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REVIEW

The deep continental subsurface: the dark biosphere

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Abstract
Although information from devoted geomicrobiological drilling studies is limited, it is clear that the results obtained so far call for a systematic exploration of the deep continental subsurface, similar to what has been accomplished in recent years by the Ocean Drilling Initiatives. In addition to devoted drillings from the surface, much of the continental subsurface data has been obtained using different subterranean "windows," each with their correspondent limitations. In general, the number and diversity of microorganisms decrease with depth, and the abundance of Bacteria is superior to Archaea. Within Bacteria, the most commonly detected phyla correspond to Proteobacteria, Actinobacteria, Bacteroidetes, and Firmicutes. Within Archaea, methanogens are recurrently detected in most analyzed subsurface samples. One of the most controversial topics in the study of subsurface environments is whether the available energy source is endogenous or partly dependent on products photosynthetically generated in the subsurface. More information, at better depth resolution, is needed to build up the catalog of deep subsurface microbiota and the biologically available electron acceptors and donors.

Keywords Deep subsurface drilling · Geomicrobiology · Dark biosphere · SLiME · Fluorescence in situ hybridization

The beginning of the deep biosphere study

Although it has been about two centuries since Darwin predicted the possibility of life in the subsurface (Darwin 1839), it was in 1926 when the first data about life at great depths were obtained (Bastin et al. 1926). Shortly afterwards, in the 1930s, microbiological studies on marine sediments demonstrated the existence of life in the oceanic subsurface (Zobell 1938; ZoBell and Anderson 1936). However, advances in this field in subsequent years were limited because of the lack of credibility by the scientific community (Lipman 1931). In addition, after observing that the combined effect of low temperatures and high pressure inhibited the growth of microorganisms in the ocean depths, the possibility of finding active life in the deep subsurface was severely questioned (Jannasch et al. 1971). The concept of life at great depths changed radically in 1979, when Corliss and coworkers revealed that in deep oceanic hydrothermal vents, animal life sustained by the chemosynthesis produced by sulfur-oxidizing microorganisms existed in an ecosystem completely independent of photosynthesis (Corliss et al. 1979). Thanks to this discovery, the study of deep biosphere in the oceanic subsurface was promoted and included in successful international drilling programs (D'Hondt et al. 2002; Oremland et al. 1982; Whelan et al. 1986).

However, the study of life in the continental subsurface was not seriously promoted until years after the discovery of the great biodiversity in the oceanic subsurface. In 1988, Ghiorse and Wilson denounced the indifference towards the possible existence of life in terrestrial subsurface environments (Ghiorse and Wilson 1988). These authors pointed out that several studies had detected microorganisms in continental subterranean locations for decades, but they had been ignored and questioned due to the high risk of contamination during the sampling (Lipman 1931). For this reason, development and use of tracers were key in providing credibility to the study of life in subsurface environments, since they allow the control of the main sources of microbiological contamination during sampling (Kieft 2010).

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Nevertheless, one of the first to speculate about the existence of an ecosystem in the continental subsurface independent of photosynthesis was Thomas Gold. Gold not only considered the subsurface as a possible habitat for microorganisms but also the possibility that life could be found in other planets (Gold 1992).

Finally, numerous studies showed unequivocally that in fact, there is great microbial diversity in both oceanic and continental subsurface, and, nowadays, we can assure that life in these environments is ubiquitous representing a large percentage of Earth's biomass.

Limitations in the study of continental subsurface

Several research groups have carried out studies of the subsurface biosphere in different locations of the planet, and their methods of sampling and analysis of samples differ depending on the studied area, the type of retrieved samples, and the available technology (Table 1).

Nowadays, thanks to advances in drilling methodologies, samples can be extracted at great depths of the Earth's crust, minimizing and quantifying their contamination by using tracers (Kieft 2010). However, very few projects have performed devoted geomicrobiological drills from the surface to collect pristine samples at different depths (Fig. 1) due to the existing mechanical and economic difficulties (Fernández-Remolar et al. 2008; Gronstal et al. 2009; Wu et al. 2015; Zhang et al. 2005). Instead, many researchers have taken advantage of subterranean "windows," both natural and artificial, for deep sampling (Table 1). These include artesian wells (Chapelle et al. 2002; Stevens and McKinley 1995), springs (Magnabosco et al. 2014; Probst et al. 2014a; Suzuki et al. 2013), underground locations for radioactive waste disposal (Pedersen 1999), underground research facilities (Momper et al. 2017a; Murakami et al. 2002), or deep mines (Onstott et al. 2003; Sahl et al. 2008). In the last case, for example, instead of using a large surface drilling machinery, a small equipment that can be deployed in limited spaces is used and samples are taken from the walls of the galleries of the mine.

