

Scientific Opportunities in the Deep Subseafloor Biosphere

A perspective from the U.S. Working Group on
IODP and the Deep Biosphere

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1. Introduction and Perspective

As one of its many scientific accomplishments, the Ocean Drilling Program (ODP) provided the first sediment samples demonstrating that microbes occur at depths greater than 850m below the seafloor. This exciting discovery set the stage for the subsequent early exploration of this entirely new ecosystem using drilling technology. Further analysis of the deep subseafloor for microbes and microbial communities will clearly yield many unexpected and exciting results. For these reasons, the Integrated Ocean Drilling Program (IODP) selected this topic as one of its major research initiatives.

Over a surprisingly broad range of subsurface depths, temperatures and pressures, the subseafloor ocean hosts an extensive microbial population comprising the deep biosphere. **Estimates of the fraction of the Earth's microbial population deeply buried in oceanic sediment and crust range from just over 10% to as much as two-thirds (2,5).** With the recognition that this pervasive subseafloor ocean may teem with microbial life come new, fundamental questions about the evolution and distribution of life and the operation of the carbon cycle. How this huge biomass survives in an environment of apparently meager resources poses basic questions for biochemistry, microbial physiology and microbial ecology. In addition, discoveries holding promise of major practical benefits and applications can be anticipated. The U.S. arm of the IODP has given exploration of the deep ocean subseafloor biosphere a high priority, and the U.S. Working Group on IODP and the Deep Biosphere was established to assist in bringing specific focus to these projects and to help in planning for broadening the participating groups. This report represents the discussions at a meeting of the Working Group held in April 2006.

The IODP panels and committees began to mentor, help develop and evaluate submitted proposals that address this major thematic area, to expedite proposals and expand participation by microbiologists. To date the review panels have approved a limited number of outstanding projects and they are being scheduled.

2. Science Drivers for Studying the Deep Biosphere: Microbial Ecology

The search for a greater understanding of deep biosphere ecosystems is hampered by truly fundamental gaps in our knowledge of biological processes and properties of these systems. For example, we know that prokaryotes are

surprisingly abundant in the deep ocean subsurface biosphere, but do not know their composition, diversity, variability, distribution or activity except in the broadest terms. We do not know whether microbial eukaryotes are present to significant depths below the seafloor, and if present, whether there might be an endemic subsurface community as opposed to penetration of shallow sediment microbial eukaryotes into the deeper biosphere. It is not known whether viruses are abundant or rare, and if present whether they play roles comparable to their ecological roles in the water column. We know that sediment composition can vary on cm to m scales (as well as larger scales) but do not know whether or how microbes respond to these variations. Given these major gaps, it would be premature to suppose that deep biosphere microbial communities are inherently more or less complex than in the water column or shallow sediments.

However, it is certain that the deep biosphere presents a novel and rich opportunity to study microbial processes and interactions in the absence of a host of confounding physical and biological processes that characterize other ocean environments. Deep biosphere ecosystems have fewer biological components than better known systems such as surface ocean waters or surficial sediments. Metazoans are absent, with profound implications for ecosystem complexity. Chlorophyll based photosynthesis is absent, reducing the variety of autotrophic processes supplying microbial communities. Perturbation and water motion are greatly reduced in the deep biosphere compared to the water column or shallow sediments, suggesting that microbial cells in the deep biosphere remain in close proximity to one another over long periods, and that the microbial economy is diffusion-based. Both properties are conducive to examining the fundamental processes by which microbes communicate and interact, a topic extraordinarily difficult to study in a turbulent water column.

