Deep Biosphere: Microbiome of the Deep Terrestrial Subsurface

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Abstract

Deep biosphere represents an unexplored realm of planetary life residing underneath the continental and oceanic crusts that constitutes majorly of prokaryotic life forms bacteria and archaea. Microbial communities which reside within various deep subsurface environments form a significant but largely unknown portion of the Earth's biosphere. While the shallow aquifer and sedimentary rock microbiome might get access to the nutrient pool available above ground, deep subterranean habitats hosted by crystalline rocks are severely constrained by the availability of photosynthetically derived nutrients. Deep subsurface microbiome underneath the continental crusts not only showed variations based on their geographic locations but also with respect to the abundance of various microbial populations and their metabolic properties. It is estimated that the deep biosphere microorganisms represent the largest pool of carbon, nitrogen, and phosphorous and constitute a critical component of biogeochemical engine of our planet. The aphotic deep dark microbial realm that has evolved possibly billions of years ago has developed unique metabolic repertoire for their survival. The deep biosphere microbiome is considered to be a portion of planetary life with extraordinary life-supporting system that works beyond our notion about biological and physical constraints. Advancement of techniques in microbial ecology has enabled us to decipher deep subsurface microbiome which resides up to several kilometers below the surface using both cultivation-dependent and cultivation-independent techniques. In this chapter, we have summarized our understanding of the deep biosphere microbiome within terrestrial subsurface. Habitability of life within the

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deep subsurface has been discussed considering the major metabolic routes deployed by the microorganisms. Cultivation-dependent and cultivation-independent studies and their requirement and outcome from various exploratory researches have been documented. Techniques used for sampling the subsurface microbiome are discussed, highlighting the role of possible contamination during drilling and subsequent postcore extraction processes. Lastly, applications of deep subsurface microbiome research in achieving better sustainability and biotechnological innovations are discussed.

Keywords
Deep biosphere · Metabolic processes · Metagenomics · Enrichment · Drilling process-contamination · CO_2 sequestration · Waste repository · Bioprospecting

8.1 Introduction

Variations and complexities of life on Earth have always surprised biologists. Even the intricacies of the simplest known organism are so pronounced that it leaves us astounded. Microbes are the earliest known life forms, which is evident from the fossil records. Microbes consist of both prokaryotes and eukaryotes, of which prokaryotes are thought to be the first living organisms on our planet. It is estimated that the number of prokaryotic cells residing on our planet ($4–6 \times 10^{30}$) (Whitman et al. 1998) is much higher than the number of planets present in our galaxy (1×10^{11}) (Cassan et al. 2012). Projections show that the total population of prokaryotes harbors $350–550 \times 10^{15}$ g carbon (accounting for 60–100% of the estimated total carbon in plants), $85–130 \times 10^{15}$ g nitrogen, and $9–14 \times 10^{15}$ g phosphorous (accounting for tenfold more nitrogen and phosphorous than plants) making them the largest pool of these nutrients in the living organisms (Whitman et al. 1998). Microbial assemblages can vary and diverge from place to place and create distinct biogeographic patterns (Green et al. 2008). Based on various evolutionary circumstances, biogeographic patterns are hypothesized to expand or regress owing to the effects of ecological and evolutionary forces at the genomic level (Ramette and Tiedje 2007). Traditional opinion on microbial biogeography has been that "Everything is everywhere, but the environment selects" (Baas-Becking 1934). However, it is debatable whether distribution of microbial populations over space results from environmental selection or if dispersal of microorganisms is restricted and affected by geographical barriers and other incidents in the geologic past (Eisenlord et al. 2012). Events in the geologic past may give rise to niche-specific diversity pattern through isolation and genetic divergence. Microbial diversity pattern often varies owing to uneven and unequal distributions of microbes. Restrictions in even and equal distribution of microbes suggest that factors shaping microbial community structure are more complex than the adaptive evolution through natural selection (Eisenlord et al. 2012).

Microbes are an important support for the Earth to function and microbial diversity is an unseen resource that deserves greater attention (Mishra 2015). Study on microbial diversity will not only help to maintain and conserve global genetic resources but also

will help us to know the unknown (Colwell 1997). Microbes reside in different spheres of our planet of which the major proportion of it inhabits the subsurface environment (Whitman et al. 1998). Permanent darkness persists in the subsurface provinces which are separated from the light-driven surface world (Edwards et al. 2012). The ecosystems which sustain in the subsurface environment are often referred to as deep biosphere. Hoehler and Jørgensen in 2013 described deep biosphere as "the set of ecosystems and their organisms living beneath the upper few meters of the solid earth surface." Extent of life in subsurface is much deeper than it was presumed earlier. It was thought that life is a surface phenomenon and sustenance of life even by the "hardy prokaryotic types" is not beyond tens of meters below the surface (Jannasch et al. 1971). In the 1990s and early 2000s, it became much more evident that life in the deep biosphere is ubiquitous and comprises a metabolically and genetically diverse microbial community (Parkes et al. 1994; Takai et al. 2001; Fry et al. 2008; Reith 2011). Nevertheless, the facts about lower depth limit of deep biosphere, energy sources sustaining microbial communities, and the link between microbial diversity/function and geochemical/geological factors remain elusive (Reith 2011). However, the knowledge that we have is that the deep subsurface is characterized by extreme conditions where the microorganisms have developed various mechanisms to deal with different physical and chemical constraints such as high pressure, high temperature, limited energy and nutrient availability, extreme acidity and alkalinity, metal toxicity, and radioactivity (Pikuta et al. 2007).

8.2 Habitability of Life in Deep Subsurface

Life in deep biosphere is often exposed to different extremes. Deep biosphere environments are generally characterized by aphotic and oligotrophic nature frequently having elevated temperatures and pressures. Other extremities include water scarcity, radiations, high salinity, and presence of degenerative substances which might be the limiting factors for sustenance of life in deep subsurface. It is thought that microorganisms that reside in such extremes must have evolved mechanisms of adaptation that makes themselves suitable to thrive under such harsh conditions (Kieft 2016). Interestingly, many of the inhabitants of these extreme environments can not only tolerate these harsh conditions but also often require those conditions for their survival (Rampelotto 2013). Knowing the extremities in the subsurface, it has also been postulated that microbial cells in deep biosphere might enter into a stage of "semi-senescence" due to severe nutrient deprivation which might extend their doubling time in the range of hundreds to thousands (Chivian et al. 2008).

Nutrient availability in the deep biosphere regime is limited and restricted. Microbes residing in such extreme habitats typically occupy the fractures or pore spaces with nutrients made available either from the rock/sediments and/or through transportation (via available interconnections) in the form of dissolved gases, solutes, or colloids (Fredrickson and Balkwill 2006). But often it has been found that sources of nutrients in the deep biosphere vary in different locations. Main sources of nutrients in deep provinces are either biogenic or geogenic in nature. Possible geological and biological processes that support the sustenance of life in the subsurface provinces mainly with

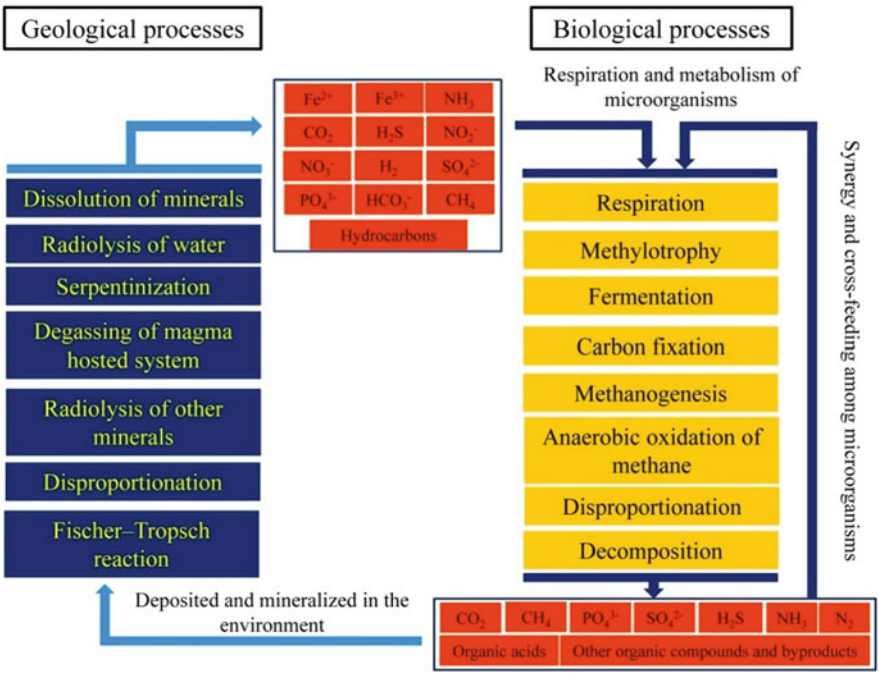

Fig. 8.1 Possible geological and biological processes that support the sustenance of life in the subsurface igneous provinces

respect to igneous provinces are mentioned in Fig. 8.1. Deeply buried organic matter can serve as a nutrient source for the subsurface life, but its presence in the subsurface environment is rare. Geologic events create an opportunity for introduction of nutrient in such oligotrophic environment. Seismicity and other processes like continental drifts create faults, fractures, and fissures which allow water to percolate in the deep subsurface. The infiltrated water from the surface not only provides the basis of life in the subsurface but also carries nutrients for microbial cells to thrive. Abiotic processes like serpentinization, radiolysis of water, oxidation of minerals, mineral dissolution, and degassing of magma-hosted systems help in the formation of different gases like H_2, CO_2, CH_4, and H_2S. These gases can be utilized by a specific group of microorganisms for their survival in the deep biosphere. Byproducts of these microbial groups are utilized by other populations for their sustenance. These kinds of ecosystems are mainly fuelled by hydrogen (which might be geogenic or biogenic in nature) and are termed as hydrogen-driven subsurface lithoautotrophic microbial ecosystems (SLiMEs) (Stevens and McKinley 2000; Nealson et al. 2005). The main stakeholders of these ecosystems are sulfate reducers, methanogens, and anaerobic methane-oxidizers. Sulfate reducers involved in these systems are obligate or facultative anaerobes which use the mechanism of dissimilatory sulfate reduction (DSR). DSR is a form of anaerobic respiration where sulfate is converted to sulfide. Microorganisms harboring sulfate adenylyltransferase (*sat*), adenylyl-sulfate reductase (*apr*), and dissimilatory sulfite reductase (*dsr*) genes are generally involved in this