It must be kept in mind, however, that the study of the subsurface biosphere through "artificial windows" is based on systems that in many cases have been previously modified by man (sometimes years before sampling), and, therefore, they are disturbed environments where microbial populations may not be representative of the native microorganisms existing in the subsurface. Perhaps a good example, among others (Moser et al. 2003), is represented by the work done by Sahl and collaborators (Sahl et al. 2008), which showed the great variation in the microbial composition of the water that flowed through wells drilled in the Henderson Mine after only 2 weeks of well isolation, that is to say, after eliminating the aeration of the water.

On the other hand, several studies confirmed that microbial communities inhabiting the rocks show a different composition from those detected in underground water (Lehman et al. 2004; Momper et al. 2017b). Hence, to obtain a true vision of subsurface environment, both types of samples should be analyzed in order to characterize the microorganisms associated to them. Most research groups have focused on the study of groundwater, since it is much easier to sample and analyze water than hard rock samples from drilled boreholes (Table 1). As a result, the data obtained, to date, from subsurface studies corresponded mainly to planktonic life. If we consider that the number of microorganisms that live attached to surfaces is up to three orders of magnitude higher than planktonic ones (McMahon and Parnell 2014), we have to conclude that the great majority of the subsurface microorganisms studied with this methodology are vastly underestimated.

Continental subsurface characteristics

According to Hoehler (Hoehler 2004), habitability of an underground environment on Earth is defined by the presence of three basic requirements: energy availability, liquid water, and moderate temperature. The deep subsurface is considered an extreme environment characterized by darkness and anaerobiosis where the temperature and pressure increase with depth (Kieft 2016). In these environments, nutrients and water availability and, therefore, the number and activity of microorganisms are controlled by geochemistry and geohydrology. On one hand, due to the shortage of organic matter, minerals are virtually the main source of substrates, either because they are biologically dissolved or because energy is released by abiotic processes. Thus, the available electron donors and acceptors are determined by the geological composition of the underground location (Jones and Bennett 2017; Rempfert et al. 2017). In addition, anaerobic metabolisms dominate the deep subsurface; thus, the obtained energy is rather low (Hoehler 2004) when compared to aerobic processes. On the other hand, rock porosity and the presence of fractures or faults in the system influence the growth of microorganisms. In those systems where the porosity is high, the flow of water and nutrients will be higher and, in addition, will present a bigger physical space, all of which promote microbial colonization (Fredrickson et al. 1997; Pedersen 2000). In addition, geochemistry and geohydrology play an important role in the formation of heterogeneous microniches, which allow the coexistence of the competitive (antagonistic) metabolisms detected in the subsurface such as sulfate reduction and methanogenesis (Jakobsen 2007).

Table 1 Examples of deep continental subsurface microbial studies and summary of their sampling and analytics methods. *N* most probable number, *PLFA* phospholipid fatty acid analysis, *qPCR* quantitative PCR, *DGGE* denaturing gradient gel electrophoresis, *T-RFLP* terminal restriction fragment length polymorphism.

