DCO Deep Life Workshop

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Deep Life Directorate White Paper:

Deep Subsurface Microbiology and the Deep Carbon Observatory

I. Goals

Earth's biota includes great numbers of largely unknown life forms within the planet's interior. The Deep Life Directorate (DLD) of the Deep Carbon Observatory (DCO) will explore the inner limits of our planet's life process and the role of deep life in controlling biogeochemical processes and climate on the surface. It aims, for the first time, to integrate continental and marine subsurface science in order to develop a global model of dark carbon fixation, and additional carbon biochemical transformations, that occur in the subsurface biosphere. An ambitious program is envisioned that combines *in situ* discovery and observation with *ex situ* characterization extending from molecules and microbes to new biomes and provinces.

The overarching goals of the DLD initiative are 1) to explore the diversity of deep life, its metabolic character, evolutionary history, and adaptations, 2) to elucidate the relationship (or lack of) between deep life and the surface biosphere, 3) to define the biologically available pools of carbon and quantify their fluxes, 4) to compare biotic and abiotic chemical processes at depth, and 5) to utilize this program as a vehicle for education, public outreach and training the next generation of scientists. More specific goals are as follows:

- *To explore the diversity of deep life and its molecular constituents.*
    - *To compare and contrast continental and marine subsurface habitats.*
    - *To characterize the biogeography of deep life.*
    - *To explore evolutionary processes and rates in the deep biosphere.*
    - *To interrogate the relationship between Earth history and the deep biosphere*
    - *To assess potential of terrestrial subsurface as an analog for life off the Earth*
- *To define the limits of life at depth (T, P, nutrients, diffusion, isolation, ΔG…).*
    - *To explore survival and cell maintenance under extreme conditions.*
    - *To measure the rates of microbial migration and community succession in the subsurface.*
    - *To discover and characterize new physiological and biochemical processes, possibly including new pathways for carbon and nitrogen fixation, bioenergetics and cell structures.*
- *To better understand linkages between the deep biosphere and lithosphere*

- *To define the role of microorganisms in the weathering of crustal rocks.*
- *To characterize new biological energy sources including those involving minerals, radioactivity, and mechanical strain.*
- *To explore microbial activities related to hydrocarbon formation and alteration.*

- *To explore the relationship between deep biosphere life and climate*
  - *To define the relationship between deep life and the global carbon cycle.*
  - *To incorporate chemosynthesis for the first time into global pictures of the carbon cycle.*
- *To differentiate between biotic and abiotic processes that impact the carbon cycle*
  - *To merge abiotic and biotic carbon cycles over geological time.*
  - *To explore subsurface prebiotic conditions and abiotic chemical transformations.*
- *To engage the next generation of scientists in deep biosphere science.*
- *To engage the public and students of all ages in deep biosphere science.*

II. Basic Science

A. Microbiology

Microbes are the principal innovators of Earth's biogeochemical cycles. Their cell numbers in terrestrial and aquatic environments exceed 5×10^{30} organisms with cellular carbon in excess of 10^{17} grams. Yet we know very little about the abundance, distribution, diversity and activity of deep subsurface microbial life. One recent study indicates that deep subsurface life can persist in complete isolation: fixing its own carbon and nitrogen and living in complete indifference to photosynthesis-derived organics and O_2. Such discoveries demand a profound recalibration of long-held principles of biology and ecology. Concepts such as primary production, competition, succession, and food webs all likely operate under different rules and constraints in diffusion-limited subsurface settings. To understand the growth, persistence, and evolution of life in this deep and dark biological reservoir we must address a number of questions. Who is down there? How far down does life extend? What is the diversity of deep microbial life? How did life get there? How fast does it grow? How quickly does it adapt to changing environmental conditions? How does it live? In what ways does it influence the release and uptake of greenhouse gases? We need to better understand the biochemistry and physiology of subsurface microorganisms. Are current models of microbial cell structure sufficient? What role do biofilms play? Does quorum sensing operate in some fashion? Do alternative mechanisms of energy transfer (e.g. direct electron transfer between cells) dominate in a world free of advection? Do subsurface microorganisms have unique adaptations that permit their activities and survival under extreme conditions (e.g. low energy, high pressure, and high temperature) and what are the limits to these adaptations? How important are necromass recycling and viral predation as agents of cell mortality and gene transfer in the evolution of subsurface microbial communities?