process. DSR is mainly observed in bacteria affiliated to *Deltaproteobacteria* (genera *Desulfovibrio*, *Desulfomonile*, *Desulfopila,* and others) and *Firmicutes* (*Desulfotomaculum*, *Desulfosporosinus*, *Desulforudis*, and others). In hydrogen-driven ecosystems, often such reducers are fuelled by geogenic hydrogen or hydrogen liberated by anaerobic methane oxidizers. The process of anaerobic methane oxidation (AOM) is restricted to the domain archaea, and most of the members are closely related to class *Methanomicrobia* (ANME-1, ANME-2, and ANME-3). AOM was also reported in other archaeal members such as *Candidatus* Methylomirabilis oxyfera, *Candidatus* Methanoperedens nitroreducens, and Marine Benthic Group D (Cui et al. 2015). All known ANME members harbor methyl-coenzyme M reductase (*mcr*) gene which is the key gene for methanogenesis, and it is postulated that *mcr* present in these archaea is responsible for anaerobic methane oxidation by a process called reverse methanogenesis (Cui et al. 2015; Timmers et al. 2017). Though AOM was first found to be coupled with sulfate reduction, later studies reported coupling of AOM with denitrification and metal ion (Mn^{4+} and Fe^{3+}) reduction (Cui et al. 2015). Anaerobic methane oxidizers in the deep subterranean environment are driven by biogenic or abiogenic methane. Biogenic methane is liberated by methanogens which are one of the prominent residents of deep biosphere. Methanogenesis is restricted to the domain archaea and mainly affiliated to seven orders (*Methanobacteriales*, *Methanococcales*, *Methanomicrobiales*, *Methanosarcinales*, *Methanocellales*, *Methanopyrales*, and *Methanomassiliicoccales*) of phylum *Euryarchaeota*. Later studies revealed that methanogenesis is phylogenetically widespread and also found in phylum *Bathyarchaeota* (formerly Miscellaneous Crenarchaeota Group), *Verstratearchaeote*, and other Candidatus groups (Vanwonterghem et al. 2016). Central role of methane metabolism is played by methyl-coenzyme M reductase complex. Substrates for methanogenesis mainly include H_2/CO_2 (hydrogenotrophic), acetate (acetoclastic), and methylated compounds (methylotrophic). In subsurface oligotrophic ecosystems, it is frequently seen that these substrates are produced by fermentative group of microorganisms. Fermentation is an anaerobic process where sugar is consumed by an organism to produce CO_2, H_2, organic acids, alcohol, or combination of either. This process is not only widespread across different taxonomic groups of bacterial domain but also found in single-celled eukaryotes such as yeast. Some of the known organisms which are prominent fermentors are *Saccharomyces* (ethanol fermentation), *Lactococcus* (homolactic acid fermentation), *Leuconostoc* (heterolactic acid fermentation), *Propionibacterium* (propionic acid fermentation), *Escherichia* (mixed acid fermentation), *Enterobacter* (2,3-butanediol fermentation), *Clostridium* (butyrate fermentation and acetone-butanol fermentation), and *Acetobacterium* (homoacetic acid fermentation). Some of the genes which play key roles in fermentation are lactate dehydrogenase (*ldh*), pyruvate dehydrogenase (*pfl*), alcohol dehydrogenase (*adh*), acetate kinase (*ack*), phosphoenolpyruvate carboxylase (*ppc*), and malate dehydrogenase (*mdh*). In addition to these pathways, denitrification and ammonification (for respiration and assimilation) are frequently observed in subterranean deep biosphere. Both these processes not only play an important role in nitrogen cycle but also are commonly coupled with processes of other subterranean biogeochemical cycles. Some of the common denitrifiers are affiliated to *Pseudomonas*, *Micrococcus*, *Achromobacter*, *Serratia*, and *Thiobacillus*. Major genes involved in denitrification

processes are nitrate reductase (*nar*), periplasmic nitrate reductase (*nap*), nitric oxide reductase (*nor*), nitrous oxide reductase (*nos*), and nitrite reductase (*nir*). Dissimilatory nitrate reduction to ammonium (DNRA) is an important ammonification process in the deep which is generally found in anoxic environment and observed in both prokaryotes (*Beggiatoa*, *Thioploca*, *Candidatus Nitrosocaldus yellowstonii*, and others) and eukaryotes (*Aspergillus terreus*, *Fusarium oxysporum*, *Cylindrocarpon tonkinense*, and others). Common marker gene used to detect bacterial DNRA is nitrite reductase (cytochrome c-552) (*nrf*A) which is reported from different subterranean deep biosphere sites (Momper et al. 2017; Lau et al. 2016).

Different physicochemical conditions in the subsurface environment suggest that chemolithoautotrophic microorganisms are the main dwellers in the subsurface provinces. S^{2-}, NO_2^-, NH_3, Fe^{2+}, and H_2 are widely available reduced inorganic compounds in the subsurface environment which can act as an energy source for the chemolithoautotrophs. Reducing inorganic compounds may be geogenic or biogenic in nature. Geogenic sources of these reducing compounds are mainly from mineral (like pyrite, phyllosilicates, etc.) dissolution, water-rock interaction, and radiolysis, whereas the biogenic sources are the products of sulfate-reducing, denitrifying, nitrogen-fixing, iron-reducing, and fermentative bacteria (Nealson et al. 2005; Chivian et al. 2008; Lau et al. 2016). Chemolithotrophs can be either obligate or facultative in nature which are phylogenetically diverse and play an important role in different biogeochemical cycles in subsurface provinces. Some of the established chemolithotrophs are *Nitrospira*, *Nitrobacter*, and *Nitrosomonas* (ammonia oxidizers); *Gallionella*, *Thiobacillus ferrooxidans*, and *Leptospirillum ferrooxidans* (iron oxidizers); *Hydrogenobacter thermophilus*, *Aquifex aeolicus*, and *Hydrogenovibrio marinus* (hydrogen oxidizers); and *Acidithiobacillus*, *Thiomonas*, and *Thiobacillus* (sulfur oxidizers). Common genes involved in chemolithotrophy are ammonia monooxygenase (*amo*), sulfur-oxidizing protein (*sox*), sulfide:quinone oxidoreductase (*sqr*), hydrogenase expression/formation protein (*hyp*), [NiFe] hydrogenase, and [FeFe] hydrogenase. These chemolithoautotrophs often derive cellular carbon from carbon dioxide. Some of these organisms also harbor genes to fix bicarbonate. There are several CO_2 fixation pathways which are observed in aphotic subterranean provinces. One of the earliest known pathways for CO_2 fixation by microorganisms is Calvin-Bassham-Benson (CBB) cycle which uses 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) to assimilate CO_2 (Bassham and Calvin 1957). Other CO_2 fixation pathways frequently reported from deep biospheres are reductive tricarboxylic acid (rTCA) cycle, Wood-Ljungdahl (WL) pathway, and 3-hydroxypropionate (3-HP) cycle (Momper et al. 2017; Lau et al. 2016; Purkamo et al. 2015). In addition to CO_2 fixation, the 3-HP pathway exclusively fixes HCO_3^-. Some of the key marker genes used to detect these pathways are ribulose-bisphosphate carboxylase (*rbc*) (CBB cycle), ATP citrate (pro-S)-lyase (ACLY) (rTCA cycle), acetyl-CoA synthase (*acs*) (WL pathway), and acetyl-CoA carboxylase (*acc*) (3-HP) cycle. Geogenic sources of carbon dioxide and bicarbonates in the subsurface provinces are mainly from degassing of magma-hosted system and dissolution of calcite minerals, respectively. Biogenic contributors of CO_2 in the subsurface ecosystems are mainly the fermentative and anaerobic methane-oxidizing bacteria. Chemolithoautotrophs can be both aerobic and anaerobic in nature. Though oxygen is limited in the subsurface provinces, presence of terminal electron

acceptors (TEA) like NO_3^-, NO_2^-, Fe^{3+}, and SO_4^{2-} allows facultative anaerobes and obligate anaerobes to thrive in the subsurface depending on the availability of TEA.

Deep biosphere is often deprived of organic carbon. Dearth of organic carbon in the subsurface environment gives us a sense that microbial life in deep biosphere has prevalent chemolithoautotrophic lifestyle, but this is not always true. Heterotrophic microorganisms have also been reported from subsurface environment (Hallbeck and Pedersen 2008; Nyyssönen et al. 2014; Purkamo et al. 2015). Metabolic intermediates or products of chemolithoautotrophic metabolism can fuel the heterotrophs in the subsurface. Heterotrophic microbial groups can also be fuelled by geogenic hydrocarbons generated by Fischer–Tropsch reaction where liquid hydrocarbons are created from carbon monoxide and hydrogen (Purkamo et al. 2016).

Considering the extremities, it is often thought that specialist groups of organisms having less diverse populations are known to reside in the subsurface environment. Though single-species ecosystem has been reported from South African Gold Mine (Chivian et al. 2008), most of the reports suggest that organisms in nutrient-deprived stressed subsurface ecosystems prefer to work in synergy. Often co-occurrence of different microbial populations in the subsurface environment is observed which substantiates the fact of cross-feeding and mutualistic behavior in the subsurface. Co-occurrence of microbial populations might also be attributed to the nutrient availability and environmental amicability where different species exploit the same resource and prefer to reside in a similar environment.

8.3 Cultivation-Dependent Studies

8.3.1 Cultivable vs Uncultivable

Investigation on deep biosphere microbiome remained more cultivation centric for a considerable period (Jannasch et al. 1971; Cragg et al. 1990; Parkes et al. 1994; D'Hondt et al. 2004; Hallbeck and Pedersen 2008). In the early 1990s, the first report on changes in microbial activity with depth, biogeochemistry, and estimation of cultured biodiversity was published (Cragg et al. 1990; Parkes et al. 1994) showing that there exists a clear link between biological activity and the availability of organic carbon and terminal electron acceptors in the deep subsurface biosphere. D'Hondt et al. (2004) reported the diversity of microbial communities and numerous energy-yielding activities that occur in deeply buried sediments of oceanic environment. Using the samples recovered from one of most representative sites for Earth's ocean (Ocean Drilling Program (ODP) Leg 201: equatorial Pacific Ocean and the continental margin of Peru) wherein the water depths range from 150 m to 5300 m, elaborate analysis of microbial activities including metabolic requirements has been reported. Sediment samples were obtained from sub-seafloor depth from 0 to 420 m, temperature from 1 to 25 °C, and age from 0 to 35 million years ago (Ma). Presence of prokaryotic cells occurring throughout the sampled sediment column was noted in every location. With culture-based methods, these investigators have shown that rates of activities, cell concentrations, and populations of cultured bacteria may vary consistently from one sub-seafloor environment to another. A major role of

photosynthetically derived substances from surface in providing necessary electron acceptors and electron donors for microbial metabolism was noted. Hallbeck and Pedersen (2008) reported that microorganisms should be considered as an inseparable part of the "hydrogeochemical modeling." They have developed and tested several culture-dependent methods to estimate the total number of microbial groups, to quantify their biomass amount in groundwater, to study their diversity, and to find out the type of metabolic profile they belong to. Recently, another interesting study with Fennoscandian shield deep subsurface groundwater samples has hypothesized that microbial communities residing in deep subsurface Fennoscandian shield are distinctive to each site or area (Purkamo et al. 2018). The role of iron-oxidizing bacterial communities and methanogenic and ammonia-oxidizing archaeal groups was identified. The role of geochemistry in shaping microbial communities and their functions were highlighted.

Isolation and characterization of microbial populations using various enrichment or direct isolation-based methods have enriched our understanding of this section of microbial world. We have looked into the 16S rRNA gene inventory within the Ribosomal Database Project Database (Fig. 8.2). With a search keyword of "Deep Biosphere," 269 sequences so far retrieved from various isolates were found. These organisms are taxonomically affiliated to six phyla with the maximum hits belonging to the phylum *Thermotogae*, followed by proteobacterial members.