Location	Reference	Sampling depth (mbs)	Sampling site	Samples	Count of microorganisms	Biodiversity analysis	Metabolic diversity analysis
Columbia River Flood Basalt (EEUU)	Fry et al. 1997; Stevens and McKinley 1995	1270	Preexisting artesian well	Subsurface water	Acridine orange stain	Hybridization of RNA in nylon membranes, cloning	Enrichment cultures
Lidy Hot Springs (EEUU)	Chapelle et al. 2002	200	Drilling and creation of artificial artesian well	Subsurface water	PCR-MPN, qPCR, FISH	Cloning	–
South Africa Gold Mines	Onstott et al. 2003	Up to 3000	Subterranean mines	Fracture water and core rocks from small perforations made in the walls of the tunnels and chiseled rock from tunnel wall and floor	PLFA	Cloning	Enrichment cultures
	Magnabosco et al. 2016			Fracture water from a drilled borehole in mine tunnel	–	Metagenomics (16S)	Metagenomics
Dabie Sulu (China)	Zhang et al. 2006; Zhang et al. 2005	5000	Drilling from surface	Rock cores and drilling water after use	Acridine orange stain	Cloning and PLFA	Enrichment cultures
Chesapeake bay (EEUU)	Cockell et al. 2012; Gronstal et al. 2009	1766.3	Drilling from surface	Rock cores	DAPI stain, MPN	Cloning, DGGE, FISH	Enrichment cultures
Outokumpu Deep Drilling Project (Finland)	Itävaara et al. 2011b; Nyyssönen et al. 2014; Purkamo et al. 2015; Purkamo et al. 2013	2516	Drilling from surface	Subsurface water	Baclight Bacterial Viability Kit, DAPI, qPCR (*dsrB* and *mcrA* genes)	DGGE, cloning, metagenomics and reverse transcription of RNA	Metagenomics, transcriptomic, qPCR, and cloning of functional genes
Mt. Simon Sandstone (EEUU)	Dong et al. 2014	Up to 1800	Drilling from surface	Subsurface water	FISH, TOPRO3, and Sybr Green I	Cloning, T-RFLP, metagenomics	Enrichment cultures, metagenomics
Regensburg (Germany)	Probst et al. 2014a; Probst et al. 2013; Probst et al. 2014b	?	Spring	Subsurface water and biofilm	qPCR, FISH	Microarrays, FISH, metagenomics (16S), cloning	Metatranscriptomics
IPB (Spain)	Fernández-Remolar et al. 2008; Puente-Sánchez et al. 2014	164	Drilling from surface	Rock cores	–	Microarray, cloning, CARD-FISH	–
Mizunami Underground Research Laboratory (Japan)	Ino et al. 2017; Ino et al. 2016; Iwatsuki et al. 2015; Suzuki et al. 2014	300	Subterranean laboratory	Subsurface water of preexisting and drilled artesian boreholes	Sybr Green I	Pyrosequencing, clonation (16S, *dsrA* y *mcrA*)	Metagenomics
Samail Ophiolite (Oman)	Rempfert et al. 2017	Up to 475	Preexisting boreholes	Subsurface water	–	Metagenomics (16S)	–

Fig. 1 Sampling the deep subsurface of the Iberian Pyrite Belt using drilling and coring techniques

The deep biosphere

The number of "intraterrestrial" microorganisms reported varies markedly depending on the studied site. The values fluctuate between 10^2 and 10^7 cells/ml or gr (Basso et al. 2009; Itävaara et al. 2011a; Pedersen 2000; Zhang et al. 2005) depending on the geology of the area, its physicochemical characteristics, and the analyzed depth. Different studies have tried to estimate the percentage of biomass inhabiting subsurface environments, but their results differ greatly from each other (Kallmeyer et al. 2012; McMahon and Parnell 2014; Whitman et al. 1998). If we consider the theoretical depth at which microorganisms could develop (5–10 km in most areas of the crust due to temperature as a limiting factor (Gold 1992)) and the number of microorganisms detected in diverse studies, the percentage of subsurface prokaryotic life has to be substantial.

The question of whether large amounts of prokaryotic microorganisms can develop in the subsurface considering the insufficient energy supply existing in many underground locations has often been posed. To survive, when energy is scarce or nonexistent, many microorganisms adopt a latent metabolic state, so it is believed that most microorganisms in the subsurface are in anabiosis (D'Hondt et al. 2002). Recent papers have shown that the number of active microorganisms increases when subsurface samples are incubated in enrichment media in the span of only few hours (Rajala and Bomberg 2017; Rajala et al. 2015), supporting the hypothesis that microorganisms may not be metabolically active in those environments and are activated after an energy source is supplied. However, according to Morita (Morita 1999), survival of DNA in a latent state is limited to around 100 years at cold temperatures, but the recovery of viable microorganisms in a geological formation dated 250 million years old contradicts these calculations (Vreeland et al. 1998). Morita offered a possible solution to this discrepancy by proposing that microorganisms could survive for long periods of time in starvation state if they were able to achieve a minimum of energy, called survival energy, to compensate the racemization of amino acids and the depurinization of DNA. This implies that subsurface microorganisms are metabolically active even when their growth rate is extremely low (Phelps et al. 1994), making it possible to talk about geological times of duplication. In addition, different studies support the idea of an active underground life, such as the detection of biogeochemical signatures (Fernández-Remolar et al. 2008), the measurements of microbial activity in different points of the subsurface (Pedersen 2012; Suzuki et al. 2014; Wouters et al. 2013), or the results of metatranscriptomic studies (Lau et al. 2016; Zinke et al. 2017).