Ultimately, these questions about microbiology can be folded into large- scale questions of ecology, evolution, diversity (phylogenetic, taxonomic and functional), biogeography, biogeochemical cycles and climate. Are evolutionary patterns recorded in the distributions and adaptations of subsurface microorganisms? What role does consortial/community behavior play? What limits productivity in these ecosystems? And finally, how do abiotic organic chemical reactions influence extant ecosystems and could they have provided the raw materials for the origin and early evolution of life?

To address these questions the DLD community must organize experimental strategies that integrate cultivation-independent, genomic approaches with physiological and biochemical studies made possible through the isolation and growth of microorganisms or consortia in the laboratory. Field measurements of geochemical and physical parameters will provide a context for interpreting the ecology of subsurface microbiology. These studies must determine and embrace appropriate temporal scales necessary to understand long-term persistence at extreme pressures and high temperatures. The success of these investigations will hinge upon developing technology for recovering pristine samples and / or deploying instruments capable of making *in situ* molecular and microbiological measurements.

B. Biogeochemistry

Microbial science closely integrates with biogeochemistry. Studies of surface life have revealed that biological activity controls elemental cycling at a planetary scale and impacts most aspects of lithospheric processing. Earth's biogeochemical engine depends upon the ability of life to harness the near limitless potential of solar energy for energy, transport and organic synthesis. However, far removed from these familiar systems, the deep subsurface provides a natural laboratory where other energy-yielding processes may sustain life. Examples include the radiolytic decomposition of water to yield H_2, O_2, and H_2O_2, with the subsequent oxidation of minerals containing reduced forms of sulfur, iron, manganese or even arsenic. In general, microbes could evolve means to harvest energy in many situations where there is disequilibrium between chemical redox states. During the course of using this energy the microorganisms in the subsurface biosphere may serve as mediators of the redox balance between the mantle and atmosphere. For example, subsurface microbiota might serve as a net sink for photosynthetically produced O_2.

Ultimately, microbial activity in subsurface environments has the potential to play a critical role in the cycling of carbon and other elements between deep and shallow environments on Earth. Carbon in marine and continental subsurface environments may derive from photosynthetically-produced organic matter, organic-bearing meteoritic or cosmic exogenous influx, and deep sources located in the crust and mantle redox reactions. In marine systems, the molecular and isotopic compositions of surface-derived organic compounds (photosynthetic - including continental debris and exogenous dust) experience a broad spectrum of diagenetic transformations before and after burial in sedimentary basins, leaving only 0.1 to 0.5 % of the original photosynthetically-derived biomass and an unknown fraction of cosmic dust. Most of these transformations are microbially mediated. This organic matter is initially recalcitrant for the subsurface biosphere, but may undergo transformations that release bioavailable components with increased burial and time, to

create an even more recalcitrant carbon-rich residue. In addition to entering the system from the top, carbon may enter inhabited subsurface environments from photosynthetically-independent pools deep within the crust and mantle. The composition of carbon from deep sources may also show a large degree of compositional variability owing to variations in redox state that may favor either CO_2 or CH_4, and abiotic synthetic processes that produce longer chain organic compounds. The composition and fluxes of carbon from deep sources likely influences microbial physiological diversity and community structure.

A primary objective of the DLD initiative is to constrain the response of phylogenetic (taxonomic), physiological and functional diversity, and community structure to variations in carbon composition and flux, which in turn are influenced by temperature, pressure, and carbon source. Characterization of heterotrophic primary producers that derive their carbon from abiotically synthesized reduced carbon pools represents a particularly exciting avenue for future research. In addition to sources of carbon, subsurface life requires additional chemical species such as nutrients (H, N, P, S, O) and transition metals. Accordingly, research efforts need to be focused towards understanding (i) the full range of elements and chemical speciation necessary to support life in the deep subsurface, (ii) their bioavailability (dissolved or bound in minerals), and (iii) the biogeochemical processes that regulate their abundance.

