Soil microorganisms play a crucial role in maintaining major biogeochemical/nutrient cycle, soil quality, and productivity. Hence, the understanding of soil microbial community structure, distribution, and their metabolic function is essential for getting a deeper insight into soil ecosystem and its health. A number of molecular methods for extracting metagenome, total RNA, protein, and metabolites from the diverse environmental samples, sequencing technology, etc. are present which help to know about microbial structure, composition, and their metabolic function in the specific environmental ecosystem. Genetic fingerprinting like ARDRA, RFLP, DGGE, and T-RFLP and omics approaches like metagenomics, metatranscriptomics, and metabolomics are essential techniques for identifying and depicting the total microbial community structure and their interactions with environmental and biotic factors. So for these molecular techniques, it is possible to identify and functionally characterize soil microbes that are not culturable in a laboratory environment. This chapter describes old and modern novel state of the art molecular techniques which proved insights into the phylogenetic and functional activities of microbial assemblages in a terrestrial ecosystem.

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Molecular Genomic Techniques
for Identification of Soil Microbial
Community Structure and Dynamics
2
Dhiraj Paul, Satish Kumar, Mrinal Mishra, Sushant Parab,
Sunil Banskar, and Yogesh S. Shouche
Abstract
Soil microorganisms play a crucial role in maintaining major biogeochemical/
nutrient cycle, soil quality, and productivity. Hence, the understanding of soil
microbial community structure, distribution, and their metabolic function is
essential for getting a deeper insight into soil ecosystem and its health. A number
of molecular methods for extracting metagenome, total RNA, protein, and
metabolites from the diverse environmental samples, sequencing technology,
etc. are present which help to know about microbial structure, composition, and
their metabolic function in the specific environmental ecosystem. Genetic finger-
printing like ARDRA, RFLP, DGGE, and T-RFLP and omics approaches like
metagenomics, metatranscriptomics, and metabolomics are essential techniques
for identifying and depicting the total microbial community structure and their
interactions with environmental and biotic factors. So for these molecular
techniques, it is possible to identify and functionally characterize soil microbes
that are not culturable in a laboratory environment. This chapter describes old and
modern novel state of the art molecular techniques which proved insights into the
phylogenetic and functional activities of microbial assemblages in a terrestrial
ecosystem.
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Keywords
Microbial ecology · Stable isotope probing · Autoradiography · Fluorescence
in situ hybridisation · DGGE · Next generation sequencing
2.1 Introduction
In soil/natural ecosystems, microorganisms including bacteria and fungi exist in a
very large number and play a very crucial role in maintaining major biogeochemical
cycles (Molin and Molin 1997; Wall and Virginia 1999), plant nutrition (George
et al. 1995; Timonen et al. 1996), plant health (Srivastava et al. 1996; Filion et al.
1999; Smith and Goodman 1999; Wright and Upadhyaya 1998; Dodd et al. 2000),
soil fertility (Yao et al. 2000; O’Donnell et al. 2007), soil structure (Wright and
Upadhyaya 1998), and degrading organic pollutants and remediation of toxic metals
(Barakat 2011). Therefore, microorganisms are key players in important ecological
processes, such as carbon, nitrogen, phosphorous, and sulfur biogeochemical cycle,
and directly influenced all lives on Earth (Garbeva et al. 2004). It is noted that 1 gm
of soil/sediment may contain l09 bacterial cell (Whitman et al. 1998). In terrestrial
environments, soil sustains as many as 4–5 10 30 microbial cells and in aquatic
environments approximately 1.2 10 29 cell (Whitman et al. 1998; Singh et al. 2009).
It constitutes 60% of the total biomass of the Earth, and it represents two to three
orders greater biomass than the total plant and animal cells (Singh et al. 2009).
Therefore, a large number of microorganisms and their genetic diversity are unex-
plored, and that is directly involved in maintaining major nutrient cycles, global
climate change, and the greenhouse effect. So understanding this unexplored genetic
diversity is a high-priority issue in microbial ecology.
The soil microbial ecology analysis does not only mean the identification of total
microbial biomass and community diversity, but it also explores microbial growth,
function, distribution, and interactions among species. Therefore, soil microbial
ecologist tries to answer fundamental questions, i.e., (1) What is microbial commu-
nity structure and composition? (2) What are the metabolic functions/functional
genes expressed so that microorganisms can run major biogeochemical cycle in
the ecosystem? (3) How do the functional activities of the microorganisms relate to
major ecosystem functions including biogeochemical cycling, energy flow, etc.?
Besides, anthropogenic activities including city development, agriculture, pesticide
use, and other pollution directly affect the soil microbial diversity. How these
changes affect surface and subsurface ecosystems is unknown. For a decade many
new molecular approaches like NGS, metaproteomics, metabolomics, etc. have
evolved that incredibly help soil microbial ecologist for better assessments of
microbial diversity and their function in the ecosystem. In-depth understanding of
microbial distribution, their function, and interaction helped in the development of
new techniques for bioremediation, energy generation processes, pharmaceuticals,
food, chemical, mining, etc. Therefore, for addressing how microbial community
structure and dynamics affect the ecosystem function, reliable and accurate
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techniques of soil microbial ecology are needed. In the following sections, we are
describing the traditional molecular techniques, current methods, and their
advantages and disadvantages which are used for studying soil microbial structure
and function.
2.2 Culture Based Techniques: Advantages and Limitation
A diverse group of microorganisms are present in the environment including soil,
and for their isolation, identification, characterization, and culture-based microbial
diversity analysis purpose, different standard culture-based techniques (Hugenholtz
2002) are available that include use of different types of growth media, namely,
Luria-Bertani, nutrient agar, tryptic soy agar, etc., for copiotrophic bacterial growth
and R2A, RAVAN, minimal media, and synthetic marine water/groundwater media
for oligotrophic bacteria. Despite a number of ways like mimicking the environmen-
tal niches (from where the samples are collected) by changing parameters like
temperature, pH, nutrient composition, and trace nutrient composition, more than
99% organisms are still uncultivable which are seen as viable under a microscope.