The sustained proximity of microbes also allows exploration of the processes that drive microbial phylogenetic and community diversity. Many familiar ecological and evolutionary processes contain the implicit assumption that organisms can interact over meaningfully long periods. For example, competition cannot structure microbial communities or produce evolutionary winners and losers unless organisms can actually compete, which in turn implies that they remain in close enough proximity to use the same resource pools. One might reasonably argue that the surprisingly rich diversity of Bacteria in the turbulent world of a water column stems from a lack of effective competition rather than a corresponding diversity of ecological

niches; i.e. multiple solutions to a given set of environmental challenges can coexist if they never have to compete. In short, stability driven by a lack of perturbation and absence of turbulence will certainly have major implications for community structure and evolutionary processes. The deep biosphere is a ready-made natural laboratory to examine these processes.

It is likely that microbial processes in the deep biosphere are stretched in both time and space, offering both challenges and opportunities for research. Unlike spatially compressed ecosystems (e.g. biofilms, surface sediments, microbial mats), energy sources in the deep biosphere are expanded in space and can be studied on meter and even kilometer scales. Microbial growth rates appear to be much lower than microbial oceanographers are accustomed to measuring. While this presents obvious challenges and requirements for development of appropriately sensitive methodologies, it also offers a unique natural laboratory to study concepts that are difficult to examine in an academic laboratory and almost impossible to address in other real world environments.

Questions uniquely answerable through Deep Biosphere research:

Consider, for example minimal energy allocation strategies; i.e. which intracellular activities are essential when a cell is barely alive? What are the implications of these absolutely necessary choices? In the absence of luxury energy levels, is DNA repair a high priority, or are mutation rates greater than in shallower environments? Are there alternative energy sources that are insignificant in the “upper world”, but worth exploiting in a minimal-energy deep biosphere? Slow growth rates also have profound implications for population connectivity. Given very slow turnover rates (but are these rates always low, or are there ephemeral bursts of rapid growth?), are deep biosphere microbes truly isolated from the upper world by slow physical transport processes? Is there genetic evidence suggesting either isolation or connection to upper-world populations? Is the deep biosphere a source or sink of genetic diversity?

The deep biosphere does not presently figure into estimates of global biogeochemical cycles, because of the lack of information regarding the biomass, diversity, structure and activity of the deep biosphere. However, even first-order calculations of the possible magnitude of biogeochemical

processes in the deep biosphere will be irrelevant to upper-world problems unless it is clear that the deep biosphere is connected to the upper biosphere over time scales and at rates that are meaningful to upper-world biogeochemical cycles.

Finally, it is almost certain that the deep ocean subsurface biosphere is not one but many ecosystems, but in the absence of any real understanding of the operation of these microbial ecosystems, we can only apply broad concepts learned from more familiar environments. We might expect that sediment composition, sediment versus basalt substrates, variations in pore water chemistry, presence of hydrocarbons, temperature, and a host of other physicochemical properties will influence microbial communities and processes- but we do not know which of these will prove most important or whether our surface-world intuitions will be applicable. For example, we know that the upper thermal limit of eukaryotic life is about 60°C, and appears to be limited by the difficulty of forming and maintaining thermostable organellar membranes. Using this upper temperature limit, we can look at an extrapolated map of sediment temperatures and conclude that there are only a few places in the deep biosphere where temperature would limit the distribution of eukaryotic life. However, that first-order evaluation is based on life as we know it and not on life in the deep biosphere. Can eukaryotes in a minimal-energy setting make the same metabolic strategy choices as their counterparts in the upper world? Can they afford to maintain organellar membranes at 60°C given that they may be barely alive, at best? The prospect that some of our intuitions regarding how microbes interact with their surroundings may be wrong or altogether irrelevant in the deep biosphere is both a challenge and a profoundly exciting prospect.

3. Science Drivers for Studying the Deep Biosphere: Evolutionary Biology

Microorganisms are known to horizontally exchange genes, a process that provides rapid genome adaptation and diversification. The deep ocean subsurface biosphere of the Earth represents a unique habitat in which to address questions regarding fundamental evolutionary biology and ecology that will yield insights into the contributions of horizontal gene flow to deep biosphere microbial community structure and function. As a group we posed a number of broad issues ranging from microbial survival, persistence and energetics to genomic innovation and the horizontal flow of genes. These are only a few of the many interesting problems in this ecosystem,

Major questions to address:

- 1) What are the genomic features and adaptations that characterize the deep marine subsurface biosphere, and promote survival and persistence?
- 2) What are the relative contributions of horizontal gene transfer and mutational innovation?
- 3) What is the effect of population size and community isolation temporally and spatially on genome evolution?
- 4) What is the effect of metabolic activity on genome evolution?