The following section describes a brief outline of the cultivation-based microbiome study and importance of getting the appropriate medium for growth and cultivation of deep biosphere organisms. In natural habitats, there exist microorganisms that can be differentiated into distinct categories based on their culturability (Madsen 2008; Stewart 2012). The small fractions of the total microorganisms that readily form colonies on agar plates are the ones that grow and are known as the cultured organisms. Microbial growth requires proper resources, especially a carbon source, nutrients, electron donors, and electron acceptors, and necessary interactions among the organisms and their abiotic environment. Cultured microorganisms are those that have been successfully isolated and purified in the laboratory. The remaining ones that do not grow on readily formulated medium are known as uncultured microorganisms. Uncultured microorganisms are the ones for which no appropriate growth medium has been

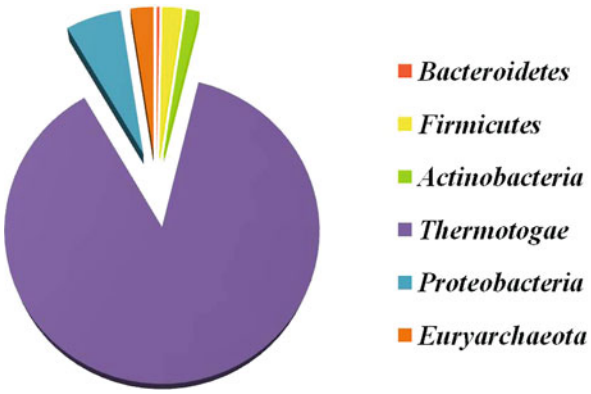

Fig. 8.2 Taxonomic affiliation of microorganisms isolated from deep biosphere

devised (Stewart 2012; Vartoukian et al. 2010). The uncultured category can be further divided into culturable and nonculturable. Culturable microorganisms are the ones that can be cultured when an optimized growth medium, which matches the organism's nutritional needs, is used for cultivation. Key physical and chemical growth conditions must also be provided. Nonculturable organisms are the ones whose physiological state prevents them from being cultured, i.e., they do not grow even when proper growth conditions are provided. Nonculturable cells are also known as dormant (Madsen 2008).

Figure 8.3 illustrates the approaches for isolation of different categories of microorganisms from deep subsurface rock samples based on their cultivability.

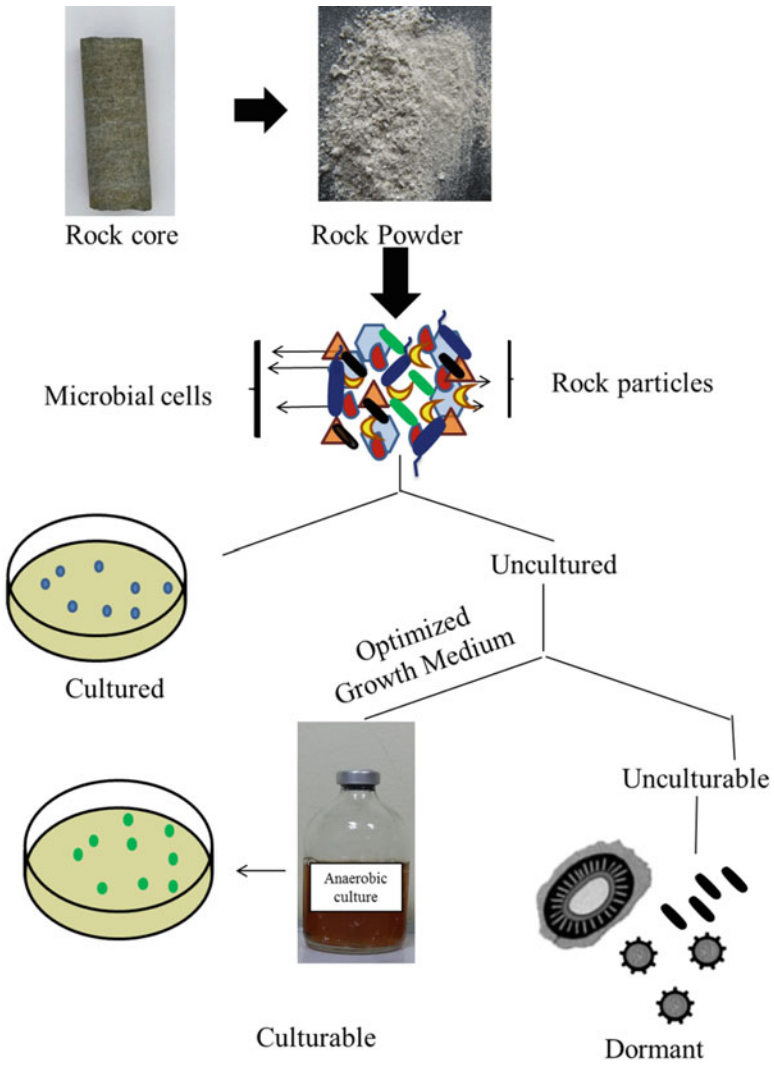

Fig. 8.3 Different categories of microorganisms (based on their cultivability) which could be studied

8.3.2 Requirements for Cultivation and Growth of Microorganisms from Deep Subsurface

For growth to happen, microbes need energy and other essential material resources which in turn can be obtained from raw materials and nutrients. A culture medium is one such preparation which provides the organism with necessary requirements of nutrients. Specialized media preparations are essential in the isolation, identification, and characterization of microorganisms from deep oligotrophic subsurface samples. Although all microorganisms need sources of energy, carbon, nitrogen, phosphorus, sulfur, and various other major and trace elements, the exact composition of a proper medium depends on the type of organism to be cultivated, as nutritional requirements of different group of organisms vary greatly. Idea about the microorganism's normal habitat can often be useful in selecting an appropriate culture medium because the nutrient requirements are very much linked to its natural surroundings (Prescott et al. 2002).

Exploring the microbial diversity in nature and finding the ecological connections between environmental geochemistry and microbial communities will help us to unveil how organisms survive and thrive in natural habitats and provide insights into the development and expansion of life on Earth. For many years, attempts have been made by researchers to use molecular techniques to find the relationships between microbial diversity and physicochemical and geochemical parameters, indicating that environmental variables such as moisture content, conductivity, pH, temperature, and concentrations of electron acceptors and donors can have strong influences on the phylogenetic differentiation among microbial cohorts in natural habitats. These studies have provided insights into the factors, both physical and chemical, that dictate what kinds of metabolisms are possible in a natural setup which in turn determines the pathways of energy harness by the inhabiting microbes. It also helped in understanding the response microbes have toward temporal shifts in the environment geochemistry. In fact, even in "extreme" environments, the distribution of microbial populations and communities is shaped by the prevailing environmental conditions (Richards et al. 2014; Stevens et al. 1993).

Conversely, microbes shape their geochemical surroundings through their metabolic interactions and growth needs, controlling every facet of redox, metal, organic, nutrient, and trace element components, which determines the geochemical and mineralogical composition of the surroundings. Microbial evolution has occurred in concert with changing geosphere conditions—microbes have been the major drivers causing shifts in the chemistry of oceans, continents, and atmosphere (Knoll 2003a, b). The role of microbes is critical for element cycling in any environmental system. A combination of different experimental approaches to interrogate microbial activity (through physiology, genetics, culturing, and microscopy) and geochemistry (aqueous, mineral, isotope geochemistry) has been developed to address these critical and significant interactions between microbes and their surroundings. Changes in the environment occur when interactions between physical

entities exceed its buffering capacity. Environmental change, in turn, feeds back on biology, creating shifts in microbiological communities. In nature, energy and nutrient flow is intricately coupled to complex geochemical reactions and processes (mineral precipitation and dissolution reactions, absorption reactions, redox reactions, etc.) that can affect the microbial growth (Istok et al. 2010). In turn, microorganisms also influence the chemical and physical properties of their surrounding environment (Ham et al. 2017). Microorganisms, residing in minute fractures in the deep crystalline crust, gain energy by following diverse metabolic processes (Kieft 2016). Microorganisms can interact with the environment acquiring different nutrients, electron donors, and electron acceptors such as molecular oxygen, nitrate, metal oxides, sulfate, sulfur, carbon dioxide, or water. Metabolic and growth interactions are not the only interaction happening in the environment. Microbes interact within themselves following different mutualistic relations, which help each of the interacting groups to survive in a particular environment and thrive in nature. Sharing of electron donors and acceptors, interspecies hydrogen ion transfer, and utilization of metabolic byproducts are some of the interactions which play a major role in the formation of a microbiome inside the deep terrestrial subsurface. Life in the deep subsurface is partially dependent on the supply of carbon and energy from the surface even though there are evidences that microbial life habituating deep in the crystalline rocks can derive its energy from autotrophic processes independent of photosynthesis and can also utilize hydrogen as an energy source (Stevens 1997; Pedersen 1999). Figure 8.4 illustrates some interactions which go on in between environment and organism and within different microorganisms residing inside the Earth's crust.

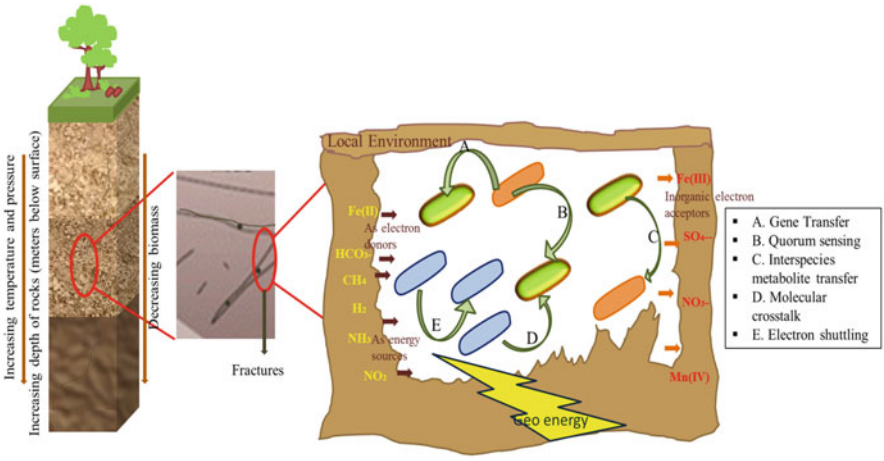

Fig. 8.4 Processes and interaction (inter cellular interaction and interaction with the local environment) that occur within the deep subsurface which fuels the biosphere

8.3.3 "Omics"-Based Technologies: A Helping Hand Toward Understanding the Organisms, Their Metabolisms, and Their Growth Requirements

In order to achieve higher levels of cultivability, necessary clues may be obtained from cultivation-independent, omics-based microbial community studies. High-throughput next-generation sequencing technologies have rapidly become a substantial tool for studying diversity and distribution of microbial ecosystems in the environment. Large-scale sampling and deep sequencing of microbial communities from different geographic regions and areas have revealed that there are specific effects of geochemical factors on the microbial diversity patterns and community composition in the environment (Liu et al. 2014; Joseph et al. 2003; Vartoukian et al. 2010). These technologies have enabled the generation of large amounts of genetic information on microorganisms without the need to grow cultures in the lab. Armed with these technologies, one can generate draft metabolic network for organisms directly from genome annotations and shed light on the procedures to enhance growth of cultivable microbes. A closer look into the 16S rRNA gene inventory within the Ribosomal Database Project Database, a search string of "Deep Biosphere" retrieved 1050 matches of uncultured microorganisms belonging to various deep subsurface regions. These organisms are taxonomically affiliated to various phyla with the maximum hits belonging to the archaeal phylum Crenarchaeota, followed by other archaeal phyla. As for the members of the bacterial domain, the maximum members belonged to the phylum *Microgenomates* (Fig. 8.5).