More than 20 phyla are present as a candidate division like TM7, OP10, OP11, WS2,
WS3, etc. which are taxonomically well defined based on their metagenomics
information, but they are still uncultivable (Schloss and Handelsman 2004). There-
fore, their ecological and industrial application is not possible due to uncultivable
nature. Bacterial phyla Acidobacteria which constitute more than 20% soil bacterial
population but very few genera of these phyla are culturable, mostly uncultivable.
Therefore, for understanding who are present in the ecosystem and what is their role
in maintaining major biogeochemical cycle in the particular ecosystem, application
of culture-independent molecular techniques is highly desirable.
2.3 Classical Molecular Methods of Microbial Community
Analyses
2.3.1 Clone Library Method
Before next-generation/high-throughput sequence-based microbial diversity analy-
sis, a most widely used technique was clone library-based analysis, where PCR
product amplified from diverse environment DNA samples is subjected to clone and
sequenced the individual clones containing gene fragments. Then for taxonomic
assignment, sequences are compared with different databases like Greengene, Ribo-
somal Database Project (RDP), SILVA, etc. Based on good-quality sequence size,
cloned sequences are assigned at a lower taxonomic level like genus and species, but
one of the limitations of this technique is being time-consuming and labor intensive.
One of the studies showed that environmental samples like soil/sediment may
require over 40,000 clones to document 50% of the richness (Dunbar et al. 2002).
Generally, using clone library-based approach, one can handle nearly about
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thousand clones at a time; therefore it gives a tiny picture of the microbial commu-
nity structure of the particular environment. Many studies are there where this
technique is used for identifying the total community structure. For example,
recently Pascual and co-worker (2016) used clone library-based techniques for
analysis of bacterial communities associated with the rhizosphere of wild plant
species found in natural settings where bacterial phyla Proteobacteria,
Actinobacteria, Acidobacteria, and Gemmatimonadetes dominated. Microbial diver-
sity analysis of hydrocarbon-contaminated sediment samples of northwest of
Bemidji, Minnesota, USA, using clone library-based analysis revealed presence of
iron-reducing Betaproteobacteria followed by Deltaproteobacteria, Smithella, and
the hydrogenotrophic Methanoregula (Beaver et al. 2016).
2.3.2 Genetic Fingerprinting Techniques
Difference in genomic or nucleotide sequences can be utilized to generate the
identity of any organisms, and the techniques, which facilitate this kind of identifi-
cation, are known as genetic fingerprinting techniques. Hence, using genetic finger-
printing techniques like ARDRA, DGGE, and T-RFLP, microbial community
profiling is done where PCR product amplified from direct environmental DNA
samples is used (Table 2.1).
2.3.2.1 Amplified Ribosomal DNA Restriction Analysis (ARDRA)
ARDRA is a method similar to restriction fragment length polymorphism (RFLP) or
an extended form of it and originally developed by Vaneechoutte et al. (1993). It was
firstly used for characterization of Mycobacterium species. Then it was used to
characterize other bacterial species also (Vaneechoutte et al. 1995). Previously
ARDRA was used for selection of clone libraries and strain typing to determine
phylogenetic groups inside a microbial community and to study microbial diversity.
This procedure involves amplification of the conserved region of 16S rRNA gene
using universal or genus-/species-specific primers through polymerase chain reac-
tion followed by enzymatic digestion of the PCR products. The restricted fragments
are segregated on agarose or polyacrylamide gel, and the emerging profile of bands
is used for grouping of the community as per genotype or for strain typing (Tiedje
et al. 1999). Generally, for 16S rRNA gene product digestion (1.5 kb), tetra cutter
restriction enzymes (e.g., MspI, HaeII) are used. Due to random prevalence of the
restriction sites, the chance of occurring specific restriction sites of tetra cutter
enzyme is 256 bp. Therefore, care should be taken during restriction enzymes
selection. The restriction enzymes that possess the same recognition sequence, i.e.,
isoschizomers, should not be used; otherwise, it will create difficulty in analysis.
Although 16S rRNA gene is a promising marker for the differentiation up to species
level, ARDRA is very much useful among the groups which have more interspecies
similarity to each other (Heyndrickx et al. 1996). ARDRA method is widely used for
the discrimination of isolates undergoing different changes and from different
environments (Błaszczyk et al. 2011). ARDRA is also helpful in finding out
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Table 2.1 Comparison among the genetic fingerprinting techniques
ARDRA DGGE RAPD T- RFLP SSCP RISA
Use of
restriction
enzyme
Yes No No Yes No No
PCR
amplification
of 16S rRNA
gene
Yes Yes Yes Yes Yes Amplification of
spacer region
GC clamp Not needed Needed Not needed Not needed Not needed Not needed
Advantage Resolving
intraspecies
similarity
Less time-consuming Very quick and easy to
assay
Rapid method Simple and
straightforward
technique
compared to
DGGE
Now automated
ARISA is used
for microbial
community
analysis
Very good for
comparing the different
samples of microbial
profile
Multiple samples are
analyzed
simultaneously
Random primer used for
amplification; therefore
no prior knowledge
about sequences are
needed
Microbial
composition
changes in
diff.
environment
Limitation Laborious,
time-
consuming
Resolution quality is not
good, not able to
classify at lower
taxonomic level of the
organisms
Low reproducibility If two or more
sequences share same
terminal restriction site,
it shows same peak on
electropherogram
Need sequencing
data for designing
specific primers
Time-
consuming and
cumbersome
It required purified DNA
for amplification
For
reproducibility,
need highly
standardized
electrophoretic
condition
Many times false
terminal restriction
fragments generated
which result in false
peaks being generated
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structural changes which are undergoing in microbial communities; however, it can’t
measure microbial diversity or identify specific phylogenetic cluster within a com-
munity fingerprinting profile (Liu et al. 1997). Major limitations of this technique are
being time-consuming and laborious, and the restriction profile obtained from
complex microbial communities is hard to analyze sometimes. Gulitz et al. (2013)
compared four water kefirs and found that they consisted of different proportions of
genera Lactobacillus, Leuconostoc, Acetobacter, and Gluconobacter. Shehata
(2012) used this method for characterization of Lactobacillus sp. from fermented
millet drink and fresh and raw cow milk.