There is little information available about the role of gene flow and evolutionary processes, in part because of low microbial biomass, low microbial activity and possibly limited and/or significantly restricted community interactions in the deep biosphere. There is a clear need to better understand the primary rules, processes and mechanisms that dictate the exchange of genomic information in the deep biosphere microbial community. What role do known mechanisms of genomic exchange play in promoting adaptation, recombination, mutation and HGT within the deep biosphere microbial community? For example, what is the relative role of viruses in the deep biosphere? Can we expect to detect comparable viral populations in the deep ocean subsurface biosphere as has been reported for the marine water column? How will the inherent spatial patchiness of the deep biosphere impact viral abundance, diversity and activity? Are there recognizable “biofilms” and what role might they play in HGT?

Similar questions arise with regard to other mobile genomic elements in the deep ocean subsurface biosphere. For example, what is the relative importance of transposons, integrons, insertion sequences and plasmids in promoting microbial genome diversification and innovation in this unique environment? What is the expected genetic diversity of such mobile genomic elements residing in the deep subsurface biosphere? What is the extent or degree of genomic diversity within the microbial populations? How important is population size in promoting genome innovation in the deep biosphere? How does the genome respond to environmental and physical challenges posed by the unique conditions in the deep biosphere (e.g., radiation)? Microorganisms in the deep biosphere exist in conditions

that pose considerable threat to DNA integrity. Examining the DNA repair capabilities and mechanisms that promote the informational integrity of DNA may yield new insights into how deep subsurface microorganisms have evolved strategies for preventing, repairing or tolerating DNA damage.

4. Subseafloor Biosphere Analyses: A Contemporary Case Study

Although the deep biosphere is a poorly known component of life on planet Earth, it also poses unprecedented challenges to existing microbiological methodologies. The complex, heterogeneous nature of the deep sediments and rock matrix, the relatively low biomass and physiological activity, and unknown genetic and physiological features of deep subsurface microbes, all present unique and significant analytical challenges. Beyond specific limitations of current microbiological techniques, studies will need to incorporate the ideas, efforts and expertise of many disciplines, including geology, geochemistry, paleoceanography, and the full breadth of the microbiological sciences. This requires coordination between fields and investigators that traditionally have not interacted.

Despite the significant challenges, several recent studies provide good examples of how transdisciplinary efforts can combine with modern methodologies to propel understanding of the deep ocean subsurface biosphere forward. A closer examination of one integrated set of studies provides a departure point for discussing what can be done currently, where significant road blocks still exist, and what potential paths lead forward to a deeper understanding of the ocean subsurface biosphere. These studies show the value of a comparative and integrated geological, geochemical and biological approach that provides new information on biomass, types and rates of activity, and phylogenetic identity of deep ocean subsurface microbes.

A series of deep biosphere studies were associated with ODP Legs 201 and 204 (2, 3, 5). Cores were collected along Peru's continental margin at water depths of 150 m to 5300 m. These cores sampled sediments from 0 to 420 m below the seafloor, spanning temperatures from 1°C to 25°C, and ages of 0 to 35 million years. This core transect specifically targeted a range of influences from waters above (e.g., from highly productive overlying waters to the oligotrophic open ocean), as well as heterogeneous and variable subsurface influences (including variable geochemical gradients and atypical

near-basement fluid flows).