The microbial populations that have been described in different subsurface locations vary widely, even at different depths of the same borehole, which might be due to the geological and physicochemical heterogeneity of the studied systems as well as the origin of the water. This variability, together with the scarcity of studied sites and the different methodologies used, makes it difficult to compare existing geomicrobiological data to extract general rules for subsurface ecosystems. However, we must consider that the existence of discrepancies among the different subsurface studies is mainly a reflection of the heterogeneity of these systems.

It is a general rule in most of the studies carried out in the continental subsurface that the number of microorganisms decreases with depth (Cockell et al. 2012; Itävaara et al. 2011b; McMahon and Parnell 2014; Moser et al. 2005), along with the frequency of sequence similarities found in the databases (Itävaara et al. 2011b). Groups of sequences different from all known microbial groups have been found that could correspond to new divisions of both Bacteria (Gihring et al. 2006; Sahl et al. 2008) and Archaea (Probst and Moissl-Eichinger 2015; Takai et al. 2001).

Although there are exceptions (Itävaara et al. 2011b), in most cases microbial diversity also tends to decrease at greater

depth (Chivian et al. 2008; Lin et al. 2006b; Zhang et al. 2005). However, what kind of microorganisms is more abundant or diverse is not clear yet, since this variable depends directly on the geological characteristics of the studied site. Generally, diversity and abundance of Bacteria is superior to Archaea (Cockell et al. 2012; Ino et al. 2016; Lau et al. 2016; Rempfert et al. 2017; Takai et al. 2001). Within Bacteria, the most common reported phyla in the continental subsurface are Proteobacteria, Actinobacteria, Bacteroidetes, and, above all, Firmicutes (Dong et al. 2014; Lin et al. 2006a; Moser et al. 2005; Onstott et al. 2003; Zhang et al. 2005), which comprise, in some cases, up to 40% of the total population in the deepest layers (Basso et al. 2009). Nevertheless, other less represented phyla have also been detected such as Deinococcus-Thermus, Nitrospirae, Acidobacteria, Chloroflexi, or newly proposed phyla, which have no cultivated members, as candidate phylum Omnitrophica (OP3) or candidate phylum Saccharibacteria (TM7), among others.

One of the great surprises of the study of the deep biosphere has been the frequent appearance of sequences belonging to microorganisms with the potential to carry out photosynthetic metabolism. Members of the phylum Cyanobacteria have been reported repeatedly in subsurface environments (Bomberg et al. 2014; Ino et al. 2017; Onstott et al. 2003; Purkamo et al. 2015; Rempfert et al. 2017; Zhang et al. 2005). These studies, however, do not offer a possible explanation for why this type of microorganisms has been detected hundreds of meters below the surface (mbs). Members of this phylum have the ability to carry out non-photosynthetic metabolism allowing them to grow in the absence of light (dos Santos et al. 2017; Mannan and Pakrasi 1993), and, therefore, they might be an active part of the underground ecosystem.

Finally, it should be noted that members of the characterized archaea found in the subsurface are very low (Takai et al. 2001). In general, members of the phylum Crenarchaeota are usually more abundant in the superficial layers of the subsurface, while members of the phylum Euryarchaeota are more common and diverse in deeper layers (Nyyssönen et al. 2014; Takai et al. 2001; Zhang et al. 2006). Special attention should be given to members of the orders Methanobacteriales, Methanomicrobiales, and Methanosarcinales, which correspond to the identified Euryarchaeota detected more often in the continental subsurface (Moser et al. 2005; Probst et al. 2014a; Purkamo et al. 2015; Rempfert et al. 2017) and suggests that the production of biological methane is an important metabolism in the subsurface.

Several studies have shown the presence of viruses in subterranean environments (Eydal et al. 2009; Kyle et al. 2008; Lau et al. 2014; Nyyssönen et al. 2014), which could be important for the microbial diversity (Eydal et al. 2009) or involved in the horizontal transfer of genes between microbial populations of the subsurface (Labonté et al. 2015). Unfortunately, the information on the subsurface viral community is still very scarce. However, perhaps one of the most surprising findings, due to the anaerobiosis of the system, has been the occasional detection of eukaryotic organisms in subsurface environments. Some studies have revealed the presence of fungal communities (Pedersen 1997; Purkamo et al. 2013; Sohlberg et al. 2015) and even new species of nematodes (Borgonie et al. 2011). The survival of the nematodes could be explained by the presence of a minimum oxygen concentration in the water. There are authors who consider the possibility that the subterranean fungal communities can develop in anaerobic conditions through a symbiotic collaboration among species (Sohlberg et al. 2015) or the existence of facultative anaerobic metabolism in these organisms (Kurakov et al. 2008). In any case, few studies have paid attention to the subsurface non-prokaryotic communities and their role in these ecosystems.