2.3.2.2 Denaturing Gradient Gel Electrophoresis (DGGE)
The technique of DGGE was originally invented by Fischer and Lerman (1980), and
for small ribosomal subunit (i.e., 16S rRNA), it was described by Muyzer et al.
(1993). For performing DGGE a gradient of chemical denaturant is formed which is
in progressively increasing concentration. PCR products have to pass through this
gradient in a polyacrylamide gel. On reaching threshold concentration, the PCR
products begin to melt and weaker melting domains melt very fast; therefore
migration slows intensely. Amplicon which has different sequence composition
will migrate differently and stop at various positions in the gradient result in the
formation of different band patterns. In DGGE, forward primer is tagged with a
GC-rich nucleotide sequence (30–50 bp) as it renders complete separation of ds PCR
product into single strand during electrophoresis. For taxonomic identification,
bands from gel are excised, reamplified, and sequenced. DGGE has been extensively
applied for elucidation of the microbial community structure and finds out changes
in microbial community and dynamics of contaminated soil, water, and many other
microcosm-based studies (Macnaughton et al. 1999; Ralebitso et al. 2000; Watanabe
et al. 2001; Cummings et al. 2003). The key advantage of DGGE is that it allows the
observance of the spatial and time-based changes in microbial community structure
and gives a clear picture of the prevailing microbial species present in a particular
sample (Malik et al. 2008). Major drawbacks of this technique are as follows:
(1) several DNA bands/fragments may have the same melting points; (2) the lengths
of the DGGE bands are many times smaller in size, that leads to difficulty in proper
taxonomic identification; and (3) due to sequence heterogeneity between multiple
rRNA operons of one bacterium, it can lead to several bands in DGGE, resulting in
overestimation of the diversity. DGGE is a powerful tool which can discriminate
among the microbial populations from different ecosystems. DGGE and
pyrosequencing can also be used for elucidation of microbial diversity or composi-
tion from different environments such as native plants or nursery-raised plants or
bulk sediment from mangrove. The results show that DGGE is a vigorous and
practical method and effective in discriminating among earlier defined groups
(Cleary et al. 2012). Ivone and Conceição (2013) used DGGE and culture-dependent
method to identify the bacterial community composition especially from tap water.
The results revealed that the members of Alpha-, Beta-, and Gammaproteobacteria
were the major lineages.
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2.3.2.3 Random Amplified Polymorphic DNA (RAPD)
RAPD is a PCR-based method, where single short oligonucleotide primers (6 bp),
i.e., arbitrarily selected, are used for PCR amplification. As primers are short sized, it
can anneal arbitrarily at many sites on the genomic/total DNA due to low annealing
temperature (~35 C) (Franklin et al. 1999). Results are obtained from random
amplification of various length products from a single reaction. Depending on the
microbial community structure/complexity, different band pattern is generated dur-
ing agarose/polyacrylamide gel electrophoresis. Unlike conventional PCR, RAPD
does not require any specific knowledge about targeting organisms. By using a
single primer of random nucleotide sequence in a PCR-based method, this process
can detect single nucleotide polymorphism. Due to its easy use, it is widely used for
genetic fingerprinting of microbial community composition and closely related
microbial species and strains. The Fasciola hepatica and F. gigantic both liver
flukes are parasitic trematodes which belong to phylum Platyhelminthes and coexist
in part of Africa and Asia. Life cycles of both are similar but possess different
transmission characteristic. McGarry and co-workers (2013) have successfully
identified these two species using RAPD-based PCR over a period of 12 years
from different countries. RAPD was also used as a typing method for Campylobac-
ter species from ducks and duck-related environmental samples from Penang,
Malaysia (Adzitey et al. 2012).
2.3.2.4 Terminal Restriction Fragment Length Polymorphism (T-RFLP)
This method (T-RFLP) is used for the exploration of complex microbial community
based on the occurrence of recognition sequence of restriction enzymes on the 16S
rRNA gene. It is one of the fingerprinting methods aimed to understand the unknown
microbial community. The method was invented by Liu et al. (1997). This method
includes fluorescent labeling of end of PCR products and restriction digestion of the
PCR products of different variants of a single gene. For amplification one or both
primers should have their 50 end labeled with a fluorochrome molecule. Fluorescent
molecules, namely, TAMARA, HEX, or 6-FAM, can be used for 50 end labeling.
The mixture of amplicon is subjected to restriction digestion by using one or more
restriction enzymes (generally tetra cutter). After the restriction digestion, fragments
are separated in a DNA sequencer either by capillary or by polyacrylamide electro-
phoresis, and the fluorescence detector in the DNA sequencer determines the sizes of
the different terminal fragments (Osborn et al. 2000). Due to the use of dye, only
fluorescently labeled terminal fragments are detected and determined, while all other
fragments are not considered. Therefore, T- RFLP method is different from ARDRA
or RFLP where all the fragments are visualized. This procedure also includes
purification of PCR product before performing restriction digestion or if a capillary
electrophoresis is being used, then before running the sample desalting is also done.
The obtained results will be found in graph form, called electropherogram, where Y
axis denotes the fluorescence intensity of each fragment and X axis denotes the
fragment size. Thus, the bands which appear on an electrophoresis gel are visualized
as a peak on the electropherogram. In a T-RFLP, each genetic variant in the original
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sample is supposed to represent as a single peak, whereas peak height and area
represent its relative abundance in a particular community.