Related objectives are (i) to measure the chemosynthetic contributions to the global carbon cycle, and (ii) to determine how abiotic processes (diagenesis, catagenesis and abiotic organosynthesis) in the deep biosphere impact deep biology, the interplay between microbes and geologic conditions (hydrates, carbonates, mineral weathering…), and (iii) to explore the connections between deep life and global climate. Addressing these objectives will involve chemical and hydrological (i.e. concentration and flux) characterization of subsurface environments, assessment of operant metabolic strategies that may take advantage of available energy sources and determination of the migration rates of microorganisms. It will be necessary to define minimum energy needs for survival and growth and the interactions and structure of microbial consortia. Ultimately, microbial activity in subsurface environments has the potential to play a critical role in the cycling of carbon and other elements between deep and shallow environments on Earth.

What are the energy sources of the deep biosphere?

Away from sunlight, microbes gain energy from chemical systems that are out of oxidation-reduction equilibrium. Some sources of disequilibria are inherited from photosynthesis in the photic world and deposited with sediments. Geologic processes including quenching, fluid movement, and tectonic juxtaposition generate others. Hydrothermal processes, which are particularly effective in moving chemical constituents from zones where equilibrium prevails to other areas where disequilibria emerge, are the primary mechanisms of ore deposition and may support subsurface ecosystems. Organic transformation reactions deep in marine sediment and continental sedimentary rock convert recalcitrant organic matter into compounds capable of supplying carbon and energy to the deep biosphere. Water-rock reactions in rock-hosted hydrothermal systems also can support abiotic organic synthesis that can undergo transport into subsurface habitats. Alternatively, conservation of energy released from *in situ* radioactive decay in

the lithosphere in the form of reductant and oxidant production from radiolysis may generate disequilibrium.

To understand the relative importance of these mechanisms, DLD will characterize the chemical context of subsurface environments and assess operant metabolic strategies that may take advantage of available energy sources. We will define minimum energy needs for survival and growth and the interactions and structure of microbial consortia. DLD will explore these interactions in order to define interdependencies and flow between the hydrosphere, lithosphere and atmosphere.

There are many unresolved questions that DLD must overcome in order to understand subsurface interactions between microbial populations, and pools of carbon and other elements. These challenges include reconciling the apparent disconnect between empirically observed carbon and energy flux and the size of microbial populations that are sustained. This disconnect is particularly apparent in deep-sea sediment and oceanic crust, where there is evidence for microbial activity, yet energy sources are extremely limited. In contrast, energy sources appear to be abundant in deep continental environments but evidence for microbial activity is limited. It will also be necessary to develop approaches for determining the bioavailability of key chemical species and the sequestration of toxins. Isotopic characterization of chemical species and biomass represents a powerful tool for identifying sources and sinks for key chemical species. Implementation of this approach, however, will require ground-truthing of abiogenic and biochemical processes that regulate the isotopic fractionation of elements such as C, H, O, and S.

How do nutrient cycles function in the deep biosphere?

Life depends on about 30 elements. Demands for carbon, nitrogen, and phosphorous, and exchange among organisms at or near the surface and with the abiotic environment set up well-known nutrient cycles. In contrast, few reports describe how these cycles operate in the deep biosphere and the identity of the major limiting nutrient(s) is unclear. In many deep subsurface environments, dissolved phosphate and nitrogen other than elemental N_2 occur at unmeasurable levels. In other subsurface environments, at least three nitrogen species occur at relatively high concentrations. These findings imply that in some subsurface environments, factors other than traditional nutrients limit biological productivity. The accumulation of metabolic waster products may inhibit nutrient cycling. Cycles in the deep biosphere of the myriad micronutrients, including metals required for enzyme function, are complete mysteries. It is possible that in some places these cycles operate completely within the deep biosphere, whereas in others, exchange may take place with the surface biosphere. Studying cases where elemental cycles link the surface and deep biospheres will reveal the extent to which the familiar biogeochemical cycles at the surface link with the deep biosphere.