Energy sources and metabolism

As mentioned, the mineralogy of the subsurface should control the availability of nutrients and the source of energy and, therefore, the operating metabolism at a given location and depth. In the subsurface, oxygen is rapidly consumed and anaerobic metabolisms, both autotrophic and heterotrophic, are dominant.

One of the most controversial topics in the study of subsurface environments is whether the available metabolic energy sources are endogenous or, on the contrary, are partly dependent on products photosynthetically generated in the surface. The most purist authors affirm that only those microbial communities capable of developing in the absence of sunlight can be considered part of the subsurface biosphere (Momper et al. 2017b; Orcutt et al. 2011). The ecosystems that operate without photosynthesis are called SLiMEs (Subsurface Lithoautotrophic Microbial Ecosystems), name created by Stevens and McKinley in 1995 (Stevens and McKinley 1995). As described by Nealson and collaborators (Nealson et al. 2005), a true SLiME system must be powered by the geosphere and both electron donors and acceptors should be continuously renewed by geological processes, and, therefore, the microorganisms that form the basis of the ecosystem must be chemolithoautotrophs. However, according to Hoehler (Hoehler 2004), to sustain life in underground environments, the mineral matrix must store enough energy and also have the potential to transfer it in a biologically accessible form.

Different studies have shown the capacity of microorganisms to use minerals as electron donors or acceptors (El-Naggar et al. 2010; Shock 2009) or to dissolve minerals such as biotite (Shelobolina et al. 2012), pyrite (Vera et al. 2013), chalcopyrite (Edwards et al. 2000), or feldspar (Rogers et al. 1998) among others (Dong et al. 2014), releasing compounds that can be used as substrates and contribute to the production

of biomass. However, in general, microorganisms need to produce extracellular agents to dissolve minerals, which imply an increase in the energy required to survive in an environment that is considered oligotrophic. Therefore, candidate environments to be considered SLiME are those in which energy is released and biologically accessible abiotically.

One of the most abundant gases in the subsurface is hydrogen, which can be generated abiotically in many different ways (Apps 1993). H_2 is one of the most commonly used molecules by chemolithoautotrophic microorganisms and is currently considered the main source of primary energy in SLiME environments. Both Stevens and McKinley (Stevens and McKinley 1995) and Pedersen (Pedersen 1997) suggested a similar model in which H_2 was the main driver of the underground biosphere in the Columbia River Basalt Group and the Äspö area, respectively (Fig. 2). According to this model, autotrophic methanogens and homoacetogenic microorganisms would constitute the basis of the trophic chain through the consumption of H_2 and CO_2. Their metabolic products, as well as the biomass obtained by these communities, could serve as the energy source for anaerobic heterotrophs and fermenters, closing the carbon cycle.

Several research groups defend the possibility that H_2 is the main source of energy for primary producers, and, until now, it is the most accepted model to explain the survival of a subsurface biosphere independent from the surface (Brazelton et al. 2012; Chapelle et al. 2002; Lau et al. 2016; Nealson et al. 2005). Data obtained from several underground ecosystems reported the presence of H_2, CO_2, and CH_4, at least in micromolar concentrations, together with the presence, and sometimes dominance, of microorganisms whose metabolism is based on the oxidation of H_2 (Itävaara et al. 2011b; Moser et al. 2005; Pedersen 2000; Wu et al. 2017), which supports this hypothesis.

However, not all authors share the view that H_2 can be a significant source of abiotic energy and argue that underground life may be, at least in part, dependent on the flow of organic carbon and energy from the surface, for various reasons. One of them is that not all the sources of energy available in the subsurface are inorganic compounds. The best examples are petroleum deposits or sedimentary rocks, where