2.3.2.5 Single-Strand Conformation Polymorphism (SSCP)
It is a simple and sensitive method to detect polymorphism in DNA. It utilizes the
variation in single nucleotide sequences of identical length that can arise under
certain conditions such as mutation or single nucleotide polymorphism. This allows
separation of different fragments due to their different conformation by using gel
electrophoresis and ultimately helps in distinguishing different sequences. The
technique was first described by Masato Orita et al. (1989). In SSCP, the environ-
mental DNA is first amplified using PCR and then denatured. After denaturation,
single-stranded DNA is separated on polyacrylamide gel (Schwieger and Tebbe
1998). Even a minute difference (often a single base pair) can result into different
secondary structures, migrating differently in the gel leading to separation of differ-
ent sequences in the form of different bands. The technique works on the principle
that under non-denaturing conditions, DNA can form different secondary structures
based on specific sequences. Molecules having a minute difference like single base
substitution may generate different conformers and migrate differently in
non-denaturing polyacrylamide gel (Sheffield et al. 1993). Goszczynski (2007;
Goszczynski and Jooste 2015) used SSCP method to check the heterogeneity of
grape wine virus A and found that it is a rapid and relatively low-cost preliminary
analysis of molecular heterogeneity of viruses. Generally, bacteria are involved in
spoilage of processed food products, but some fungi are also responsible for this.
Dorn-in et al. (2013) tested the presence of fungi in heat-processed meat product
using PCR-SSCP. The result shows the presence of Aureobasidium pullulans,
C. tropicalis, C. zeylanoides, and Pichia membranifaciens and/or species such as
Guignardia mangiferae, Lewia infectoria, and Lasiodiplodia theobromae. Pure
cultures of Pseudomonas fluorescens, Sinorhizobium meliloti, and Bacillus subtilis
have also been successfully differentiated using SSCP (Schwieger and Tebbe 1998).
2.3.2.6 Ribosomal Intergenic Spacer Analysis (RISA)
In this method PCR amplification of a region of 16S rRNA gene known as an ISR,
i.e., intergenic spacer region, is generally done. Spacer region is found between large
23S and small 16S subunit of rRNA operon. A significant heterogeneity in ISR
region in terms of nucleotide and length is noted. RISA fragments can be generated
with the help of oligonucleotide primers which are complimentary to 23S and 16S
rRNA genes. The resulting PCR products will be a mixture of fragments
representing many dominant community members. These fragments are
representing most of the dominant bacteria in an environmental sample. RISA is
used originally to explore microbial diversity in soils. It is also used for monitoring
microbial community composition in anaerobic treatment plants or bioreactors
(Ciesielski et al. 2013). Besides, an automated form of RISA, i.e., ARISA, was
used for bacterial community composition analysis of freshwater system (Fisher and
Triplett 1999). Although RISA is one of the virtuous methods for the analysis of
microbial community composition, the limitation of this method is the same as
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conventional PCR like primer mismatch, annealing timing, DNA concentration and
quality, etc. ARISA techniques were used to detect the presence of different types of
Clostridium species in raw tank milk and curd used for cheese production in dairies
situated in different parts of Northern Padan Plain (Feligini et al. 2015).
2.4 Modern Molecular Methods of Microbial Community
Analysis
2.4.1 Stable-Isotope Probing (SIP)
Stable-isotope probing (SIP) technique has become the state of the art in microbial
ecology for identifying and detecting microorganisms that are actively involved in
specific metabolic processes and elemental fluxes taking place in environmental
samples in order to effectively link the taxonomic identity with function (Vogt et al.
2016). This is the most widely employed technique to identify and characterize
active community members or specific functional groups of microbial communities
that are capable of utilizing specific isotopic-labeled substrates. SIP basically tracks
the incorporation of heavy stable isotopes by incubating an environmental sample to
substrates containing 13 C, nitrogen ( 15 N), 3 H, and 18 O that are assimilated into
microbial biomass of environmental samples. The isotopically labeled carbon/
hydrogen/nitrogen from the substrate gets incorporated into the biomass (particularly
DNA, RNA, and proteins) of the active microorganisms in the sample and serves as
biomarkers of active community members. After stable isotopes have been
assimilated in the environmental sample and metabolically active cells, the label
goes into their biomass including DNA, RNA, lipid, and proteins. These labeled
biomolecules serve as biomarkers, which are recovered and analyzed using various
techniques like fingerprinting, microarrays, clone libraries, metagenomics, and next-
generation sequencing (Uhlik et al. 2013). Depending on the type of the isotopic
label incorporated and biomarker recovered as a target in a particular study, the SIP
can be categorized as DNA-SIP, RNA-SIP, and protein-SIP. However, DNA-SIP,
using 13 C-based isotopic labels, has been the most extensively used strategy to
decipher the microbial populations with a defined function in different sorts of
environmental samples (Radajewski et al. 2002; Neufeld et al. 2008; Antony et al.
2010; DeRito et al. 2005; Zhang et al. 2016). The advantage of the DNA-SIP-based
studies is that the recovered labeled DNA after the SIP experiment can be subjected
to several downstream analyses. A great wealth of significant genetic information is
provided by the metagenomes of organisms which include characterization of
metabolism-related functional genes and also the ribosomal genes using various
fingerprinting-based approaches (e.g., denaturing gradient gel electrophoresis
(DGGE), clone libraries, metagenomic libraries, and high-throughput sequencing).
A typical DNA-SIP experiment begins with incubation of an environmental sample
with labeled substrate in microcosm, maintaining the environmental conditions to
the mimicking extent. Incubations for DNA-SIP can be performed either in vitro
using laboratory microcosms constructed from field-collected samples (Wald et al.
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2015; Paes et al. 2015) or in situ using incubation directly in soil or sediment (Liou
et al. 2008; Key et al. 2013). Substrate concentration and exposure time always
remain to decide critical factors and hence must be for sufficient time so that
detectable amount of label gets incorporated in the sample. However, care must be
taken to avoid excessive labeling, as the label may rapidly spread, via trophic
interactions and cross-feeding (Neufeld et al. 2007). The control samples exposed
to the unlabeled substrate should always be included in order to confirm that the
DNA recognized as being isotopically labeled is truly the result of labeling with
stable isotope and not due to the difference in GC content. The outline flowchart of a
representative DNA-SIP experiment is presented in Fig. 2.1; however, the details of
all the requirements, steps involved in DNA-SIP protocol, and critical technical
considerations can be found in Neufeld et al. (2007) and Dunford and
Neufeld (2010).
