in laboratories or without any prior information about the microbial community. (Ghazanfar et al., 2010; Gosal & Mehta, 2015; Riesenfeld et al., 2004) Metagenomics is widely known as environmental genomics, community genomic and bacterial Eco genomic as well. The metagenomic study does not involves the PCR based methods which only amplifies the targeted genes, as such techniques provides information about genetic diversity based on the gene's amplification only. Metagenomic study deals with the sequencing and analysis of entire microbial genetic composition as the whole genome sequencing of a pure bacterial culture.

Metagenomics study involves the construction of metagenomic libraries which could be form by

1) Generation of suitable DNA fragments and ligation of fragments into the suitable cloning vector (BAC, Cosmid, fosmid etc.)
2) Transformation of recombinant vector in to suitable bacterial host
3) Screening of successfully transformed host which possess the specific sequence. (Daniel, 2005)

Metagenomic libraries usually contains the small DNA fragments (ranges 2 - 3kb) provides sustainable coverage of environmental metagenome than the larger fragments. (Panigrahi et al., 2018; Riesenfeld et al., 2004) Specially the larger fragment metagenomic libraires (ranges 100 - 200kb) are suitable to study the multigenic biochemical pathways. The metagenomic libraries can be screen by both ways, either sequenced based screening or functional based. The sequenced based screening requires high throughput sequencing while another one is based on phenotypic expression on the selective medium. (Handelsman, 2004) Usually the frequency of active gene to express phenotype is very low. There are some other limitations of metagenomic study are, the isolation of metagenomic DNA from extreme condition such as high contaminated soil, sludge, wastewater, polluted sediments etc. is challenging in terms of quality. (Cowan et al., 2005) Significantly the extreme environments usually possess low microbial population density which directly affect the metagenomic library construction as direct extraction of metagenomic DNA from environment will not provide sufficient genome. To overcome with such drawbacks, it requires PCR - amplification using phi29 polymerase. (Abulencia et al., 2006) Metagenomic research in useful for discover the unknown bacterial diversity in extreme environmental conditions; it could also helpful to discover the novel isolates and genes. (Feng et al., 2018) uses metagenomics to analysis the microbial community structure and function of Cd contaminated soil. The detailed study reveals the various mechanism developed by the microbial species to tolerate heavy metals. It also shows that the Cd contamination decreases taxonomic species of microbes in soil and changes the microbial profile as well (Hemmat - Jou et al., 2018) . Similar approach was done by (Jung et al., 2016; Kim et al., 2021; Zafra et al., 2016) to estimate the microbial community and their function in petroleum hydrocarbon contaminated soil. (Sangwan et al., 2012) In another approach of metagenomic analysis was used to examine the microbial composition in pesticide contaminated soil. (Regar et al., 2019) further study indicates that the microbial diversity and richness in contaminated soil is less as compare to the non - contaminated soil. The major phyla were Proteobacteria, Actinobacteria, Firmicutes, Acidobacteria and Bacteroidetes. Metagenomic analysis is subsequently applied for analysis of functional diversity of microorganism in heavy metal

contaminated sites (Gupta et al., 2017; Robas Mora et al., 2020) hydrocarbon contaminated site (Auti et al., 2019; Baek et al., 2009; Cunningham et al., 2020) .

Metatranscriptomics

Metatranscriptomics is an advanced molecular approach to study the microbial diversity from an environmental sample which deals with extraction of RNA and treated with DNase to remove any residual of DNA. This study includes the amplification of mRNA and cDNA synthesis along with bioinformatic analysis which helps to understand the biogeochemical and ecological processes of microbes in the environment. (Dash & Das, 2018) . Metatranscriptomics also provides valuable data regarding the microbial responses as changing in environmental conditions via changing their expression in targeted genes. Moreover, this technique is suitable to study the microbial community of contaminated site which changes frequently. However this technique have major challenge as prokaryotic microbial cells does not have polyA tailed mRNA (Panigrahi et al., 2018) results into difficulty in obtaining complementary DNA or cDNA. (Yu & Zhang, 2012) were obtained around 2.4Gbp DNA dataset which provides the information about the major ammonia monooxygenase producing group of organisms i. e., *Nitrosomonas* and *Nitrosospira* were highly abundant, there are some another sequence obtained which belongs to the unculture group of bacteria like ammonia - oxidizing beta proteobacterium. (de Menezes et al., 2012) for the first time reveals that how soil microbial community response in the presence of contaminants. Before this study the microbial responses to pollutant were measure on the basis of change in microbial diversity or identifying the changes occurring in specific genes which are available on the basis of cultured method. (R. Sharma & Sharma, 2018) used similar approach to estimate the microbial community structure and function of agriculture soil contaminated with pesticides and heavy metals. In another study (P. K. Sharma et al., 2019) provides information about the molecular markers which can act as strong indicator of metal and pesticide contaminants to study the microbial diversity. This study indicates that the archaea play major role in nitrification process as compare to the bacteria in such contaminated soil.