What is fundamentally different across the biotic fringe?

Different parameters must limit life under different conditions. These parameters include temperature, pressure, physical connectivity, water, and availability of carbon, energy and nutrients from rock and subsurface fluid (e.g. liquid CO_2 and hydrocarbon). Exploring the limits of life on a planetary scale through studies of continental and marine subsurface

microbiology will identify conditions that are not compatible with biological processes. At present, we do not know where the biotic fringe is or which of the many possible factors define it in subsurface environments. If we can demonstrably cross the biotic fringe into sterile conditions without contamination, then we can examine the rates of abiotic reactions, mineral and fluid compositions, and organic transformations across this profound spatial transition within the Earth.

One potentially informative and socially relevant manifestation of the biotic fringe may be hydrocarbon reservoirs. An intriguing research target in the DCO program is to understand the biogeochemical and abiotic diagenesis of deeply buried hydrocarbon (oil, gas and coal) reservoirs. Knowledge of deep carbon cycles in the continental margin hydrocarbon system is very limited. Deeply buried hydrocarbons may function as a geobiological reactor system that releases life-sustaining energy and organic nutrients. The transformation and transport mechanisms of organic compounds could influence population size, diversity, activity and functioning of the deep subsurface microbial ecosystem. The DLD will seek to identify fluxes of both thermogenically and biologically mediated carbon compounds within the deep biosphere and their contribution to the carbon budget for shallow subsurface and surface biospheres. These studies will also aid understanding of how the past events and processes (e.g., anaerobic oceanic events, tectonic erosions) affect living and fossil bio-signatures.

Understanding biogeochemical and geomicrobial characteristics of CO_2-rich, biodegraded hydrocarbon reservoirs will also provide information about the possibility of carbon sequestration into the deep subsurface environments: (CO_2 capture and sequestration in the deep subsurface is a possible mechanism for reducing atmospheric concentrations of anthropogenic green-house gas and moderating future climate change). Knowledge of (i) the deep cycling of sequestered carbon, as well as (ii) the *in situ* behavior of CO_2 and its potential physical, geochemical and biological interactions is very limited. For example, we do not understand how liquid or supercritical CO_2 spatially penetrates into various lithostratigraphic settings or how CO_2 reacts with minerals, recalcitrant organic matter and with life in the deep subsurface. Nor can we predict the impacts of long-term CO_2 storage on biogeochemical carbon cycling and the deep biosphere on different time scales.

Addressing these issues will require *in situ* and *ex situ* (bio)geochemical, geophysical, hydrogeological and microbiological studies, as well as the technological developments for deep high-pressure/temperature drilling/sampling techniques and novel experimental approaches. Three-dimensional time-transient simulations based on multiple experimental and *in situ* logging data will provide new insight into the prediction of future carbon cycling and climate change.

III. Strategies

The overarching strategy of the DLD initiative will include organizing and coordinating field sampling campaigns *in situ* measurements and experiments, and complementary carefully planned laboratory investigations. By matching these studies with modeling, the relationships between different subsurface microbial communities, their role in carbon cycling and the conditions under which they exist, can be deciphered. A first step for DLD is to locate and characterize diverse subsurface communities in a range of globally