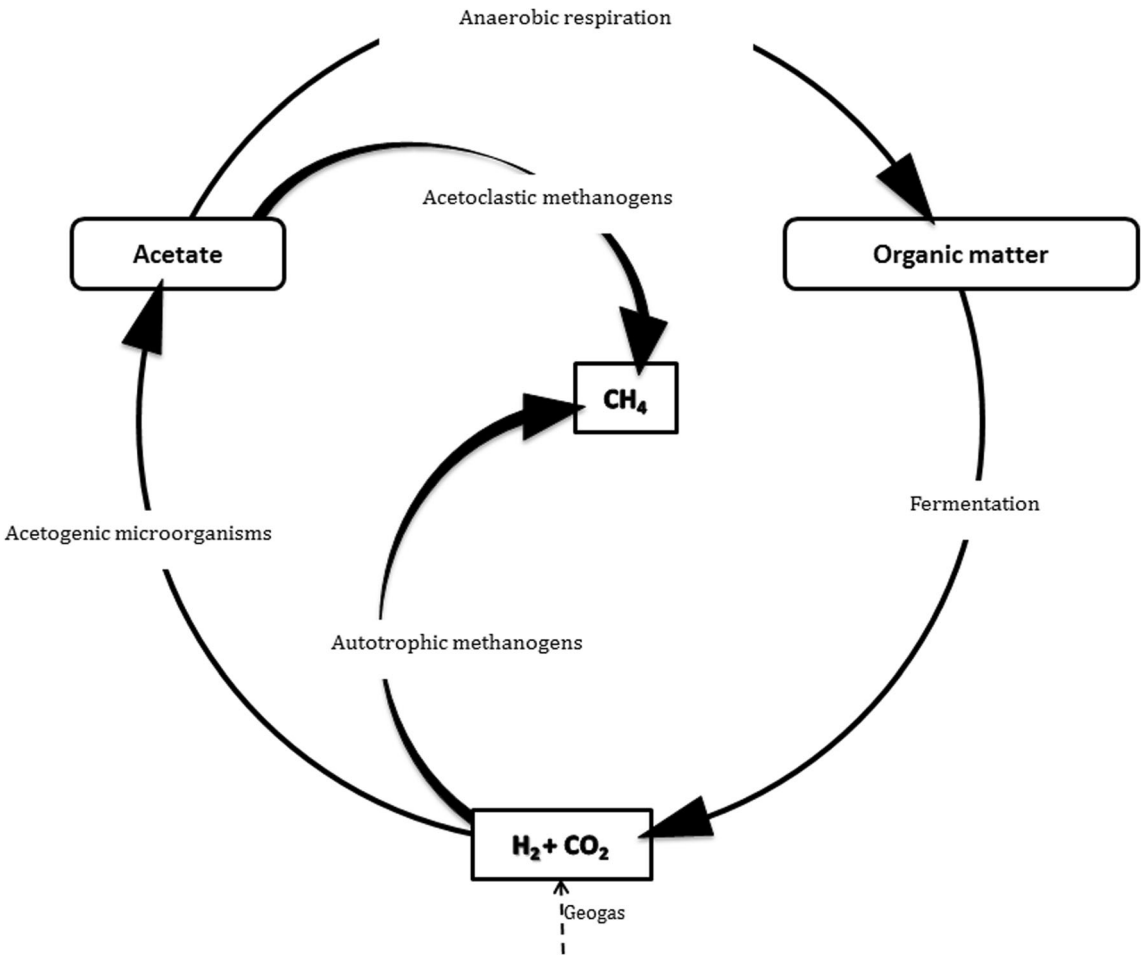

Fig. 2 Proposed model in which hydrogen is the principal energy source for primary production in SLiMe environments. Figure modified from Pedersen (1997)

the presence of organic matter is indisputable (Fredrickson and Balkwill 2006). On the other hand, the subsurface is not a completely isolated system since water percolating through pores and fractures from the surface may contain small amounts of organic matter that can contribute feeding the system. In addition, heterotrophy is a widely represented metabolism in the subsurface, and heterotrophic microbial populations are more diverse and, on occasions, more numerous in these environments than the lithoautotrophic ones (Breuker et al. 2011; Purkamo et al. 2015). To date, the existence of a truly SLiME community has not yet been unequivocally demonstrated in the continental subsurface.

In subsurface environments, other lithotrophic metabolisms have been detected that do not require H_2 or reduced organic compounds as an energy source. Among these are the oxidation of reduced sulfur compounds (Amend and Teske 2005; Gihring et al. 2006; Lau et al. 2016), iron (Sahl et al. 2008; Shelobolina et al. 2012; Swanner et al. 2011) and nitrogen (Lau et al. 2016; Nyyssönen et al. 2014; Swanner and Templeton 2011). In addition, other less common metabolisms have also been detected such as the oxidation of arsenic (Sahl et al. 2008; Zhang et al. 2005), manganese (Moser et al. 2005), or methane (Ino et al. 2017; Lau et al. 2016; Nyyssönen et al. 2012).