(Baldrian et al., 2012) for the first time observed that from single eukaryotic functional gene can be helpful to analyze the microbial diversity. They collected different samples from different soil horizon and analyze for the *chbI* gene. The study indicates that almost all *chbI* gene were associated with all cellulolytic fungal community in all horizon. Meta transcriptomics can be also implemented for comparative study of rhizosphere microbiome and plants. Similarly, another approach was carried out by (Žifčáková et al., 2016) for the analysis of microbial activity of forest soil in summer and winter and results indicates that different enzyme activity microbial community and their biomass is decreasing contents of bacterial and fungal biomass with depth and increases content of Ectomycorrhizal fungi. Further analysis provides detailed information about microbial community functions were differently occurs in different soil horizon. In litter zone the major activities were carried out by fungi, whereas in soil it was done by bacteria. The study confirms that the activity of decomposition is higher in summer due to high availability of C compounds while fungal contribution decreases in winter.

(Turner et al., 2013) They used 3 - 5 biological replicated to overcome with lack of biological replicates. They observed that oat and pea plants exert higher eukaryotes as compare to wheat plants which indicates that oat or pea cropping can improve the soil fertility during crop rotation. Furthermore, this study also provides information that pea plants increase the nitrogen status while oat plants reduce the disease incidence. Detailed study indicates that cereals cropping can enriched in cellulose degrades while legumes was enriched with H2 oxidizer.

Metatranscriptomic approach was carried out for comparative study of two different arctic peat soil microbiota. (A. T. Tveit et al., 2014) study indicates that the top peat horizon was dominated by the *Alpha* and *Deltaproteobacteria, Acidobacteria, Planctomycetes and Actinobacteria* and as dept increases the *Actinobacteria* increases while *Acidobacteria* community decreases. Further study leads to the aromatic and cellulose degradation decreases with increasing depth which indicates that the *Actinobacteria* were involve in cellulose and hemicellulose degradation while *Proteobacteria* in phenolic compounds. Detailed study identified that the major groups of methanogens like *Methanomicrobiales, Methanobacteriales, Methanosaetaceae, and Methanosarcinales.* (A. Tveit et al., 2013) found that the acetoclastic methanogens was highly active and abundant in lower layer while hydrogenotrophs was at upper layer.

The microbial community of acid mine drainage was analyzed by (Chen et al., 2015) by combining study of metagenomics and meta transcriptomics. This study provides detailed blueprint of gene transcriptome involved in microbial community which exhibits in acid mine drainage. The study indicates that the microbial gene profile was closely related to the physiological characteristic of acid mine drainage system. The detailed analysis shows that the major group of microbial community involve in AMD system was *Acidothiobacillus ferrivorans* and *Leptosprillium ferrodiazotrophum* this microorganism possesses several genes which help to assimilate the nitrogen, carbon and phosphate. Some other gene which help in survival at low pH and high stress conditions.

Metaproteomic

Metaproteomics is also called as environmental proteomics which involves the qualitative and quantitative analysis of entire proteins expressed in the microbial community. Metaproteomics combined with other omics technologies to enhance the analysis such as metabolomics and transcriptomics. The development of metaproteomic is fecillited by various other techniques where the mixture of proteins from environmental sample is separated by 2D electrophoresis (Wilmes & Bond, 2004) or high - performance liquid chromatography and the information was analyzed by compute tools. LC - MS/MS used to identify and quantify thousands of peptides. (Aziz et al., 2021)

The combine study of metagenomic and metaproteomic help to identification and prediction of proteins using the

metagenomic sequencing data. SIP/MAR - FISH methods are useful to study the structural and functional analysis of microbial communities and their specific biogeochemical cycles such as nitrification, methane oxidation. Compare to this study, proteomics provides significant approach to investigate physiology of microbial community, qualitative as well as quantitatively. (Panigrahi et al., 2018)

Metaproteomics study reveals the critical information about the microbial community such as protein - protein interaction, protein abundance which is not delivered by other DNA or RNA molecular techniques. (Keller & Hettich, 2009) The major challenge with this approach is to extraction on total protein from environmental sample cause extraction of other organism's protein leads to complication in taxonomic characterization of proteins. To overcome with this problem, the environmental sample need to treat with ultracentrifugation and then lysed separated through 1D and 2D electrophoresis. (Keller & Hettich, 2009)