This kind of "omics"-based approach can help us in knowing the organisms residing in a particular habitat and hence help in formulating specific growth medium to

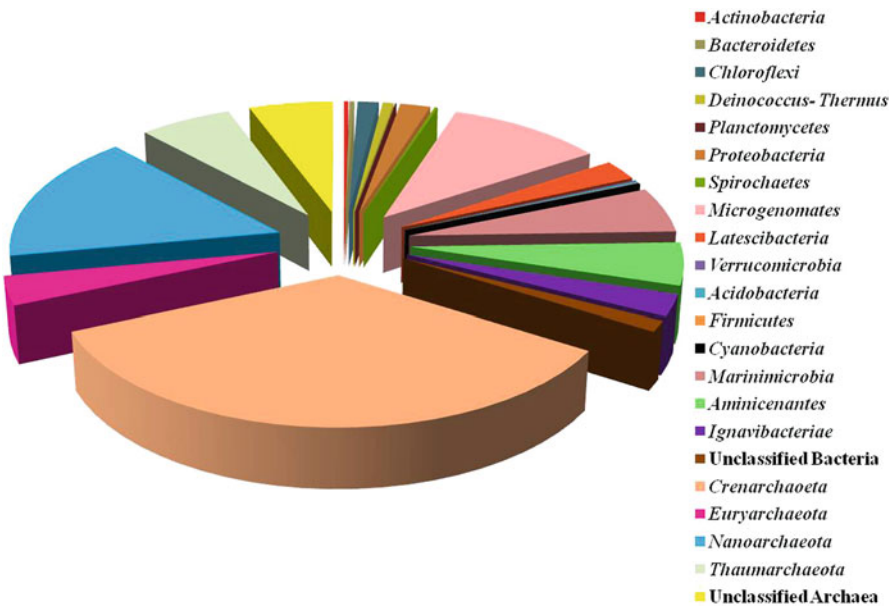

Fig. 8.5 Taxonomic affiliation of uncultured microorganisms from deep biosphere

cultivate the uncultured population. Isolation of pure microbial cultures and cultivating them in the laboratory on defined medium is used to characterize the metabolism and physiology of organisms fully. But, as it is said that it is "easier said than done," identifying an appropriate growth medium for a novel isolate remains a challenging task. Even organisms with sequenced and annotated genomes can be difficult to grow, despite the ability to build genome-scale metabolic networks that connect genomic data with metabolic function. The term the "great plate count anomaly" was coined by (Staley and Konopka 1985) to describe the difference in magnitude between the number of cells from environmental samples that form colonies on agar media and the numbers countable under the microscope. One of the most significant explanations for the "great plate count anomaly" is that many of the microbial species that can grow in natural settings are not adapted for growth in media containing high concentrations of complex organic carbon, i.e., the medium components are inadequate. It can also be such that the species that would otherwise be "culturable" may fail to grow because of the inability to adjust to the conditions found in the medium used for the plate counts. These microorganisms may need oligotrophic or other fastidious conditions to be successfully cultured. These microbial strains maybe are common in nature but can only be cultivated by specialized techniques (Spiegelman et al. 2005). An important requirement of culture-dependent study is the growth medium. For proper growth of most of the organisms present in an environmental sample, the media for growth should be similar in nature to the surrounding habitat. The medium should more or less mimic the physiological, chemical, and environmental conditions of the ecosystem, for example, geochemistry of the rock samples and environmental conditions of the site should be studied. The hydrologic and geologic properties of the samples should be adequately understood to predict the distribution and physiologies of the microorganisms throughout the depth and also the mechanisms involved in their colonization (Colwell et al. 1997).

8.3.4 Medium Formulation Based on Extensive Study of Local Geochemistry

The repertoire of prokaryotic life found in the subsurface and sub-seafloor biosphere by cultivation-independent molecular methods is much greater than obtained by standard laboratory culture methods (enrichment setups and isolation procedures). Also, populations obtained so far using cultivation-based methods represent only a very small subset of those revealed by molecular methodologies and culture-independent studies (D'Hondt et al. 2004). Yet methods for analyzing microbial metabolic processes and their outcome are being developed, tried, and tested in situ conditions (Hallbeck and Pedersen 2008). Medium formulations, enrichment culturing, and different other isolation procedures are being used from the early days of this deep subsurface research to peek into this world of unknown habitants and study them. According to Stevens et al. (1993), geochemical processes which may be interdependently controlled with microbiological processes can contribute toward formation of a specific condition of the sampling site. To stay alive, grow, and propagate, microorganisms transform several components present in their local environment, between different reduced and oxidized

states. Microbiological growth and enhancement depend on the energy sources and electron acceptors present (Madigan et al. 2006). Organic carbon (including methane) and reduced inorganic molecules (including H_2) are possible energy sources in the subterranean environment (Hallbeck and Pedersen 2008). Table 8.1 highlights different examples of case studies where formulation of medium was done after extensive study of local geochemistry of the deep subsurface regions.

8.3.5 Nature of Organisms Recovered from Diverse Deep Terrestrial Subsurface Environments Through Enrichment Studies

Study of the environment deep beneath the Earth's surface may provide an opportunity in understanding the mechanism which helps organisms to survive in extreme and apparently nonfavorable conditions. There is a lot of evidence which supports the presence of life which is ubiquitously distributed deep inside the Earth's crust. It has also been suggested that this life is dependent on lithogenically and geogenically produced energy compounds to sustain their existence (Colman et al. 2017). The rock minerals play a critical role in providing the different growth elements which in turn helps in sustenance of life in this extreme habitat. This biosphere consists of a diverse group of organisms which mostly follow the anaerobic mode of respiration. Depending on the type of mineral which predominates, organisms like sulfate reducers, iron reducers, nitrate reducers, and acetate producers can be found. Presence of acetoclastic and hydrogenotrophic methanogens can also be seen in this biosphere.

Study of microbial community in rock-hosted deep terrestrial subsurface environment is limited. Among the published literature that has discussed about deep subsurface biosphere, some of the works were selected. Major deep biosphere culture dependent studies undertaken in Asia took into consideration parts of Japan and China. Chinese Continental Scientific Drilling Project at China is one of the deepest (2026 m) and earliest explored subsurface site in Asia (Zhang et al. 2005). Subsurface environment of this site was mainly dominated by proteobacterial members. The presence of *Bacteroidetes* and *Planctomycetes* was also observed. Iron-reducing bacteria were observed which thrived in thermophilic and alkaliphilic conditions. As per reports by Fukuda et al. (2010), several studies were conducted in mine environments and established Underground Research Laboratories (URL) in Japan. They suggested the presence of members of *Proteobacteria* and *Firmicutes* which could survive in alkaliphilic conditions. Piceance Basin, western Colorado, USA, North America, was explored to search for microbial communities where presence of anaerobic thermophilic sulfate-reducing bacteria was reported (Colwell et al. 1997). Similar studies were reported in basaltic aquifers of Snake River Plains (Lehman et al. 2004). These studies revealed presence of bacterial members which included heterotrophs, hydrogen oxidizers, iron reducers, etc. Subsurface sedimentary rocks of Antrim Shale harbored methanogenic communities (Waldron et al. 2007). On exploration of deep mine environment of North America, like

Table 8.1 Case studies dealing with formulation of proper growth medium based on geochemistry of samples

Local geochemistry	Media formulated	Organisms cultivated successfully/types	References
Alkaline pH, presence of sulfate, iron TIC>TOC, temperature around 29 °C	Artificially formulated. Containing five different electron acceptors (O_2, Fe(III), NO_3^-, SO_4^{2-}, HCO_3^-) and four groups of Electron donors (fermentation products, monomers, polymers, aromatics) in a mineral salts medium at pH 9.5 Incubation at 30 °C	Sulfate reducing bacteria (SRB)/Nitrate reducing bacteria (NRB)/Iron reducing bacteria (IRB)/ bacterial populations utilizing varied carbon substrates	Stevens et al. (1993)
Near-neutral pH, has presence of sulfate, manganese, iron, hydrogen, high TOC	Medium formulated which mimicked the in situ groundwater chemistry for optimal microbial cultivation Medium for NRB, SRB, IRB, Manganese reducing bacteria (MRB), Autotrophic acetogens (AA), Autotrophic methanogens (AM), Heterotrophic acetogens (HA), Heterotrophic methanogens (HM), etc., pH around 7	SRBs/NRBs/IRBs/ MRBs/acetogens and methanogens	Hallbeck and Pedersen (2008)
Contains sulfide, sulfate, nitrate, iron, methane, H_2/CO_2	MG1 medium was supplemented with 20 mM acetate and a headspace composed of N_2:CO_2, medium MG2 with the gas mixture H_2: CO_2 and medium MG3 was supplemented with a solution composed of propionate: Butyrate: Methanol and headspace composed of N_2:CO_2. The basal medium was same for all. Incubation of cultures was done at 30 °C	*Proteobacteria*, *Actinobacteria*, *Rhizobium*, *Nocardioides, Desulfovibrio, Brevundimonas*, etc.	Leandro et al. (2018)
Presence of sulfate, CH_4, acetate, H_2/CO_2 Temp: 29 °C in	MJS medium for cultivation of methanogens with methanogenic substrates	ANME–I organisms	Ijiri et al. (2018)

(continued)

Table 8.1 (continued)

Local geochemistry	Media formulated	Organisms cultivated successfully/types	References
sediments and around 60 °C in porewater	like H_2/CO_2, acetate, formate, etc., pH around 7 and temperature 25 °C/ 55 °C		
Alkaline pH, temp 10–20 degrees, low organic carbon content, sulfate conc. Increased with depth. NH_4^+ ions were present in detectable amounts	Artificial medium formulated for growth and enrichment of aerobes, obligate and facultative anaerobes, pH 7.5. Incubation was done at 20 °C	Obligate anaerobes, SRBs, etc.	Pedersen and Ekendahl (1990)

Lupin Au mine (Canada), Onstott et al. (2009) reported the presence of organisms which can reduce sulfate as a part of their metabolism or can tolerate high salt concentration. Fennoscandian shield which is present in the northern part is the most studied location in the continent of Europe. Many investigations related to deep biosphere have been done in this location that has increased our understanding in the field of deep biosphere. Äspö Hard Rock Laboratory and Outokumpu deep borehole are the prominent deep biosphere sites of the Fennoscandian shield. Lubin copper mine, in Poland, is also one site where the microbiome of the subsurface has been studied. The mechanisms by which these organisms adapt to such environments are studied extensively in this site. Organisms found here are mostly mesophilic in nature and can survive in high pH. The microbiome broadly consists of methanogens and sulfate reducers (Hallbeck and Pedersen 2012; Kotelnikova and Pedersen 1997; Rajala et al. 2015; Dziewit et al. 2015; Rajala and Bomberg 2017; Purkamo et al. 2017). Many ultradeep mines and gold mines in the African continent have been explored to study about the deep subsurface organisms. When native organisms from the samples were enriched under different conditions using specific medium or using supplements in the sample itself, a variety of organisms could be reported. Organisms belonging to archaeal and bacterial lineages which can sustain in extremes of temperature and pH were found to be prevalent in these environments. Methanogenic organisms were also reported (Lazar et al. 2017; Onstott et al. 2003; Kieft et al. 2005; Lin et al. 2006). An elaborate details about different organisms that are identified from these selected study sites based on cultivation-dependent studies has been provided in Table 8.2.