Several studies suggest the superiority of RNA as biomarker in SIP (RNA-based
SIP or RNA-SIP) due to the remarkable attributes of RNA like higher synthesis rate,
direct reflection of cellular activity, replication-independent turnover in the cell, and
more responsiveness to environmental conditions (Manefield et al. 2002; Whiteley
et al. 2007). RNA-SIP is technically more demanding as RNA-SIP requires a more
rigorous procedure for gradient evaluation than DNA-SIP. RNA-SIP cannot be
performed in cesium chloride (CsCl) gradient media, as CsCl itself precipitates at
the buoyant density required for rRNA, and hence, the use of cesium trifluoroacetate
(CsTFA) can be a better choice for gradient formation (Rickwood 1992; Manefield
et al. 2002). RNA-SIP has been successfully used to decipher the active community
Fig. 2.1 An outline of a typical DNA-SIP experiment
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members in various types of environmental samples like bioreactor sludge
(Manefield et al. 2002), paddy soil (Lueders et al. 2004), rice rhizosphere (Lu and
Conrad 2005), and grassland soil (Rangel-Castro et al. 2005).
Nucleotide-based SIP approaches like DNA-SIP and RNA-SIP strictly require an
incorporation of around 20% 13 C label (Radajewski et al. 2000), while protein-SIP-
based approach is 200-fold more superior in sensitivity and can detect incorporation
levels of 13 C below 1% (Taubert et al. 2011). In a typical protein-SIP-based study,
the environmental samples are incubated with stable isotopes (13 C, 15
N, 36 S) which
are subsequently incorporated into the amino acids, peptides, and proteins. The
amount of atoms replaced by their heavy isotopes changes the natural isotope
composition of the labeled peptides. The rate of incorporation of stable isotopes is
used for assessing the metabolic activity of the corresponding species (Jehmlich
et al. 2008, 2012). The incorporation of a heavy isotope in the proteins is detected by
high-resolution mass spectrometry (HRMS) and nano-secondary ionization mass
spectrometry (nano-SIMS). The subsequent analysis takes into account the relative
isotopic abundance (RIA), and calculation of the RIA is either done by analysis of
the distribution of different isotope patterns of the peptides or based on features of
the peptide mass such as the relation of the parent mass to the first two digits (Seifert
et al. 2012).
2.4.2 FISH (Fluorescence In Situ Hybridization)
FISH is an excellent technique for reliable and rapid identification of
microorganisms from environmental samples, and prokaryotic cells can rapidly be
identified without cultivation using FISH. FISH involves hybridization of
oligodeoxynucleotide complementary to ribosomal RNA sequences (rRNA-targeted
nucleic acid probes) that have phylogenetic group-specific sequence signatures. In
laboratory protocol of FISH, whole cells from environmental samples to be studied
are often fixed by ethanol or paraformaldehyde treatment, and their 16S or 23S
rRNA is hybridized with fluorescently labeled taxon-specific oligonucleotide
probes. The labeled cells are viewed by scanning confocal laser microscopy
(SCLM) (Hill et al. 2000). The abundance of ribosomes (104–105) per cell and
consequent abundance of rRNA gene in bacterial cell, apparently observed lack of
lateral gene transfers, and a good length of about 1500 and 3000 nucleotides for 16S
and 23S, respectively, serve as a basis for hybridization of group-specific fluorescent
probes complimentary to rRNA gene. Further, the parameters, such as probe length,
GC content, and targeted region of the gene, are the crucial factors for deciding the
sequence of the correct species and genus-specific 16S/23S rRNA probes. A
workflow of the typical FISH experiment on environmental samples is presented
in Fig. 2.2. As in FISH experiment, whole cells are hybridized with group-specific
probes, the artifacts and bias introduced due to the DNA extraction, PCR artifacts,
and cloning are avoided (Felske et al. 1998). FISH has been successfully applied to
study the microbial community composition of different environmental samples
(Müller et al. 2016; Kindaichi et al. 2016), and several studies are reported in soil
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samples. For instance, Zarda et al. (1997) used Cy3-labeled rRNA-targeted oligonu-
cleotide probe “EUB338” to study the community structure of pristine forest soil of
“Hau” (an aquic eutrochrept bulk soil) and revealed predominance of microbial
members of α- and δ-subdivision of Proteobacteria and the Planctomycetes in
targeted soil samples.
Fig. 2.2 A flowchart of the fundamental steps involved in a typical FISH protocol
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2.4.3 Microautoradiography
Microautoradiography involves the incubation of the environmental sample with a
radiotracer for labeling of the microbial cell using radioisotopic compounds like soft
beta emitters ( 3 H, 14 C, 33 P) or strong beta emitter like 32 P (Rogers et al. 2007). After
incubation, the samples are fixed in paraformaldehyde or ethanol and washed to
remove surplus radiotracer. The radiolabeled substrate is subsequently taken up by
individual cells and releases beta decay particles that are used to develop the pattern
using a radiation-sensitive emulsion silver halide emulsion. Excited silver ions will
precipitate as metallic silver and will appear black grains after the development of
the film. These beta decay particles reduce the silver ions in silver halide crystals to
silver atoms generating silver grain clumps adjacent to radioactive cells. These silver
grain clumps, so developed, can be easily seen clearly using transmission light
microscopy/bright-field microscopy/phase-contrast microscopy/LSM (laser scan-
ning microscopy). Microautoradiography is often combined with fluorescence in
situ hybridization (MAR-FISH) which uses oligonucleotide probes for identification
of the microorganisms in order to link the key metabolic features to the identity of
the microorganism (Ouverney and Fuhrman 1999). The key determinant of a
successful microautoradiography experiment is the exposure times which may
range from a few hours to days and weeks depending on many factors which include
the decay rate of the radioisotope used, substrate concentration labeled, isotope
uptake rate, etc. Further, the cross-feeding of the labeled tracer may sometimes
give erroneous interpretation. For instance, anticipated incorporation of labels spe-
cifically targeted for glucose-consuming bacteria, in certain conditions, can be
incorporated and can cross-feed to other nontarget bacterial groups who cross-feed
on labeled products produced by certain glucose-consuming bacteria and may give
false-positive MAR signal. The microautoradiography, either alone or in conjunc-
tion with FISH, has been used to decipher the many aspects of soil microbial
community structure and functions (Varró et al. 1986; Rogers et al. 2007; O’Donnell
et al. 2007; Karbin et al. 2015).