Metaproteomic analysis is at early stage (Mattarozzi et al., 2017; Wang et al., 2011; Wilmes & Bond, 2004) is limited to certain level of analysis due to major drawback in protein extraction, protein separation and its identification. The bioinformatic tools were also limited to analysis the huge data generated from Metaproteomics. (Williams et al., 2010) metaproteomic approach has been used to analyze the microbial community responses to the changes in environmental factors such as temperature, xenobiotic and many other. (Lacerda & Reardon, 2008)

The detailed combine study of metaproteomic using 2D gel electrophoresis and de novo sequencing with MS leads to find out more than 100 proteins from different microbial association within Cd contaminated site. (Lacerda & Reardon, 2008) Metaproteomics also used to detection and characterization of novel isolated, protein and genes which are involved in the biodegradation of toluene and ethyl benzene in anaerobic conditions. (Kühner et al ., 2005) . (Wilmes et al., 2008)

Uses 2D electrophoresis in combination with MALDI - ToF and quadrupole ToF MS to identify the microbial protein involve in the removal of phosphorous from activated sludge. Similar approach was carried out by (Chourey et al., 2013) to examine the metaproteomes of microbial community of uranium and nitrate contaminated site.

Metabolomics

Metabolomics is the study of small molecules generally known as metabolites (such as amino acids sugars, lipids etc.) present in the cells, tissues or organisms. It is the study of substrate and products of biological reactions which are majorly influenced by various parameters such as genetic and environmental factors as well. MS ana NMR are the advanced technologies which are combinedly used for the study of metabolomics. (Jones et al., 2014; Panigrahi et al., 2018) (Jones et al., 2014) combined the modified methanol - chloroform - water extraction with ^{1}HNMR spectroscopy to analyze the microbial metabolic profile living in the former mine site. They also used PCA (principal component analysis) to identified the microbial metabolic profile whether exposed to organic or inorganic pollutants. Similarly, another approach was carried out by (Song et al., 2020) to estimate the difference between metabolite profile and relationship of pepper rhizosphere soil and bulk soil under the plastic greenhouse vegetable cultivation. The PCA analysis shows that the metabolite profile of rhizosphere was different from the bulk soil. The detailed study indicates that the most of the metabolite were higher in the rhizosphere soil as compare to the bulk soil, these is due to the plants roots release metabolite in the rhizosphere soil. (Cheng et al., 2018; Song et al., 2020)

The combining study of NMR spectroscopy and GC - MS/LC - MS are very effective to analyze the metabolites. (Withers et al., 2020) uses similar approach to study the soil metabolome and to understand the relationship between physiochemical soil indicators and its metabolome. Detailed analysis shows that the metabolome may influenced by the environmental factors such as oxygen, moisture, pH, temperature, Na contents etc. moreover single GC - MS is not sufficient to analyze the complete metabolome, it requires coupling of advanced techniques to study in depth of microbial molecular pathways and their interaction with environment. In another study (H. Zhang et al., 2020) used silver nanoparticles (AgNPs) to analyze the microbial community and their metabolite profile in the presence and absence of AgNPs. They used to combine approach of GC - MS along with 16S rRNA sequencing to understand the AgNPs response. Study shows that the silver nanoparticle alters both bacterial community and richness. Furthermore, it also induces some critical parameters which helps in the C, N, P cycling and antimicrobial activities. This study can be useful for analyze the impact of other environmental/ anthropogenic factors to the environment.

The establishment of other advanced omics technologies implies to understand detailed mechanisms of soil microbial community and their metabolite profile. Combine study of Illumina HiSeq sequencing with UPHLC - MS along with KEGG analysis helpful to examine metabolite and microbial profile of Cd contaminated rhizosphere soil. The study indicates that the inoculation of *Bacillus subtilis* enhanced the microbial biodiversity in the rhizosphere soil along with induces nutrient cycling and stress tolerance. (Li et al., 2021) similar approach was carried out by (An et al., 2021) to study the soil biodiversity and metabolite profile after polymer amendment in Cd contaminated soil. The detailed study indicates that after applying polymer amendment Cd concentration was decreases in the soil as well as increases the soil microbial niche and bacterial abundance and improve metabolic profile of soil microbiota.

2. Conclusion

All the modern approaches that are available today have some advantages and limitations as well, not a single one can provide the complete assessment to study complete microbial diversity. Advanced metagenomic and sophisticated techniques continuously modified and developed to understand the bacterial community based on previously phenotypic and genotypic studies. The combination of different molecular techniques will provide better understanding the phylogenetic, genetic diversity, ecology, evolution and taxonomy of the largest group of

living organisms e. g., Prokaryotes. There are n number of questions still need to be resolve (e. g., how many microbial species are there on earth? how microbial species performed their metabolic activities under different biological and physiochemical stress conditions?) the modern "omics" technologies provides significant information about interaction between gens, proteins and biological factors will help to determination of new sight to study the environmental microbiology.

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