Table 8.2 Details of the selected terrestrial deep biosphere sites based on culture-dependent and enrichment-based studies

Area	Sample and depth	Medium	Temperature and pH	Organisms/taxa identified (isolation or via sequencing)	References
Asia: Chinese continental scientific drilling Project	Rock samples, 529–2026 m	Minimal medium M1 and FWA-Fe (III) medium	Thermophilic and alkaliphilic	*Proteobacteria*, *Bacteroidetes*, *Planctomycetes*	Zhang et al. (2005)
Asia: Mizunami Underground Research Laboratory, Central Japan	Groundwater, 1148–1169 m	Groundwater supplemented with different nutrients	Mesophilic and alkaliphilic	*Proteobacteria*, *Firmicutes*, *Brevundimonas* spp., *Thauera* spp.	Fukuda et al. (2010)
North America: Piceance Basin, Colorado	Sandstone, 856–2096 m	PTYG and TSA	Mesophilic and thermophilic	*Desulfotomaculum nigrificans*	Colwell et al. (1997)
North America: Antrim Shale	Sedimentary rocks, 302–520 m	DSMZ medium 141 and 318	Mesophilic	*Methanocorpusculum bavaricum*, *Methanomicrobiales*	Waldron et al. (2007)
North America: Lupin Au mine, Canada	Saline fracture water, 890–1130 m	Low nutrient marine broth/0.1× tryptic soy broth	Subpermafrost psychrophilic	*Desulfosporosinus, Halothiobacillus, Pseudomonas*	Onstott et al. (2009)
North America: Snake River Plain Aquifer	Basaltic core and fracture water, 63–134 m	R2A medium and nitrate mineral salts medium	Mesophilic and alkaliphilic	*Pseudomonas, Burkholderia, Brevundimonas, Acidovorax, Hydrogenophaga, Xanthobacter, Alcaligenes, Aurobacterium, Flavobacterium, Rhodococcus, Rhodobacter, Nocardia, Paenibacillus,* and *Micrococcus*	Lehman et al. (2004)
Europe: Swedish repository for spent nuclear fuel	Granitic groundwater, 112–978 m	Formulated medium for isolation of SRB, NRB, IRB, MRB, AA, AM, HA, HM	Mesophilic and alkaliphilic	*Methanosarcina*-like organisms, *Methanohalophilus*-related organisms, *Methanobacterium*	Hallbeck and Pedersen (2012)
Europe: Aspo hard rock laboratory	Granitic groundwater, 68–446 m	Groundwater-based medium	Mesophilic and alkaliphilic	*Methanobacterium*	Kotelnikova and Pedersen (1997)

(continued)

Table 8.2 (continued)

Area	Sample and depth	Medium	Temperature and pH	Organisms/taxa identified (isolation or via sequencing)	References
Europe	Fracture fluid, 500 m	Fracture fluid supplemented with methane or methanol	Mesophilic	*Desulfobulbus, Desulfobacterium, Desulfovibrio*, γ-proteobacterial group, methanotrophs	Rajala et al. (2015)
Europe: Lubin copper mine, Poland	600 m	Luria-Bertani medium, with required supplements	Mesophilic	*Pseudomonas, Brevundimonas* sp. LM17 and LM18, *Ochrobactrum* sp. LM19, *Paracoccus* LM20, *Sinorhizobium* sp. LM21, *Achromobacter* sp. LM16, *Psychrobacter* sp. LM26, *Stenotrophomonas*	Dziewit et al. (2015)
Europe: Outokumpu, Finland	180 and 500 m	Fracture fluid supplemented with methane or methanol	Mesophilic	*Betaproteobacteria*, *Clostridia*, *Bacteroidia* and *Anaerolineae*, *Gammaproteobacteria* (mainly genus *Pseudomonas*), with *Clostridia*, *Betaproteobacteria*, *Alphaproteobacteria* (*Rhodobacter*)	Rajala and Bomberg (2017)
Europe: Outokumpu deep drill hole	Fracture fluid, 967 m	Fracture water supplemented with different nutrients	Mesophilic	*Alphaproteobacteria*	Purkamo et al. (2017)
Africa: Hainich CZE	Carbonate/ siliciclastic rock, 8.5–69 m	Groundwater supplemented with different nutrients	Mesophilic	*Thaumarchaeota*, *Woesearchaeota*	Lazar et al. (2017)
Africa: Ultradeep mines (South Africa)	Rock, air, service water, 3200 m	Sulfolobus medium Fe(III)-reducing media	Mesophilic and thermophilic	*Proteobacteria*	Onstott et al. (2003)
Africa: Gold mine, Witwatersrand Basin (South Africa)	Groundwater, 3100 m	Basal salts medium amended with various combinations of electron donors and electron acceptors	Thermophilic and alkaliphilic	*Alkaliphilus crotonatoxidans, Alkaliphilus transvaalensis*	Kieft et al. (2005)
Africa: Mponeng gold mine (South Africa)	Groundwater, 2800 m	Groundwater supplemented with different nutrients	Thermophilic/ alkaliphilic	*Proteobacteria*, *Firmicutes*, *Methanobacteria*	Lin et al. (2006)

8.4 Cultivation-Independent Studies

8.4.1 Why Culture-Independent Studies Are Necessary?

Even two decades back, scientists were more eager to cultivate bacteria from the environment and study their characteristics. This was one of the key steps to understand their ecological role and biogeographic pattern in different environments. Designing media and mimicking environmental conditions was one of the important tasks for the microbial ecologist. Sometimes, it would take years to understand the key nutrient and the conditions required for isolating a particular type of microorganism. Question about limits of life in different extremes was one of the important topics that stormed the scientific community. In deep biosphere, which is one of the toughest places on our planet, exploration of deep life and other deep life initiatives as a part of different Integrated Ocean Drilling Program (IODP) and International Continental Scientific Drilling Program (ICDP) surfaced up and later became key components of these initiatives. Even one of the greatest initiatives for deep life research was part of the decadal goals set by Deep Carbon Observatory (https://deepcarbon.net). Cultivable approaches for different deep biosphere studies often took time and gathered limited knowledge about adaptability and sustenance of life in such extremes. Among different questions that remained unanswered or partly answered, the following ones are the most important with respect to deep life (Colwell and D'Hondt 2013; Kieft 2016):

(a) What are the processes that define the diversity and distribution of deep life?
(b) What are the environmental limits of life?
(c) How do the microorganisms in the deep subsurface interact with different global biogeochemical cycles?

Answering such questions becomes difficult and more challenging using cultivation-based approaches. Advent of metagenomics-based studies created unprecedented opportunities to investigate and understand the deep biosphere. Earlier, metagenomics studies were mainly focused on targeted gene sequencing using clone library approaches. Often this method took longer time but gave an overview of the structural and functional profiles of a community in such extremes. Other methods like denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) were developed to get a better impression of microbial community pattern in diverse environments (Schütte et al. 2008). Some of the main drawbacks of these methods were that it would take a huge number of sequencing of cloned targeted DNA which is often expensive, and dominant microorganisms are much more revealed as compared to rare microbiome. With the advent of sequencing technologies, next-generation sequencing (NGS) came in being which not only reduced the sequencing cost and time but also gave a better overview of the microbial community structure and function. Sequencing through NGS technologies can bring out the rarest taxa in a microbiome since the

depth of sequencing technologies has increased immensely. NGS also gave an additional advantage of massively parallel sequencing which saved a lot of time.

Application of NGS in deep biosphere study has been applied in two main ways to get an overview of the biodiversity. One of the two ways is through targeted DNA-based amplicon sequencing, whereas the other way is through shotgun metagenomics approach. Each of the methods has their own merits and demerits. Amplicon sequencing gives an overview of the overall microbial population with respect to particular amplified gene fragment. The most used marker gene for biodiversity study is through targeting hypervariable regions of the 16S rRNA gene. The data pool generated by amplicon sequencing is much smaller than that generated during shotgun whole metagenome sequencing and gives us an overview about the community from a single perspective. Since this method requires primer-based amplification through PCR, often the dominant microorganisms are revealed and biases are created for some primer sets used during sequencing. Shotgun whole-metagenome sequencing is much more robust and gives us a better assessment of the microbial community and function (binning and reconstruction). It not only helps to understand the possible biogeochemical cycles in the deep biosphere but also helps to predict probable interactions and behavior pattern among microorganisms in a community.

8.4.2 Microbial Ecology of Igneous Provinces

Igneous provinces are often characterized by low microbial biomass due to the oligotrophic nature of the rocks and associated environments. Knowledge about their functional potential is limited. Though studies in the subterranean igneous environments are limited, investigations in different seafloor basalts and surface environments of igneous provinces give us an overview about the microbial communities. The presence of Mn-oxidizing bacteria in basalts from Loihi Seamount and neutrophilic Fe-oxidizing bacteria in oceanic basaltic glass were reported by Edwards et al. (2003) and Templeton et al. (2005), respectively, whereas microbial communities from basaltic glasses of the Knipovich Ridge, Arctic, consisted mainly of heterotrophs and some chemolithotrophs (Thorseth et al. 2001). Iron-reducing bacteria were cultured from Arctic Ridge seafloor basaltic glasses, and the presence of other organisms belonging to the *Proteobacteria*, *Chloroflexi*, *Firmicutes*, *Actinobacteria*, and *Crenarchaeota* of unknown physiology were also reported (Lysnes et al. 2004). Microbial community diversity of two volcanic terrestrial glasses of Valafell and Dómadalshraun lava flow, Iceland, was mainly dominated by *Actinobacteria* followed by *Proteobacteria*, *Acidobacteria*, and *Cyanobacteria* (Kelly et al. 2010). In another report, dominance of *Proteobacteria* was found in another Dómadalshraun site, and dominance of *Actinobacteria* was observed at Hnausahraun site, Iceland (Kelly et al. 2011). *Betaproteobacteria* consisting of nonphototrophic diazotrophs such as *Herbaspirillum* spp. and chemolithotrophs such as *Thiobacillus* dominated the microbial communities of Fimmvörðuháls Lava Flow, Eyjafjallajökull, Iceland, but dearth of photosynthetic

groups possess a contrast to the microbial communities of older Icelandic lava flow (Kelly et al. 2014). Bacterial communities of hot, anoxic crustal fluids within Juan de Fuca Ridge flank subsurface basalt at boreholes U1362A and U1362B were represented by lineages of phylogenetically unique *Nitrospirae*, *Aminicenantes*, *Calescamantes*, and *Chloroflexi*, whereas less abundant archaeal community was dominated by unique, uncultivated lineages of Marine Benthic Group E, Terrestrial Hot Spring Crenarchaeotic Group, *Bathyarchaeota*, and relatives of cultivated, sulfate-reducing *Archaeoglobi* (Jungbluth et al. 2016).