It is unknown if these alternative sources of reducing power could be sufficient to sustain an underground chemolithotropic ecosystem where H_2 levels are insufficient, but according to thermodynamic models, these reactions could provide enough energy to maintain it (Amend et al. 2003; Osburn et al. 2014).

Methodologies used in the continental subsurface sample analysis

There are several reviews that cover the procedures recommended for drilling and contamination control thoroughly (Kieft 2010; Wilkins et al. 2014). In this section, we will focus on those methodologies used for the analysis of subsurface samples, with special emphasis on new developments. Because the deep subsurface is strictly anaerobic, care should be taken to avoid contact of the drilled cores with atmospheric oxygen during generation of samples (Kieft 2010) (Fig. 3). Most deep subsurface geomicrobiological data has been generated by diverse conventional techniques (elemental analysis by TXRF and ICP-MS, mineral identification by XRD, stable isotopes fractionation, ionic and gas chromatography, enrichment cultures, isolation of microorganisms, 16S rRNA gene cloning, massive sequencing, metagenomics, retrotranscriptomics, immunological detection, and fluorescence in situ hybridization, between others) (Table 1), each one with its own limitations. This is the reason for recommending the use of complementary techniques. Convergent results from methodologies based on different

Fig. 3 Generation of samples from drilling cores in strict anaerobic and sterile conditions using a N_2:H_2 gas mixture for an efficient removal of O_2

principles should be more reliable than those obtained by only one. The most important limitation in sample analysis is related to the amount required for many methodologies that only generate information, often very distant from the real conditions in which microorganisms operate in the subsurface. This bulk information cannot provide insights into the coexistence in the same sample of competitive metabolic activities, such as the presence of methanogens and acetogens, or metabolic activities that are unable to operate in the detected bulk conditions, such as methanogenesis or sulfate reduction at positive redox potentials. Only the occurrence of compartmentalization into microniches, which allow the existence of different optimal conditions in close proximity, would make it possible for these antagonistic metabolisms to jointly operate in the subsurface solid matrix.

To gain information on subsurface compartmentalization, microscopy methodologies are very useful. Scanning electron microscopy (SEM) allows the detection of different mineral substrates with biological structures to be correlated through elemental (EDAX) and morphological analysis. But this technique does not identify the microorganisms and consequently does not predict the type of metabolisms involved, and it cannot be of use for subsurface water analysis. The adaptation of rRNA-targeted fluorescence in situ hybridization (rRNA-FISH) to the study of microorganisms associated to semisolid substrates (CARD-FISH) was an important innovation in the study of sediments (Hoshino et al. 2008), and it can be of use for subsurface water analysis. Its implementation in solid rock subsurface samples required the development of protocols able to overcome the problems generated by the autofluorescence of many mineral substrates (Escudero et al. 2018). It is easy to envisage a near future where FISH-based methodologies will play a significant role in clarifying the ambiguous bulk results generated by comparative sequence analysis, which, as mentioned, only can give general diversity

information due to the size of the required samples to extract analyzable nucleic acids. Of course, sequence information is necessary for the selection or design of adequate new fluorescence probes. Recently, a wide range of FISH procedures have been developed targeting not only rRNA but also mRNA or single genes (Moraru and Amann 2012). It can be predicted that these procedures will give interesting results when applied to more complex environmental samples, such as those from the deep biosphere.

The use of confocal laser scanning microscopy (CLSM) greatly improved the imaging of microorganisms occupying different focal planes within the solid mineral substrate. A new generation of CLSM should improve the quality of 3D images making it easier to identify diverse functional microniches by combining specific CARD-FISH probes with different fluorophores. The recent introduction of super-resolution microscopy will go far to overcome the limitations of applying fluorescent methodologies to complex subsurface environmental samples (Moraru et al. 2010).

Although it has been suggested that oligotrophic subsurface ecosystems cannot afford to generate biofilms due to their energetic cost, recently it has been shown that biofilms also play an important role in the microbial ecology of the deep subsurface (Fig. 4) (Escudero et al. 2018). The combination of fluorescence in situ hybridization techniques with fluorescence lectin binding assay (FLBA) should improve the characterization of the extracellular polymeric substances (EPS) that interconnect subsurface microniches (Escudero et al. 2018). The correlation of FISH and Raman spectroscopy would be another option to facilitate the identification of microorganisms associated to different mineral substrates.