2.4.4 DNA Microarrays
Deoxyribonucleic acid microarrays (also called DNA chips) were originally devised
for the studies of differential gene expression in health-related issues, but their
applications have also been extrapolated for the environmental studies like differen-
tial gene expression in response to environmental pollutants (Letowski et al. 2003).
The forms of nucleic acid microarray chip, so-called GeoChips and PhyloChips,
have been used to probe the microbial communities in various environmental
samples (Asuming-Brempong 2012). In a DNA microarray experiment, expression
of thousands of genes can be compared in two contrasting situations at the same time
on the same chip. The basic principle of DNA microarrays involves hybridizations of
two complementary, single-stranded regions of two DNA molecules, i.e., target and
probe DNA molecules (usually either one is labeled with fluorescent dyes like Cy3
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and Cy5), retrieved from two contrasting conditions. The hybridization events can
be detected, owing to the labeling of the bound complementary target, by high-
resolution scanning or imaging. A DNA microarray experiment is typically like the
tradition nitrocellulose membrane hybridization experiment, but the probe and target
relation in DNA microarray are reversed. In tradition hybridization experiments, the
free probe (which is known) is usually labeled and the fixed target DNA (unknown)
is not, while in a DNA microarray experiment, fixed DNA target (known sequences)
is unlabeled and target DNA (unknown) is the labeled one. In microarray
experiments, the testing substance is attached on the chip; hence some researcher
prefers to call it a probe (by analogy to conventional hybridization experiments), as
free DNA that we are querying is labeled (Zhou and Thompson 2002). In a typically
DNA microarray experiment, the various experimental parameters like optimal
probe concentration, its length, type of probe to use (i.e., oligonucleotide or
amplicon), detection, and specificity limits to be expected have to be optimized,
and further the interpretation and normalization of data to be reported are deciding
factors and have to be considered while executing the experiment. The sensitivity of
microarrays is always a critical factor. The oligonucleotide microarray displays
higher sensitivity as reported by Small and co-workers, in their study on unpurified
soil extracts. They were able to detect the G. chapellei SSU rRNA gene using
0.5 μg of total RNA extracted from soils (Small et al. 2001).
2.4.5 Isotope Arrays
The isotope array is a very important technique in microbial ecology which is ideally
suited to screen the microbial populations consuming particular substrates, even if
no previous knowledge is available about such microbes. The technique basically
involves incubating an environmental sample with a labeled substrate like 14 C-
labeled substrate, followed by the extraction of RNA from the samples, which is
then labeled with a fluorophore and used to hybridize with an oligonucleotide array
that targets 16S rRNA gene of the bacteria of interest (Adamczyk et al. 2003). Post-
hybridization, the array is scanned for fluorescence and incorporation of the radio-
active isotope in order to determine which community members have incorporated
the 14 C isotope into their RNA. The same probe which gives positive fluorescence
signal and displays the incorporation of radiotracer can be retested by applying them
with FISH-MAR with the same environmental samples (Hesselsoe et al. 2009). In
their pioneer study based on combination of microarrays with the uptake of radioac-
tive substrates, Adamczyk et al. (2003) were able to establish 14 CO 2 fixation
activities of the ammonia-oxidizing bacterial population within a complex activated
sludge community, with identification and function of microorganisms in activated
sludge. This method first time showed great isotope arrays as a technique that could
simultaneously detect the composition and activity of specific populations of bacte-
ria within wastewater treatment communities.
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2.4.6 Quantitative PCR
Quantitative PCR, also called real-time polymerase chain reaction, is essentially
based on analysis of specific DNA from an environmental sample which provides
the estimate of number of genomes of a particular microbe per unit volume of the
sample (Bustin et al. 2005; Smith and Osborn 2009). It also allows the quantification
of the number of target genes in a community sample. Unlike conventional PCR, in
the qPCR, a specific targeted DNA sequence is amplified and quantified simulta-
neously in real time, with progress of amplification reaction. The value so obtained
corresponds to the number of genome in water sample, but does not give directly the
number of cells. Several bacteria contain more than one copy of marker gene as
rRNA genes and hence complicate the analysis. qPCR uses either intercalating
fluorescent dyes such as SYBR Green or fluorescent probes (TaqMan probes,
molecular beacons, scorpion probes, etc.) in order to measure the accumulation of
PCR amplicons in real time as the amplification progresses. Several primers for the
amplification of 16S rRNA, 5.8S rRNA, ITS gene, and functional genes (amoA,
pmoA, norS, and dsrA) specific to ammonia oxidizers, methane oxidizers, and
sulfate have been designed and employed in PCR-based quantification of soil
bacterial and fungal microbial communities (Fierer et al. 2005; Foti et al. 2007). In
a qPCR-based study, Kolb et al. (2003) reported the abundance of total
methanotrophic population and specific groups of methanotrophs in a flooded rice
field soil by qPCR assay of the pmoA genes.
2.4.7 Microbial Lipid Analysis
Phospholipid fatty acid (PLFA) analysis has been successfully used as a culture-
independent method of assessing the structure of microbial communities in different
environments (White et al. 2003; Keinänen et al. 2004; Goupil et al. 2015; Yao et al.