distributed geological settings. We can make significant progress over the next 1-2 years through meta-analyses of microbial population structures in the deep biosphere. There are substantial collections of existing marine and limited continental subsurface samples within the scientific community that could be analyzed immediately with the best current technology. However, fresh samples that are quickly frozen to preserve their RNA signatures will be required to address specific hypotheses and questions. Selection of field sites should be guided by opportunities to sample key locations as identified in the global survey of pertinent locations. A community-centered ICoMM approach comparable to the International Census of Marine Microbes (ICoMM) is immediately feasible, and facilitates immediate responses to high-priority action items (rare and/or perishable samples and cruise opportunities). This approach should facilitate access and research support for individual investigators (1-10 yr) that will grow the program from the bottom up. By targeting the breadth of such settings, including the end members that consist of microbial subsurface oases and hot spots and end-members where life is impoverished and exists at the fringe of survivability, this objective will provide access to the full inventory of microbial diversity. It will lay the ground-work for guiding necessary levels of sequencing efforts in shot-gun metagenomic investigations in order to describe the metabolic potential of a subsurface microbial community. The shotgun metagenomics with increased resource requirements relative to population structure studies will capitalize on investments by the DOE in high-throughput sequencing facilities. On analysis of a sufficient number of subsurface microbial communities it will be possible to design primers and take advantage of digital qPCR technologies to economically and efficiently measure mRNA populations as proxies for metabolic activity.

An equally important objective is to match the predicted physiologies with geochemical and geophysical settings that are conducive to the survival of microbial life in the subsurface. This effort will lead to a more comprehensive understanding of the integration of deep life survival and Earth processes that involve the cycling of carbon. Progress toward a predictive understanding of biotic function in this context will require a variety of computational efforts, including (i) quantitative examination of the thermodynamic constraints on subsurface life, (ii) annotation of metabolic potential from genome sequencing and (iii) integration of metabolic network modeling and reactive transport modeling analogous to those used to describe bioremediation of uranium in a shallow subsurface system.

Studies of deep subsurface habitability must also invest in detailed chemical and isotopic characterization of subsurface environments. Although numerous analytical techniques will be necessary to accomplish this goal, a strong emphasis should be placed on high-sensitivity techniques that can resolve compositional transformations (e.g. high-resolution mass spectrometric approaches). It will also be necessary to measure energy and chemical fluxes that sustain microbial communities. Similar efforts will also be needed to measure integrated microbial and environmental fluxes. Success in this objective will require an understanding of the extent to which the microbial communities transform and transfer energy and chemicals in subsurface environments. Important examples in subsurface environments include the consumption and production of H_2, CO_2, CH_4, CO and low molecular weight hydrocarbons such as formate, and acetate. These activities could be implemented within a 1 to 10 year timeframe. Additional strategies that will be required in

the same time frame to document subsurface habitability and microbial activities include field sampling, *in situ* and laboratory measurements of physical and chemical properties, modeling, and *in situ* and laboratory experiments.

The success of these investigations will depend upon the ability to detect and identify unique trophic systems in the subsurface and the novel interactions that occur among different microbes and between microbes and minierals in subsurface environments. New isolation and DNA extraction technologies may be required to study rock-hosted biofilms and single cells subsisting in a range of subsurface settings where mineral-microbe interaction is expected to be intimate and specialized. Novel imaging technologies, such as nanoSIMS and combinatorial spectral imaging to detect multiple phylotypes in FISH experiments may improve understanding of microbial distribution and spatial aspects of major chemical transformations. Improving the sensitivity of mass spectrometry, including compound-specific isotopic analysis of Intact Polar Lipids (IPL) would provide yet another means to detect and recognize biomass in the subsurface. It will be important to collect robust data that are both directly related to these communities and to the chemical and physical conditions of the environments in which they exist. Such data need to be of high quality and pertinent to modeling approaches that can predict key biogeochemical processes in these earth systems. With the advent of high-throughput molecular biology characterization strategies, including single-cell genomis, we can improve our ability to predict *in situ* functions of these deep ecosystems. However, many high-throughput strategies require large quantities of biomass which will be difficult to recover from many target habitats. Accordingly we must also adopt approaches that use of sophisticated molecular characterization tools (e.g., metagenomic, transcriptomic, proteomic methods and IPL analysis) to study subsurface communities that occur as single cells or rock-hosted microcolonies.

