Technological challenges to understanding the microbial ecology of deep subsurface ecosystems

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Terrestrial subsurface geomicrobiology is a new frontier in environmental microbiology. It seeks to determine whether life can be sustained in the absence of solar radiation. Subsurface ecosystems are also intriguing astrobiological models useful in the re-creation of life in early Earth scenarios or ascertaining its possible existence on other planetary bodies. Although Darwin predicted a deep subsurface ecosystem in 1839 (Darwin, 1839), it was first observed in a study of basalt aquifers less than 20 years ago (Stevens and McKinley, 1995). Whether the source of electron donors and acceptors in this ecosystem is mediated by solar radiation or the contamination from the drilling fluids required to drill can be controlled and mitigated is still being hotly debated. Despite the progress that has been made in recent years, information concerning microbial abundance, diversity and sustainability in the deep subsurface is still scarce, mainly because of the methodological limitations of studying micro-niches dispersed in solid matrixes.

Most of the geomicrobiological results generated by deep subsurface drilling projects have been acquired by a combination of techniques [mineral identification by XRD, elemental analysis by ICP-MS and TXRF, stable isotopes fractionation, ionic chromatography, gas chromatography, enrichment cultures, 16S rRNA gene cloning, massive sequencing, metagenomics, immunological detection, fluorescence *in situ* hybridization (FISH)], many of which were applied to samples that, because of size requirements, give only global environmental information and do not represent the real conditions in which these microorganisms operate. This bulk information cannot provide insight into the possible coexistence of antithetic metabolic activities, e.g. iron oxidizers and iron reducers, or metabolic activities that are unable to operate in these global conditions, e.g. methanogenesis or sulfate reduction at positive redox potentials. Only the existence of micro-niches in close proximity allowing the existence of different optimal conditions would make it possible for these metabolisms to function. This is not the case in solution, where the conditions are homogeneous.

Scanning electron microscopy allows the presence of diverse mineral substrates to be correlated with identified biological structures through elemental analysis (EDAX) and morphology, but the metabolic status and the type of functional metabolism of these biological structures cannot be determined using these techniques. The adaptation of rRN-targeted FISH (rRNA-FISH) to the study the microorganisms associated to semisolid substrates [catalysed reporter deposition-FISH (CARD-FISH)] was an important breakthrough in the microbial ecology study of sediments. Even though this technique has not been used intensively in the identification and quantification of microorganisms in continental drilling projects, it is obvious that in the near future, it will play a significant role in clarifying the ambiguous results generated by more conventional techniques such as comparative sequence analysis, which, as mentioned earlier, require sample sizes and preparation methodologies that average or destroy compartmentalization.

Fluorescent oligonucleotide probes were originally designed to target rRNA for identification of microorganisms in environmental studies. Recently, a wide range of FISH procedures have been developed targeting not only rRNA, but also mRNA or single genes (Moraru *et al.*, 2010). It is easy to foresee that these procedures will be applied to more complex environmental samples including those from the deep biosphere.

Among the observational methodologies, 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 will improve the quality of three-dimensional images, making it easier to identify diverse functional micro-niches by combining specific CARD-FISH probes with different fluorophores. The introduction of super-resolution microscopy resulting in improved resolution of light microscopy will go far to overcome the limitations to applying fluorescent methodologies to environmental microbiology (Moraru and Amann, 2012).

Biofilms also play an important role in the deep subsurface microbial ecology. I can envision that the combination of FISH techniques (CARD-FISH) with CLSM and atomic force microscopy would improve the characterization of the extracellular polymeric substances that interconnect subsurface compartments.

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 will make it possible to phylogenetically identify microbial cells and quantify substrate uptake simultaneously, giving access to basic information now lacking about the deep subsurface world.

An important limitation in deep subsurface microbiology is that most of the physico-chemical variables used to describe the ecosystem come from data generated during the drilling campaign. In some cases, the evolution of these variables have been followed with multi-level diffusion samples (MLDS) installed at different depths after the well is cased with perforated PVC that allows the water table to move freely. The problem with this technique is that after the MLDS are installed and removed several times, most of the water column is mixed and its original steady state is lost. The design of a set of probes that can measure and send data in real time on physico-chemical variables (temperature, conductivity, redox potential, pressure, gases, concentration of different electron donors and acceptors) without disturbing the water column is a technological challenge for the future. Although this methodology may not yet give detailed information on micro-niche level functioning, it would follow the evolution of environmental conditions along the length of the well and provide information critical to our understanding of the interactions among the different functional activities detected by complementary methodologies.

Finally, the identification of the deep subsurface viral populations will help ascertain if high genetic exchange rate between phages to hosts is comparable with that observed in other extreme environments and lead to study complete infection cycles to evaluate the importance of the viral charge in the adaptation to this extreme environment.

References

Darwin, C. (1839) *Voyages of the Adventure and Beagle*, Vol. III, *Journal and remarks*. London, UK: Henry Colburn, pp. 1832–1836.

Moraru, C., and Amann, R. (2012) Crystal ball: fluorescence in situ hybridization in the age of super-resolution microscopy. *Syst Appl Microbiol* **35:** 349–352.

Moraru, C., Lam, P., Fuchs, B.M., Kuypers, M.M.M., and Amann, R. (2010) GeneFISH – an in situ technique for linking gene presence and cell identity in environmental microorganisms. *Environ Microbiol* **12:** 3057–3073.

Stevens, T.O., and McKinley, J.P. (1995) Lithoautotrophic microbial ecosystems in deep basalt aquifers. *Science* **270:** 450–454.