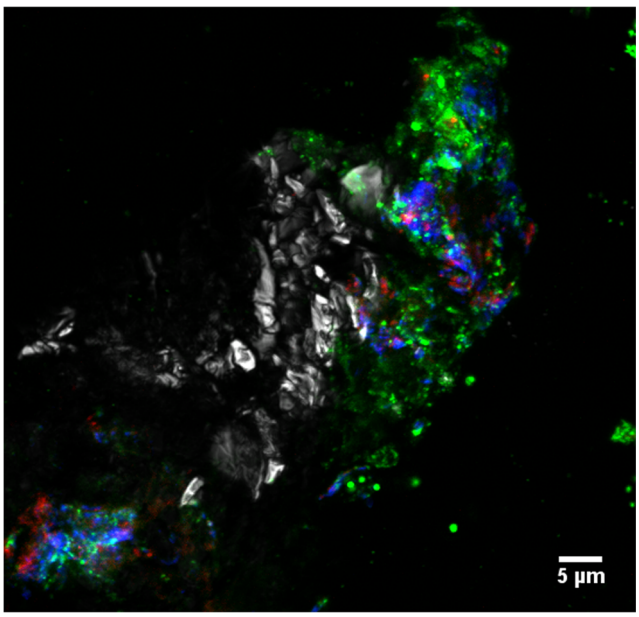

Fig. 4 Hard porous rock subsurface biofilm at 139.4 mbs of the Iberian Pyrite Belt. In red, members of Bacteria domain detected with EUB338 I-II probe; in blue, members of Archaea domain detected with ARC915 probe; in green, internal and nonreducing terminal α-D-mannosyl and α-D-glucosyl groups of EPS detected with ConA lectin; in gray, reflection. Scale bar, 5 µm

The ability of NanoSIMS to measure stable isotopes as well as radioisotopes with suitable half-lives can be used to image metabolically active microbial cells within complex communities. Furthermore, coupling NanoSIMS with halogen in situ hybridization (HISH) will make it possible to phylogenetically identify microbial cells and quantify substrate uptake simultaneously, giving access to basic information about the deep subsurface world that is still missing (Musat et al. 2008).

Conclusions

Although economic constraints have limited the amount of information from devoted geomicrobiological drilling studies, it is clear that the results obtained so far call for a systematic exploration of the deep continental subsurface, similar to what has been accomplished in recent years by the Ocean Drilling Initiatives. In addition to devoted drillings from the surface, most of the continental subsurface data have been obtained using different subterranean "windows" (artesian wells, springs, underground locations for waste disposal, underground research facilities, and deep mines) with the correspondent limitations.

The emerging picture is that there is a variable number of cells in the subsurface, probably related to the geology and hydrology of the studied system. In general, the number and diversity of microorganisms decreases with depth, and the abundance of Bacteria is superior to Archaea. Within Bacteria, the most common phyla detected so far correspond to Proteobacteria, Actinobacteria, Bacteroidetes, and Firmicutes. Within the Archaea, methanogens are recurrently detected in most analyzed subsurfaces. One of the most controversial topics in the study of subsurface environments is whether the available energy source is endogenous or partly dependent on photosynthetically generated products in the subsurface. Although H_2, which can be generated abiotically, seems to be among the electron donors most used by chemolithoautotrophic microorganisms, other lithotrophic metabolisms making use of reduced sulfur, iron, and nitrogen have been also detected in the subsurface. More information at a better depth resolution is needed to build up the repertoire of subsurface electron acceptors and donors biologically available in the deep subsurface. Several studies reported the presence of viruses in subterranean environments, but a more systematic evaluation is needed to assess their role in horizontal gene transfer among microbial populations. Similarly, a thorough analysis will be needed to verify the reported presence of fungi as members of the dark biosphere. Even though the methodologies for drilling and control of contamination are well established, the procedures for the taxonomic, functional,

and metabolic analysis are rather diverse and a reflection of the rapid evolution of available methodologies. In any case, the use of complementary techniques is strongly advisable because it can help to sort out the most important elements of the system. Of the many methodologies used for sample analysis, those based on fluorescence in situ hybridization are of particular interest because they allow a resolution at the microniche scale, which cannot be obtained by most of the currently available methodologies due to the large volumes of sample required, consequence of the low cell number existing in deep low porous rocks.

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