2016). The membranes of microorganisms have phospholipids which contain fatty
acids; these phospholipid fatty acids are potentially useful signature molecules and
are used to obtain microbial community fingerprints. Further, phospholipids are
known to be present exclusively in cell membranes which are rapidly degraded
following cell death and hence serve as important indicators of active microbial
biomass. PLFA (phospholipid fatty acids) can be easily extracted from microbial
cells in soil, and their fatty acid methyl esters (FAMEs) are accepted taxonomic
discriminators for species identification which serve the basis for elucidating pres-
ence and abundance of a particular microbial group in community PLFA profiling.
PLFA profile of a particular soil type can be used to link it with soil associated with
particular cropping practices (Zelles et al. 1995) and can also be used to track
pollution (Frostegard et al. 1993) and changes in soil quality (Reichardt et al.
1997; Bossio et al. 1998; Petersen et al. 1998). Bossio et al. (1998) studied two
contrasted soil regimes, i.e., organically managed soils and soils receiving synthetic
fertilizers and pesticides, and observed significantly different PLFA profiles in two
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regimes with greater diversity and abundance of aerobic bacteria, cyanobacteria, and
methane-oxidizing bacteria in organically managed soil.
2.4.8 Cutting-Edge High-Throughput Sequencing for Community
Structure Analysis
The high-throughput sequencing is definitely cutting the edge for community struc-
ture which is allowing us to get deeper insights into microbial community structure
and their function in maintaining the major biogeochemical cycles of different
ecosystems including soil, water, deep biosphere, etc. The sequencing technology
has come a long way since the days of electrophoresis in the 1970s. With the advent
of Maxam-Gilbert method also known as chemical sequencing method and Sanger
chain termination method in 1977, scientists gained the ability to sequence DNA in a
reliable manner. This method prevailed from the 1980s until the mid-2000. Because
of its comparative ease and reliability, it is the method used in first-generation
technology.
While these “first-generation” sequencing platforms are considered high through-
put for their time, the short read massively parallel sequencing technique is a
different approach that revolutionized sequence capabilities and launched “next
generation” into genomic science. NGS is a term used to describe a number of
various modern sequencing methods like Roche/454 GS, Solexa/Illumina, SOLiD,
and Ion Torrent. It is because of NGS that researchers can now analyze thousands to
tens of samples in a single year. NGS applies to genome sequencing/resequencing,
transcriptomics (RNA-sequencing), ChIP sequencing, and epigenome characteriza-
tion. The first NGS technology to be commercialized in 2005 is the pyrosequencing
technique of 454 Life Sciences (now Roche) (Margulies et al. 2005). After that,
Solexa/Illumina (2007), Sequencing by Oligonucleotide Ligation and Detection
(SOLiD) by Applied Biosystems (2007), and Ion Torrent Personal Genome Machine
(PGM) (2010) are commercialized (Bentley 2006; Metzker 2010). Besides, Qiagen-
intelligent bio-systems sequencing, polony sequencing, and a single-molecule detec-
tion system (Helicos BioSciences) were also developed. Here, we discuss the five
platforms, i.e., 454, Illumina, SOLiD, Ion Torrent, and PacBio, which are routinely
used for high-throughput sequencing over the past decade.
2.4.8.1 Roche/454 GS
The method was developed by 454 Life Sciences (2004) and then overtaken by
Roche Diagnostics. This sequencing method is based on the “sequencing by synthe-
sis” principle. Here a single-stranded DNA is replicated to dsDNA by a polymerase
enzyme. The enzyme sequentially added bases at the end of the DNA fragment. This
process takes place inside the sequencing machine which contains many picoliter-
volume wells. Each well contained single bead and sequencing enzymes. The
sequencing process begins with the fragmentation of dsDNA into smaller fragments
of DNA around 400–600 base pairs with the help of some restriction enzymes
(Metzker 2010). Adapters (short sequence of DNA) are attached to the DNA
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fragments, and tiny resin beads are added to the mixture. The adaptor sequences
complementary bind with template DNA which helps DNA fragments to bind
directly to the beads. The DNA fragments are polymerized numerous times by
polymerase chain reaction on each bead. Beads without sequence are filtered to
remove, and the remaining DNA-containing beads are placed into wells on a
sequencing plate for sequencing. Nucleotides are added to the wells in turns of
one type of base at a time, i.e., A’s followed by C’s, G’s, and T’s. After single base
incorporation into the nascent DNA, the chemical signals, i.e., light generated by
luciferase enzyme, are converted into light that is recorded by CCD camera. The
intensity of light varies proportionally with the consecutive number of nucleotides
being analyzed (Mardis 2008). To decide the sequence of the original piece of DNA,
this pattern of light intensity is plotted on the graph.
2.4.8.2 Solexa/Illumina
This technology is worked based on sequencing-by-synthesis method using revers-
ible dye termination nucleotides. Along with DNA polymerase, all four fluorescent
label nucleotides are added consecutively to the flow cell channels to sequence
millions of clusters on the flow surface. The DNA is randomly fragmented
(200–600 base pairs), and adapters are ligated to the end of the fragments. Unlabeled
nucleotides and DNA polymerase are added to join the DNA strands which create
“bridges” between dsDNA. Using heating, dsDNA is denatured into single-stranded
DNA. The denaturation step leaves several millions of dense clusters of DNA that
are produced in each flow channel. After that, the sequencing cycles are begun by
adding primer, DNA polymerase, and four labeled reversible terminators (Mardis
2008). Using laser excitation, the emitted fluorescence from each cluster are captured
and bases are identified. In Illumina sequencing, DNA sequence is analyzed base by
base, making it a highly accurate method (Kozich et al. 2013).
2.4.8.3 Life Technologies SOLiD
Sequencing by Oligonucleotide Ligation and Detection (SOLiD) technology is
developed by Life Technologies (2006). SOLiD is done by ligation and dual base
encoding. The high accuracy of the SOLiD system allows analysis of samples across
a wide range of applications. Two types of sample preparation method are present in
SOLiD, i.e., fragment library (single DNA fragment) and mate-paired library (two
DNA fragments). In both libraries, DNA is sheared into specific size and adapters are
ligated to both the ends. There are millions of unique molecules in the library which
represent the entire target sequence. In emulsion PCR, all the molecules associated
with beads are clonally amplified. On the SOLiD system, the template-attached
beads are combined with a universal sequencing primer, ligase, and a large pool of
di-base probes. The di-base probes consist of a set of four fluorescently labeled
nucleotides. The complementary probe hybridizes to the template and is ligated.