Microbial diversity of crystalline granitic bedrock system was studied at Äspö Hard Rock Laboratory (HRL), Sweden, in which nitrate-, iron-, manganese-, and sulfate-reducing microorganisms along with acetogens and methanogens were suggested to be part of such anaerobic and oligotrophic environment (Pedersen et al. 1993; Hallbeck and Pedersen 2008). Microbial diversity of deep-granitic-fracture-water in Colorado was mainly represented by *Nitrosomonadales* in the oxic borehole, whereas dominance of anaerobic bacteria was observed in plugged borehole (Sahl et al. 2008). In the same study, sequences from 1740 m-deep granitic core were represented by *Proteobacteria* (primarily by *Ralstoniaceae*) and *Firmicutes*. In the Chinese continental scientific drilling project, 16S rRNA gene analysis revealed that *Proteobacteria* dominated the microbial community of ultra-high-pressure rocks, and most of the organisms were related to nitrate reducers from a saline, alkaline, and cold habitat (Zhang et al. 2005). Microbial communities in the deep crystalline rock system of Fennoscandian shield were represented by highly diverse group of bacterial and archaeal populations with versatile metabolic capabilities for hydrogen-driven carbon cycling, reduced carbon compound assimilation, and nutrient cycling (Nyyssönen et al. 2014). In contrast to the hydrogen-driven lithoautotrophic systems, Purkamo et al. (2015) reported dominance of carbon assimilation by heterotrophic groups like *Clostridia* in Outokumpu deep scientific drill hole.

8.4.3 Deep Biosphere Studies of Terrestrial Subsurface

The terrestrial deep biosphere of our planet consists of diverse habitat ranging from deep aquifer system, mines, caves, and other sedimentary and igneous provinces. Though different natural environments and man-made infrastructures are present, investigations in the deep subsurface are often restricted by inaccessibility of samples from deep environments. Scientific drillings are frequently required to study the deep biosphere at greater depths (Gold 1992). Different studies are conducted to investigate microbial ecosystems of the deep subterranean environment. Some of the major study locations are marked in Fig. 8.6. Most of the deep biosphere studies involved groundwater or fracture fluid samples. Study of rock-hosted microbiome of the deep terrestrial subsurface is limited (Fig. 8.6). Details of selected study sites marked on the world map include investigations of deep biosphere of four continents, viz., North America, Europe, Asia, and Africa.

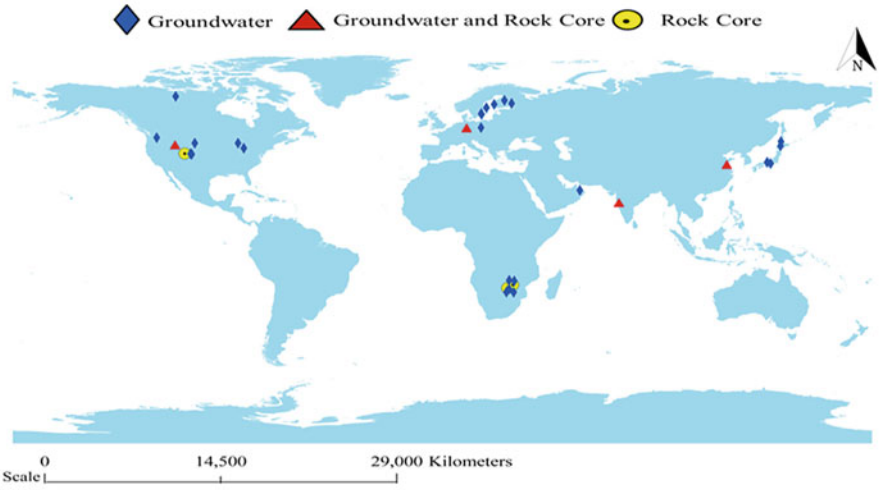

Fig. 8.6 Major subterranean deep biosphere sites from around the globe (map not to scale)

8.4.3.1 North America

There are several deep biosphere studies from different parts of North America. One of the first studies was done in the deep basaltic aquifer of Columbia River Basalt (CRB) where lithoautotrophic microbial ecosystem devoid of photosynthetic inputs was observed (Stevens and McKinley 1995). This was the first study to hypothesize hydrogen-driven ecosystem in subsurface province. Subsequent study in CRB reported the presence of sulfur-reducing bacteria (SRB) and metal-reducing bacteria from two deep anaerobic, alkaline aquifers (Fry et al. 1997). Microbial communities from deep low-biomass sandstone of Piceance Basin, Western Colorado, USA, were also explored where presence of anaerobic bacteria (mainly iron-reducing and fermentative bacteria) was reported (Colwell et al. 1997). Similar exploratory studies were done in basaltic aquifers of Snake River Plains (Newby et al. 2004; Lehman et al. 2004; O'Connell et al. 2003). These investigations revealed presence of both bacterial and archaeal members which included heterotrophs, methanotrophs, ammonia oxidizers, hydrogen oxidizers, iron reducers, propanotrophs, and phenol oxidizers. Exploration of methylotrophic and methanogenic communities in the subsurface sedimentary rocks of Antrim Shale suggested that local subsurface environment governed the microbial community structure (Waldron et al. 2007). Microbial community structure and functions were explored in different deep mine environments of North America, viz., Henderson mine (USA), Homstake mine (USA), and Lupin Au mine (Canada). One of the first extensive studies of deep biosphere in deep mine environments of North America is in Henderson mine located in Colorado (Sahl et al. 2008). This study revealed presence of *Firmicutes* and *Proteobacteria* where inorganic carbon fixation was proposed to be an important microbial metabolism. Iron-reducing *Gallionella* sp. was also observed in this subsurface environment. Later study in Henderson mine focused on N_2 fixation

and nitrification processes of the subsurface where presence of different genes involved in nitrogen cycling was correlated with NH_4^+ concentration and importance of NH_4^+ as an energy source was assessed (Swanner and Templeton 2011). Microbial community structure in Homstake Gold mine was dominated by proteobacterial members where distinct microbial communities in two different sites were observed (Rastogi et al. 2009). Investigation of subsurface microbiome at Lupin Au mine reported the presence of *Desulfosporosinus*, *Halothiobacillus*, and *Pseudomonas* as the dominant bacterial groups where sulfate reduction and sulfide oxidation via denitrification were found to be the most thermodynamically favorable processes (Onstott et al. 2009). Elaborate studies were also conducted regarding microbial community structure and function in Sanford Underground Research Facility (SURF). Bacteria were found to be more dominant over archaea in this subsurface environment where microorganisms are thought to derive energy from the oxidation of sulfur, iron, nitrogen, methane, and manganese (Osburn et al. 2014). Later detailed metagenomic study at SURF focused on energy and carbon metabolism where sulfate and nitrate/nitrite reduction were found to be the most common putative energy metabolism and energy-efficient Wood-Ljungdahl pathway was the most common autotrophic carbon fixation pathway (Momper et al. 2017).

8.4.3.2 Asia

Major deep biosphere studies in Asia covered parts of Japan, China, and Oman. Chinese Continental Scientific Drilling Project at Donghai, China, is one of the earliest and deepest (2026 m) explored subsurface biosphere studies in Asia (Zhang et al. 2005). Subsurface environment of this site was mainly dominated by proteobacterial members. *Bacteroidetes*, *Planctomycetes*, and Candidatus taxa were also observed. Presence of thermophilic, alkaliphilic, and iron-reducing bacteria was observed in the fluids, whereas rock-hosted microbiome harbored mesophilic and psychrophilic microorganisms.

In Japan, several studies were conducted in mine environments and established Underground Research Laboratories (URL). One of the first deep biosphere studies in Toyoha mine in Japan suggested presence of thermophilic SRB in the deep mine environment (Nakagawa et al. 2002). Later study in oligotrophic aquifer near Tono Uranium Mine, Japan, demonstrated the utility of $\Delta\ ^{13}C$ PLFA and $\Delta\ ^{14}C$ PLFA in understanding microbial carbon cycling in the deep subsurface environment (Mills et al. 2010). Carbon sources used by bacterial population in sedimentary versus igneous host rock were ascertained in this study. Another study on microbial diversity of deep subsurface fault-bordered aquifer in the Miocene formation suggested coexistence of methanogens and SRB (Shimizu et al. 2007). Two different aquifers were investigated in this study, where one of the aquifers was dominated by archaeal groups (sequence related to *Methanoculleus*), whereas the other aquifer was predominated by bacterial members such as *Bacteroidetes*, *Firmicutes*, and *Deltaproteobacteria*. Exploration of microbial diversity in ultra-deep granitic groundwater aquifer at Mizunami URL revealed that *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria*, and *Firmicutes* were the major residents in this subsurface environment (Fukuda et al. 2010).

Later study at Mizunami URL suggested prominent shift in microbial diversity over different time periods (Ino et al. 2016). In the same study, NanoSIMS analysis was also conducted which confirmed the presence of active microbial population in the deep granitic groundwater. Extensive investigation on microbial community structure and function was also conducted at Horonobe URL where presence of diverse microbial lineages including phyla that did not have any cultivated representatives was reported (Hernsdorf et al. 2017). Majority of microorganisms in this ecosystem could metabolize H_2 via Ni-Fe hydrogenase and Fe-Fe hydrogenases, and it was postulated that these microorganisms could also catalyze carbon, nitrogen, iron, and sulfur transformations. Among different other deep biosphere investigations in Asia, exploration of microbial habitability in Oman hyperalkaline peridotite aquifers is an eminent one (Miller et al. 2016). Microbial investigation in gas-rich hyperalkaline fluids suggested that low-temperature H_2 and CH_4 generation, coupled with the presence of electron acceptors such as NO_3^- and SO_4^{2-}, drives the deep biosphere within Oman ophiolite. In India, extensive studies have been conducted to explore the subsurface microbial community structure and function of deep granitic-basaltic environments at different depths (60–1500 meters below surface) of Koyna-Warna region of Deccan traps (Dutta et al. 2018a). Metagenomic studies revealed distinct microbial communities residing across different subterranean provinces of Koyna-Warna region. Microbial diversity of the deep Deccan also suggested partitioning of interrelated microbial guilds on the basis of rock geochemistry where synergy was observed across different microbial classes (Dutta et al. 2018a, b).

8.4.3.3 Europe

The most studied location in Europe is the Fennoscandian shield which is present in the northern part of Europe. Several investigations of subterranean deep biosphere in this location have widened our knowledge in the field of deep biosphere. Äspö Hard Rock Laboratory (AHRL), Outokumpu deep borehole, and Olkiluoto are the prominent deep biosphere sites of the Fennoscandian shield.

One of earliest studies in AHRL assessed the diversity of methanogenic archaea and homoacetogenic bacteria (Kotelnikova and Pedersen 1997). In this study, it was postulated that deep granitic groundwater from AHRL is inhabited by autotrophic methanogens and acetogens, which may produce methane and acetate at the expense of subterranean H_2 and HCO_3^-. Another study at AHRL reported the presence of nitrate-reducing bacteria (NRB), iron-reducing bacteria (IRB), manganese-reducing bacteria (MRB), and sulfate-reducing bacteria (SRB) in the deep subsurface where methanogens and acetogens were also observed (Hallbeck and Pedersen 2008). One of the recent studies at AHRL reported an extensive investigation of three subsurface aquifers through metagenomic approach (Wu et al. 2015). Two of the major findings of this research are (i) phylogenetically distinct microbial community subsets were observed across different aquifers and (ii) microbial communities having small cell size also had a tendency to have smaller genomes than their closest sequenced relatives which might be due to physiological adaptation to life in highly oligotrophic deep biosphere groundwaters.