In the first of a series of reports associated with ODP Legs 201 and 204, D'Hondt and colleagues reported evidence for metabolically diverse and active microbial communities at depths as great as half a kilometer below the seafloor (2). These investigators used geochemical indicators of microbial activity to infer some of the mechanisms by which sub-seafloor microbes survive at great depths below the seafloor. Microbial cell counts decreased with sediment depth. The distributions of sulfate and nitrate, and other microbial metabolic byproducts - carbon dioxide, ammonia, sulfide, methane, manganese and iron - provided a picture of how microbial activities are partitioned in the deep sediments, and indicated which processes may be most important. One big surprise was the observation of "upside down" redox profiles, with oxidants emanating from the sediment basement, enabling microbial respiration (usually found near the sediment surface), to occur at great subseafloor depths. These respiratory activities can potentially drive manganese and iron cycling in deep ocean subsurface microbial communities, in a microbial "bucket brigade" of cascading respiratory electron shuttles that pass electrons through various sources and sinks. The results indicated the presence of physiologically more diverse and active deep-sediment microbiota than previously supposed. Also surprising was the co-occurrence of deep sediment methanogenesis, and manganese and iron reduction, within zones of high sulfate. Typically, sulfate-reducers are expected to out compete methanogens under these environmental conditions.

Two subsequent studies (3, 5) partly addressed which microbes dominate deep ocean subsurface energy cycling. The vertical and geographic distribution of prokaryotes in ODP Leg 201 and 204 cores (some of which contained methane hydrates), was examined by cloning ribosomal RNA genes to identify the taxonomic affinities of resident microbes. In a comparative study contrasting hydrate-versus non-hydrate containing sediments, the rRNA profiles of *Archaea* suggested the predominance of one specific archaeal group implying their importance in methane and sulfate cycling (1). Comparisons of several cores suggested different associations of archaeal and bacterial groups in methane hydrates, versus non-hydrate bearing sediments. Elevated microbial numbers were found in the sulfate methane transition zone on Leg 201 cores, with two quite specific archaeal groups dominating. These and other data are indicative of elevated metabolic activity in some regions of the deep biosphere, and suggest that

tight linkages exist between geochemical conditions and microbial community structure and identity (1, 2, 4). Another study using a single-cell approach, fluorescence in situ hybridization (FISH), demonstrated the presence of ribosomal RNA in individual cells. These data suggest that a large fraction of sub-seafloor prokaryotes is living – a hypothesis that still requires much more thorough testing (5).

Integrated studies such as those described above provide an early model for in depth probing of the deep ocean subsurface biosphere. The geological, biogeochemical and molecular perspectives applied were well-integrated and useful for establishing initial models for the inner workings of the subsurface biosphere. These studies also highlighted some of the real challenges associated with deep ocean subsurface biosphere work. Many of the available techniques for measuring microbial biomass, activity and identity in these remote locations are not easily applied, or are simply not yet well developed. Reports of cultivated isolates are not always corroborated with molecular or activity data, for reasons that are not well understood (2). The use of nucleic acid-based sequencing techniques has met with limited success, but challenges still exist for obtaining sufficient DNA for some of the more advanced genome-enabled techniques (3, 5). Sub-sediment hard rock basement habitats with lower biomass and a challenging matrix may prove to be even more problematic.

5. Obstacles to Further Progress in Deep Biosphere Microbiology

Several first order challenges must be addressed immediately to enable the proposed science objectives. These include: (1) technical development of new and improved methodologies for the measurement of microbial biomass, activity and diversity in the anticipated sample materials, (2) cultural integration of microbiology into the mostly geology/geophysics/geochemistry based IODP activities, and (3) the establishment of the necessary educated workforce.