After fluorescence is measured, the dye is cleaved from the 50-phosphate group, and
50 end is available for further reactions. This process is repeated several times
(approximately seven cycles) to yield a 35 bp long read. The previously synthesized
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strand is removed. A new primer is hybridized, and base addition and the ligation
cycles are repeated.
2.4.8.4 Benchtop Sequencers
The next-generation sequencing (NGS) portfolio was expanded by Life
Technologies; this system also uses the technology of sequencing by synthesis.
But this technology differs from the previous one; instead of fluorescence it
measures the H+ ion release during base incorporation. Chemical signals are directly
transferred into digital information in Ion Torrent PGM machine. The first step in Ion
Torrent PGM workflow is library construction. A library of DNA fragments is
generated that is flanked by Ion Torrent adapters. The DNA fragments generated
during library preparation are amplified onto Ion Sphere particles (beads). Amplifi-
cation is accomplished by emulsion PCR, and Ion Sphere particles coated with the
template are deposited in the chip wells. The template-loaded chip is placed on the
Ion Torrent PGM sequencer. The data in Ion Torrent PGM runs through signal
processing and base calling algorithms associated with individual reads. Individual
bases are introduced one at a time and are incorporated by DNA polymerase. For
each base incorporation, a proton is released that results in pH change. Every micro-
well of the PGM contains approximately one million copies of DNA. The pH change
in every individual well is detected by ion sensor, which transforms the chemical
changes into digital information. The chip records two bases if the voltage is doubled
by the detection of two identical nucleotides. The generated output files of Ion
Torrent system can be viewed and downloaded in sff, fastq, or sam/bam data
formats.
2.4.8.5 Single-Molecule Real-Time Sequencing/Pacific Biosciences
Owing to single-cell sequencing technology, now it is possible to sequence a single
stretch of DNA molecule extracted from a single or unique cell. Till date genome
sequencing has mainly become possible from a large number of template DNA
extracted from a culture of homogeneous bacterial population rather than a single
cell. The technology does not need any prior PCR amplification of DNA fragments.
The single-molecule real-time (SMRT TM ) DNA sequencing technology was
industrialized by Pacific Biosciences. This technology enables a new paradigm in
the genomic analysis by delivering longer reads and built-in flexibility. SMRT
sequencing is built upon two key innovations, i.e., zero-mode waveguides
(ZMWs), where light is illuminated at the bottom part of the well in which a template
and DNA polymerase, phospholipid nucleotides complex is immobilized. SMRT
sequencing relies on the principle of the single-molecule real-time sequencing
performed in SMRT cells having millions of ZMWs. A ZMW is a cylindrical
hole, hundreds of nanometers in diameter, performing a thin metal supported by a
transparent substrate. The ZMW provides the world’s smallest light detection
volume. The DNA template is extended by the DNA polymerase with the fluores-
cently labeled dNTPs, and fluorescent tag which previously incorporated nucleotide
is then cleaved off. The CCD camera captures this signal in a real-time PCR. This
process runs in simultaneously/parallel up to thousands of ZMWs, which make up
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the SMRT cell. All the advantages and disadvantages of these next-generation
techniques are presented in Table 2.2.
2.5 Conclusion and Future Scope
The constant improvement of molecular techniques, sequencing technology, and
bioinformatics revolutionizes the field of soil microbial ecology, i.e., the identifica-
tion of total microbial community structure, understanding the link between diver-
sity and community structure and function. Almost a decade of the study in
metagenomic techniques showed its ability to identify novel and rare unculturable
organisms and their function in maintaining the major biogeochemical cycle and soil
quality. Due to the higher throughput of data produced by “omics” studies, these
approaches are gaining momentum, and more and more reported scientific studies
are centered on these high-throughput approaches. However, the conventional
Table 2.2 Pros and cons of all the next-generation sequencing platform
Technologies Pros Cons
Roche/454
GS
Low error rate Medium/high start-up costs
Must run at a large scale
Relatively high costs per baseMedium read length (~400–
600bp to 1 Kb) It has difficulty in distinguishing the number of
bases in a run of identical bases (such as
AAAA)
Cheaper and faster
Solexa/
Illumina
Low error rate Must run at very large scale
Lowest cost per base Short read length (50–150 bp)
Tons of data Runs take multiple days
High start-up costs
De novo assembly difficult
SOLiD Relatively accurate because
each base is interrogated
twice
Potential for error propagation across reads due
to two-base encoding and sequential ligation
High instrument cost
Short readsHigh throughput and low
cost per base Relatively long run time
Independent lanes can be run
on 5500XL
Ion Torrent
PGM
Low start-up costs Read lengths only ~100–200 bp so far
Scalable (10–1000 Mb of
data per run)
Low error rate
Fast runs (<3 hrs)
Medium/low cost per base
SMRT/
PacBio
Can use single molecule as
template
Medium/high cost per base
High start-up costs
Potential for very long reads
(several 10Kb+)
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techniques of microbial community analysis still remain important in the view that
many findings of the high-throughput studies need to be validated and substantiated
using conventional techniques like qPCR, FISH, and autoradiography. However, the
integrated view of community dynamics can be generated by system biology
approach which requires integration of data from various conventional and
“omics” approaches, i.e., metagenomics, metatranscriptomics, metaproteomics,
and meta-metabolomics. Since all different omics approaches provide biological
information available at different levels, hence all the approaches have their own
advantages and limitations discussed in-depth in further chapters. Therefore,
complementing all the “omics” approaches with on another provides a better insight
into functional and the physiological state of the microbial communities of a system.
Therefore, use of multi-omics approaches for answering microbial ecology-related
question and improvement of bioinformatics pipeline for the large data analysis and
interpretation will be highly desirable in the future.
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