In the deep groundwater of Olkiluotu (OL), presence of NRB, IRB, SRB, MRB, acetogenic bacteria, and methanogens was observed (Haveman et al. 1999; Pedersen et al. 2008). It was found that at OL, fracture-filling minerals were a better indicator of microbial populations than was groundwater chemistry (Haveman et al. 1999). Results also suggested that anaerobic methane oxidation may be a significant process in subsurface groundwater of OL (Pedersen et al. 2008). A later study at OL focused on methanogenic and sulfate-reducing microbial communities of deep granitic groundwater (Nyyssonen et al. 2012). Higher abundance of *dsr*B was found in samples having higher SO_4^{2-} concentration, and SRBs were mainly affiliated to different orders of *Deltaproteobacteria*. Results imply that sulfate reduction, methanogenesis, and anaerobic methane oxidation may also take place in this environment. One of the recent deep biosphere studies at OL reported that microbial communities varied with depth, salinity gradient, and sulfate and methane concentrations (Bomberg et al. 2015). In this study, the highest bacterial diversity was observed in the sulfate-methane mixing zone (SMMZ), whereas archaeal diversity was highest in the lowest boundaries of SMMZ.

Among other sites in Europe, most number of studies have been conducted at Outokumpu Deep Borehole (ODB) located in Finland. One of the first studies at ODB explored the microbial diversity of the deep groundwaters where microbial diversities were found to be varying as a function of depth and microbial community composition was linked to geochemistry of groundwater (Itavaara et al. 2011). Similar results were obtained in the subsequent studies at ODB (Purkamo et al. 2013; Nyyssonen et al. 2014). In 2014, Nyyssonen et al. reported the presence of chemoheterotrophic, chemolithoheterotrophic, thiosulfate-reducing, sulfite-reducing, and fermentative groups in the deep groundwaters of ODB where *Proteobacteria*, *Firmicutes*, and *Tenericutes* were found to be the most abundant bacterial phyla, whereas hydrogenotrophic methanogens were the most abundant archaeal groups. Another important study at ODB reported the prevalence of heterotrophic microbial groups (such as *Clostridium*) throughout the drill hole water column which was studied using marker genes for carbon assimilation, methane production, and methane consumption (Purkamo et al. 2015). Subsequent study explored the responses of microorganisms (residing in deep groundwater of ODB) to C-1 compounds (Rajala et al. 2015; Rajala and Bomberg 2017). It was found that dormant microbes from the deep became active in presence of C-1 substrates and suitable conditions.

Some of the other studies in Europe include an investigation on the role of plasmids in adaptation of bacteria in subsurface environment (Lubin copper mine, Poland); exploration of microbial diversity at high-pressure deep subsurface environment (Pyhäsalmimine, Finland); examining the microbial diversity and functionality of archaeal, bacterial, and fungal population of deep Archaean bedrock fracture aquifer (Romuvaara, northern Finland); and assessing the archaeal diversity of subsurface carbonate−/siliciclastic-rock environment (Hainich CZE, Germany) (Dziewit et al. 2015; Miettinen et al. 2015; Lazar et al. 2017; Purkamo et al. 2018).

8.4.3.4 Africa

Microbial ecology of deep biosphere of Witwatersrand basin in South Africa is widely explored. Ultra-deep gold mines in this region have provided an easy access to the deep subsurface. One of the first studies in the deep gold mines of Witwatersrand basin focused on the archaeal diversity (Takai et al. 2001). In this study, novel archaeal lineages, viz., SAGMCG and SAGMEG, were reported for the first time. Later studies focused on overall microbiome of the deep subterranean provinces of Witwatersrand basin. Comparative analysis of microbial diversity across subsurface rock, service water, and air of a 3.2 km deep gold mine was conducted to analyze the chance of contamination, and it was found that contamination of rock cores by service water was negligible accounting for less than 0.01% contamination (Onstott et al. 2003). A later study in similar environment reported presence of different H_2 generating processes, namely, serpentinization, oxidation of ferrous silicate minerals, and radiolysis of water which could fuel the microbial community in the deep (Kieft et al. 2005). The findings of this study have significance for other deep subsurface environments on Earth and possibly for those of other planetary bodies as well. Presence of *Firmicutes*, *Proteobacteria* and *Euryarchaeota* was also reported from ultra-deep gold mines where thermophilic sulfate-reducing *Firmicutes* were observed which could sustain on geologically produced SO_4^{2-} and H_2 generated in the deep (Borgonie et al. 2015; Gihring et al. 2006; Lin et al. 2006). One of the investigations in similar subterranean environment of Witwatersrand basin focused on nitrogen cycling in the deep where an array of genes related to nitrogen cycling were observed from metagenomic analysis and evolutionary relationship between surface and subsurface genes of microorganisms was assessed which suggested that subsurface habitats have preserved ancestral genetic signatures (Lau et al. 2014). With the advancement of technology and analytical tools, studies in South African subcontinent became more intricate and informative. One of the eminent and recent studies in deep environments of Witwatersrand basin focused on metabolic networks and trophic structures of microbial communities using metatranscriptomics, metaproteomics, and thermodynamic modeling (Lau et al. 2016). This study revealed that deep subsurface community in this oligotrophic environment is dependent on syntrophy where sulfur-dependent autotrophic denitrifiers are the dominant group. One of the other recent studies focused on carbon metabolism at Precambrian continental crust of Tau Tona gold mine where the energy-conserving Wood-Ljungdahl pathway was found to be the most abundant carbon fixation pathway (Magnabosco et al. 2016). This study also revealed that *Firmicutes* and *Euryarchaeota* were the most abundant members in the metagenome which is in line with previous studies in similar environment (Gihring et al. 2006; Lin et al. 2006) (Fig. 8.6).

8.5 Techniques for Sampling the Subsurface

Deep drilling is required either from the surface or from a pre-existing subsurface site, e.g., in deep mines for obtaining deep subsurface samples for microbiological as well as geological investigations (Kieft 2016; Wilkins et al. 2014). Selection for the appropriate drilling and coring methods is decided on the basis of geological formation(s) to be sampled and also on the scientific aims of the project. Basically, there are three types of drilling techniques, namely, (1) hollow-stem augering, (2) cable-tool drilling, and (3) rotary drilling using a drilling fluid (for acquiring the sample from deeper depth and crystalline environments), which are used. They use portable drill rigs or larger rigs for deeper drilling, which are assembled on site (Kieft 2010). The underground mining industry uses small drill rigs that can be deployed in the limited space of mine tunnels to drill through rock to depths of 3000 m or more (Sahl et al. 2008). Since drilling is innately quite messy, detection and removal of contamination from the subsurface samples have been a necessity for characterizing the microbiology of these habitats (Phelps et al. 1989; Kieft 2016). Soil, atmospheric, and human-associated microorganisms, material from overlying formations sloughed off in the borehole, chemical contamination from the atmosphere (including O_2), hydrocarbons used for lubrication, etc. can be potential contaminants during deep drilling (Kieft et al. 2007). Quite some time back, Pedersen et al. (1997) had reported the presence of *Acinetobacter*, *Methylophilus*, *Pseudomonas*, and *Shewanella* in drilling-related equipment. Since then, there have been much advancement in the drilling, coring, and sampling technologies such that samples can be extracted aseptically from deeper environments (>3 km depth) (Lin et al. 2006; Moser et al. 2005; Onstott et al. 1998). Techniques have been devised for aseptic handling of samples and their proper storage (in freezing conditions) in the absence of oxygen to preserve oxygen-sensitive anaerobes (Kieft 2016). Online gas analyses can be performed onsite during scientific drilling to recognize biologically active zones (Erzinger et al. 2006).

Another important aspect of deep drilling is the use of drilling fluid (gaseous, liquid, slurry, or foam) during sampling from deep subsurface for intact recovery of deep subsurface rock cores (Kieft et al. 2007). Drilling fluids lubricate and cool the drill bit and maintain the hydrostatic pressure during the drilling operations (Kieft 2016). These fluids can be problematic, especially when drilling fluids with organic additives (bentonite based) are used (Struchtemeyer et al. 2011). These drilling fluids are one of the most prominent sources of microbial contamination in deep subsurface study (Kieft et al. 2007; Kieft 2010). Drilling fluid is expected to possess microorganisms that originate from the surface and are carried to depth during drilling operations. Solute and particulate tracers which include fluorescent dyes, LiBr, and perfluorinated hydrocarbons (Table 8.3) can be added to the drilling fluid. Later, the subsurface samples can be quantified by different analytical methods to detect the presence of these tracers in the subsurface samples in order to determine the extent of contamination from drilling fluids (Phelps et al. 1989; Kieft 2016). It may be quite possible that the subsurface samples are tracer-free, but there might be still a chance of microbial contamination. Hence, the microbial communities in the

Table 8.3 Tracers used for contamination assessment in subsurface drilling

Sl. No.	Nature and area of sample	Type of drilling fluid	Type of tracer	References
1.	North hydrothermal field, mid Okinowa trough (IODP 331), sand, gravel, and clay	Guar gum (0.8%) or seawater gel mud + 5% bentonite + 0.1% sodium hydroxide + 0.1% lime + barite + surface seawater containing guar gum mud	Perfluoromethylcyclohexane (C_7H_{14}) [PFT], 1 mg L^{-1} Fluorescent microspheres (0.5 μM diameter)	Yanagawa et al. (2013)
2.	Waikato area, North Island, New Zealand (Cenozoic sediments)	Bentonite + a small quantity of Pac-R polymer + water	Fluorescent microspheres (8.4%)	Kallmeyer et al. (2006)
3.	Western Pacific during ODP leg 185, Pigafetta Basin, South China Sea (sediments and igneous rocks)	Surface seawater	perfluoromethylcyclohexane 1 mg L^{-1}. [PFT] fluorescent microspheres, 10^{10} spheres mL^{-1} (0.5 μM diameter)	Smith et al. (2000)
4.	The Mineral Park mine, Arizona, igneous rocks	Sacramento Valley groundwater + sodium hypochlorite (60 mg L^{-1})	Fluorescent microspheres	Lehman et al. (2001)
5.	The Savannah River plant (SRP), North Carolina (sediments)	Bentonite	Potassium bromide (900 mg L^{-1}), rhodamine (20 mg L^{-1})	Phelps et al. (1989)
6.	Deep anaerobic aquifer of Atlantic coastal plain of South Carolina, (Myrtle beach and Florence) (Subsurface sediments)	Local groundwater	Barium (10 mg L^{-1}) Fluorescent microspheres, 10^5 spheres mL^{-1} (1 μM diameter)	Chapelle and Lovely (1990)
7.	ODP leg 201 (subsurface sediments)	Seawater	Perfluorocarbon tracers (PFTs) (1 mg L^{-1}) Fluorescent microspheres 10^{10} spheres mL^{-1} (0.5 μM diameter)	House et al. (2003)
8	Precambrian granitic bedrock in SE Sweden	Groundwater	Fluorescein (500 mg L^{-1}) in water	Pedersen and Ekendahl (1990)
9	Äspö HRL, Baltic coast, Sweden	Groundwater	Fluorescent dye, uranine	Pedersen et al. (1997)
10	Opalinus clay, Switzerland	Compressed air (up to 9 m and pure N_2 for the next 6 m) was used for drilling	Fluorescent microspheres 10 mL of 3.5×10^{11} particles mL^{-1} (0.4 ± 0.01 μM diameter)	Gascoyne et al. (2007)