1. *Deep Biosphere Techniques for Microbiology:* Although adequate methods currently exist for study of high biomass, high metabolic activity sedimentary ecosystems (e.g. bacterial cell densities of 10^8 cm⁻³ or greater), most of the deep biosphere habitats are expected to be relatively low biomass, low activity biomes, especially those beneath the sediment column. Traditional methods such as direct microscopy or the use of specific

radiolabeled electron donors and acceptors have detection limits that may be insufficient to assess microbial properties in many deep biosphere samples. Even when present at acceptable concentrations, inefficient extraction of key biomarkers (DNA, RNA, phospholipids, ATP) may preclude an accurate identification or an accurate quantitative assessment of microbial properties. Finally, the physical characteristics of the deep biosphere habitat may select for novel metabolic processes and novel microorganisms that will require the development of equally novel methods of detection. It is highly likely that microbial rate and process studies will require in situ or remote measurement techniques to test and account for the possible effects of pressure, temperature and ambient chemical gradients. This represents an enormous challenge for the relatively remote and sometimes inaccessible deep biosphere habitats.

Recommendation #1: Fundamental microbiological technique development is critical to continued progress and deserves broader financial support. This should begin immediately so that the new/refined methods can be developed and tested in the laboratory prior to use in the field. STP Recommendation 0507-07 is an excellent beginning but much more needs to be done. Funding for this phase of technique development could come from new public-private partnerships, or from multi-agency programs (e.g., NSF-DOE) that would benefit from these developments.

Recommendation #2: Once developed and tested, the new methods should be thoroughly documented and included in the expedition and curation standard operating procedures to ensure a routine and reliable data return that includes complementary metadata. Expedition operation budgets should be increased to ensure that the new techniques will be implemented routinely. A training course in microbiological methods should be developed and required for shipboard support individuals responsible for sample collection and analysis.

Recommendation # 3: Develop novel methods for the remote detection and characterization of microbial activities in the deep biosphere. These efforts should proceed in conjunction with similar objectives of ocean observing initiatives (e.g. OOI) for use in deep sea observatories.

2. Cultural integration of microbiology into the mostly geology/geophysics/geochemistry based IODP activities.

Many of the important scientific questions regarding the existence and function of the deep biosphere are inherently interdisciplinary and there needs to be a more solid common ground for collaboration. This must start with a greater awareness of the opportunities for microbiologists in IODP, including but not limited to participation in drilling expeditions, access to archived samples, and funding. The establishment of an onboard microbiology laboratory was a giant first step and this needs to be supplemented with staff microbiology technicians and the establishment of a land-based repository for microbiological samples, preferably near an internationally recognized center for microbiological research. This should be accompanied by a data archival system that is appropriate for microbiological data, including development/adoption of standard bioinformatics tools.

Recommendation #4: Increase awareness of opportunities for microbiological research of the deep biosphere by outreach activities at non-traditional venues, including the annual meetings of interdisciplinary professional societies, as well as specialist societies such as the American Society for Microbiology, the Society of General Microbiology, the Society for Industrial Microbiology and the International Society of Microbial Ecology.

Recommendation #5: Establish a permanent archive/repository for IODP samples that is compatible with downstream microbiological analyses. These must be preserved in a manner that is suitable for the intended purpose, mostly likely frozen and stored at -80°C.

3. Training and workforce development: The study of the deep biosphere is a relatively new and specialized transdisciplinary field of study. Most scientists currently involved in IODP have traditional backgrounds in geology, geophysics, geochemistry or paleoceanography. The few microbiologists currently involved in the program are trained in biological oceanography. Many of the most important scientific questions regarding the deep biosphere require a broad based knowledge of geology, chemistry and microbiology, but cross-training in these areas of expertise is rare.

Future progress will require the training of a new cadre of individuals who are able to develop collaborations with others who complement their own expertise. In this regard, directed co-mentoring of students or postdocs, or specialized cross discipline sabbatical (or other) training, would be invaluable for closer ties between and among individuals who otherwise work in separate disciplines.

Recommendation #6: Establish a formal training program that would provide opportunities for individuals who are interested in the deep biosphere. This could take the form of a postdoctoral fellowship program for directed cross-training or perhaps a multi-disciplinary short-course. In either case, effective mentoring will be the key to success.

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