11	Ashfall tuff samples Rainier Mesa, Nevada test site (deep subsurface rocks)	Not mentioned	Lithium bromide in DF (26 mg L^{-1})	Haldeman et al. (1995)
12	Lake Towati sediment	Towati lake water	SPL-N fine grind fluorescent pigment Dispersion (DayGlo, Cleveland, OH) [pigment content 45%, size 0.25–0.45 μM] – 1×10^9 particles per mL drilling fluid	Friese et al. (2017)
13	Lake Chalco	Chalco lake water	A whitish tracer with light blue fluorescence under UV Excitation (day Glo SPL-594 N, RADGLO AFN-09) 1×10^9 particles per mL drilling fluid	Friese et al. (2017)
14	Natural gas wells, Texas	Bentonite (maximum) + cellulose + nut hulls + cedar fiber + xanthan gum + barite + lignosulfonate	Not mentioned	Struchtemeyer et al. (2011)

drilling fluid and in the subsurface rock cores can be analyzed by 16S rRNA gene-based microbial diversity analysis and compared as a further test for drilling-induced contamination (Miteva et al. 2014; Dong et al. 2014; Yanagawa et al. 2013). Subsurface rock cores can be tested for the presence of different allochthonous hydrocarbons that may be derived from the drilling equipment or drill additives (Kallmeyer et al. 2006). Drilling fluids can support the growth of extremely high densities of microbes, e.g., 10^8 cells ml^{-1} (Beeman and Suflita 1989; Kieft et al. 2007). Coker and Olumagin (1995) obtained different bacterial and fungal genera in drill cuttings, viz., *Staphylococcus*, *Acinetobacter*, *Serratia*, *Clostridium*, *Nocardia*, *Bacillus*, *Actinomyces*, *Micrococcus, Pseudomonas, Penicillium*, *Fusarium*, etc. Miteva et al. (2014) reported the presence of microorganisms mostly found in crude oil- or hydrocarbon-contaminated environments (hydrocarbon-degrading *Firmicutes* and other bacterial genera *Pseudomonas*, *Acinetobacter*, *Massilia*, *Paracoccus*, *Agrobacterium*, etc.) in the hydrocarbon-based drilling fluid (Estisol 240 and Coasol) used during NEEM Greenland ice core drilling project. Presence of *Betaproteobacteria* and *Gammaproteobacteria* and *Bacteroidetes* in guar gum- and seawater gel-based (bentonite plus sodium hydroxide) drilling fluid was reported during deep-sea drilling and coring by the D/V *Chikyu* (IODP expedition 331 and *Chikyu* shakedown expedition CK06–06) (Yanagawa et al. 2013; Inagaki et al. 2015). Interestingly, some researchers have also reported drilling fluid as carriers of deep subsurface microbial communities (Struchtemeyer et al. 2011; Zhang et al. 2006; Masui et al. 2008). Hence, there is a possibility that drilling fluids may contain signatures of subsurface microbial community.

A major problem encountered during deep subsurface research is "postcore extraction contamination" from laboratory reagents (i.e., extraction kits, Taq polymerase, or buffers) (Salter et al. 2014). It is extremely indispensable to maintain controls at each and every level of coring and postcoring processes. These controls must be analyzed for their microbial diversity to further distinguish between contaminants and subsurface microbial communities. Postcoring laboratory controls (reagent blanks, etc.) have been analyzed for the presence of potential contaminants, and a comprehensive review on this aspect has been already published (Sheik et al. 2018; Salter et al. 2014). *Betaproteobacteria* and *Gammaproteobacteria* were mostly encountered bacterial classes in the laboratory controls followed by *Actinobacteria*, *Alphaproteobacteria*, *Firmicutes*, and *Bacteroidetes*. *Pseudomonas*, *Propionibacterium*, *Acinetobacter*, *Ralstonia*, and *Sphingomonas* were the major genera found in laboratory blanks (Sheik et al. 2018 and reference therein). Advanced computational techniques have been developed that enable us to identify and filter out the "contaminant microbial populations" from the deep subsurface sequences (Jørgensen and Zhao 2016; Labonté et al. 2017; Reese et al. 2018; Sheik et al. 2018, and reference therein). But, removal of these contaminant microbial communities must be done with utmost care as it may remove some taxonomically novel microorganisms present in deep subsurface. Also, there have been many reports of taxonomically similar groups present in surface as well as deep subsurface (Struchtemeyer et al. 2011; Zhang et al. 2006; Yanagawa et al. 2013; Moser et al. 2005; Gihring et al. 2006; Davidson et al. 2011). Hence, identification of

contaminants from deep subsurface microbiological studies becomes a separate and an important area of research. A database can be developed for the microbial communities obtained from drilling fluids and controls encountered during deep drilling and postcoring processes to sort out the true representatives of the deep biosphere from imposters represented by contaminants (Sheik et al. 2018).

8.6 Applications of Deep Subsurface Research

The deep biosphere offers huge potential for the discovery of various new aspects of life, and important revelations are made with each and every new opportunity to probe the subsurface (Kieft 2016; Kallmeyer et al. 2012; McMahon and Parnell (2013). The deep subsurface research has various applications starting from hazardous waste disposal (nuclear wastes), CO_2 sequestration, and extraction of various metabolites from deep subsurface extremophiles (extremozymes and extremolytes) for biotechnological purpose. This section briefly discusses various applications of deep subsurface research.

8.6.1 Deep Subsurface as Nuclear Waste Repositories

Deep boreholes drilled through the Earth's crust are an efficient disposal source for high-level nuclear wastes. This concept (deep borehole disposal, DBD) has been around for about 40 years (Schwartz et al. 2017). Researchers from the United States (US), the United Kingdom (UK), and Sweden have periodically examined DBD as a potential alternative to a mined repository (Schwartz et al. 2017 and reference therein). One of the biggest advantages of DBD as identified by researchers from Sweden is waste deposited in deep boreholes at 3–5 km depth would exist in a moderately torpid, density-stratified hydrogeologic arrangement as compared to more active shallower flow systems in a mined repository. Also, future glaciation, earthquakes, or human intrusion would be much less likely to disturb the waste at those depths (Ahall 2007). It is a secure way of disposing nuclear materials, since the deep depth of disposal in a small borehole provides a "formidable physical barrier" to the future retrieval of materials for spiteful purposes (Hippel and Hayes 2009). Additionally, there is no release of radionuclides through groundwater (Beswick 2008). A concept of DBD developed by the US Department of Energy (DOE) envisages disposal of radioactive waste in boreholes up to 5 km deep, completed in crystalline basement rock in containers and bentonite, concrete, and other materials would seal the upper 3 km of each borehole to isolate the waste from the biosphere (Brady et al. 2012). Utilization of granite and other crystalline rocks is advantageous since layers of argillaceous rocks at depth tend to be relatively unfractured, usually providing a natural barrier to groundwater flow, and these rocks have very high mechanical strength and they might resist borehole deformation during deep drilling (Brady et al. 2009).

8.6.2 Deep Subsurface CO_2 Sequestration

CO_2 can be stored in the deep subsurface in different types of formations. Since the last 40 years, CO_2 has been injected for improved oil recovery mainly in the USA and Canada (Firoozabadi and Cheng 2010; Benson and Cole 2008). Currently, 70,000 tons of CO_2 is injected worldwide per day for enhanced oil recovery (EOR). CO_2 can be even stored in underground depleted oil and gas reservoirs (Bouquet et al. 2009). Another well-accepted method for geological CO_2 sequestration is its storage in deep saline aquifers, because saline aquifers have larger storage capacities than other geological formations. Different trapping mechanisms include geological trapping, hydrodynamic trapping, and geochemical trapping (solubility trapping and mineral trapping). Mineral trapping which involves mineralogical reactions between dissolved CO_2 and formation rock is safer and more economical in the long term. Interactions among rock, water, and CO_2 initiated in the aquifer with CO_2 injection play a vital role in CO_2 sequestration in saline aquifers (De Silva et al. 2015). This process is extremely slow and can be made faster using deep subsurface microorganisms which harbors enzymes to aid the process. Even the injected CO_2 can be converted to methane by methanogens harboring in the deep subsurface (Gniese et al. 2013). Mu et al. (2014) displayed the alteration in microbial diversity as well and metabolism due to CO_2 injection in the geo-sequestration experiment at 1.4 km-deep Paaratte Formation of the Otway Basin, Australia. A general shift from *Firmicutes* to *Proteobacteria* was observed in the groundwater before and after CO_2 injection in the aquifer. Microbial reactions might have some favorable and unfavorable effects on CO_2 sequestration in deep boreholes (Ménez et al. 2007). Hence, it is extremely important to deduce the microbiology as well as the geochemistry of the deep borehole site before its use for CO_2 injection (Mu et al. 2014; De Silva et al. 2015).

8.6.3 Deep Subsurface as a Source of Novel Bioactive Compounds

The Earth's deep continental crust has geologically varied morphology with extreme conditions (temperature, pressure, pH, etc.) which makes it almost impossible for life to survive (Fredrickson and Balkwill 2006). Nevertheless, it is long been known that "deep subsurface" of the Earth hosts a diverse array of ecosystems which harbors a diverse population of extremophilic microbial life (Whitman et al. 1998; McMahon and Parnell 2013; Kieft 2016). These extremophiles harbor many novel bioactive compounds (extremozymes and extremolytes) which have potential applications in industries to produce biotechnologically important products in a cost-effective manner (Coker 2016). The most notable example is DNA polymerases obtained from thermophiles *Thermus aquaticus*, *Pyrococcus furiosus*, and *Thermococcus litoralis*, also known as Taq (Tindall and Kunkel 1988), Pfu (Lundberg et al. 1991) and Vent (Mattila et al. 1991), respectively. These extreme microbes are also known producers of extremozymes such as proteases and lipases, combined with the glycosyl hydrolases, which account for more than 70% of all enzymes sold

(Li et al. 2012). *Thermoanaerobacterium saccharolyticum* have shown tremendous applications in producing large quantities of biofuel (ethanol) and minimizing other side reactions/products (Basen et al. 2014). These thermophiles are even utilized in the production of hydrogen through anaerobic fermentation and hydrogenases. *Acidithiobacillus*, *Ferroplasma*, *Sulfolobus*, and *Metallosphaera* are widely utilized in biomining (removal of insoluble metal sulfides or oxides by using microorganisms) (Podar and Reysenbach 2006; Vera et al. 2013). Extremophiles are producers of a host of antibiotics, antimicrobial peptides (diketopiperazines), antifungals, and antitumor molecules (Littlechild 2015). Commercial success of DNA polymerase, enzymes, biofuels, and biomining obtained from extremophilic microorganisms proves that these extremophiles and their metabolites (primary and secondary) have an extensive foothold in biotechnology. New high-throughput technologies are the need of the day to produce most extremophiles/extremolytes on a large scale required by industrial processes (Coker 2016).

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