IV. Site Selection

A critical component of the Deep Carbon Observatory, including the DLD, is the selection of locations for study. Although the DCO aspires to a global understanding of subsurface carbon cycling processes, resource availability dictates that this goal be addressed by intensive study of a limited number of sites. Our current knowledge of the deep biosphere relies upon studies during the past two decades of a few marine and continental sites. These field sites may be prime candidates for extending our knowledge by applying more resources to existing sites. However, a new program with a decadal or longer view and with the overarching goal of understanding subsurface carbon cycling on a global scale, including microbially mediated processes, should take a fresh look at site selection. One approach to systematic site selection would be to generate a matrix to evaluate the relative merits of a wide range of subsurface geological environments and habitat types. These various environments can then be evaluated for their potential to address the scientific goals and questions outlined by the program. A list of possible environments could begin with the matrix of Colwell and Smith (2004), which includes categories such as subduction zones, passive continental margins, cratons, stable continental plateaus, and others. This approach could be expanded, especially to include more marine environments. The matrix

would also likely include examples of specific candidate sites for each environmental category, e.g., Yellowstone as an example continental mantle hotspot. Examples of attribute scores that could rank these environments include: (1) potential for addressing goals and questions posed by multiple groups within the DCO, (2) global area extent or total global volume, (3) estimated total carbon content and fluxes, (4) potential for biological activity, as a function of fluid flux, geochemical parameters (electron donors and acceptors, etc.), (5) age ranges for rock units and for microbial ecosystem, (6) depth and geothermal gradient, (7) technical challenges to access and study, (8) current level of knowledge, (8), and (9) potential to address societal concerns. To further assure that site selection reflects programmatic goals, each of these attributes could be assigned a weighting factor. For example, an environmental category might score high for estimated carbon pool size, carbon fluxes, and volumes but low on other attributes. Such a situation might signal an important knowledge gap that should be addressed, despite the challenges.

Subsurface Environment Category	Specific sites as example(s) representing a category	Attribute 1, Weighting factor _____	Attribute 2, Weighting factor _____	Attribute 3, Weighting factor _____	Etc.	Total score.
Subduction zone						
Passive continental margin						
Craton						
Table continental plateau						
Etc.						

Table 1. Possible general scheme for site selections for the Deep Carbon Observatory.- Based upon: Colwell, F.S., and R. P. Smith. 2004. Unifying principles of the deep terrestrial

and deep marine biospheres. Pp. 355-367. In: The Subseafloor Biosphere at Mid-Ocean Ridges, Geophysical monograph Series, AGU.

V. Technologies

Sampling and *in vivo* measurements represent a daunting challenge for DLD. Deep subsurface microbiology investigations must develop means to (1) obtain high-quality, clean samples for laboratory investigations, cultivation attempts and single-cell gelnomics; (2) characterize *in situ* chemical and physical conditions and *in situ* biological activity; (3) detect the low density populations, low rates of activities, and improve detection sensitivity for biological, chemical and physical parameters; and (4) measure alternating and phase changes or conditions in the deep marine and terrestrial subsurface.

Many newly evolving approaches and technologies can be used to meet these challenges. To illustrate this point, we provide a few examples of promising technologies in the next several paragraphs.

One promising approach for obtaining "clean" samples is to improve contamination tracers and clean sampling technologies by using longer chain length perfluorocarbons as tracers that are analyzed by LC/MS. This effort can be accomplished within <1yr. Other approaches, such as developing a laser method for cutting sediment or rock will take longer to evaluate (1-3 yr). It will be important to develop drilling, monitoring, and sampling tools and materials that operate under high temperature and pressure. For example, over the next few years it should be possible to employ horizontal-to-vertical (HTV) for sample collection at downhole pressure. On a longer time horizon, it will be possible to develop downhole or *in situ* sensors for measuring H_2 and other gases or chemicals at low concentration and their stable isotope composition. Over a 5-10 year timeframe, deep subsurface microbiology should seek to establish and optimize *in situ* microbial observatories that could utilize instrumentation such as CORKs (Circulation Obviation Retrofit Kits), SCIMPI (Simple Cabled Instruments for Measuring Parameters *in situ*), and MULEs (Mobile Underground Laboratory for Experimentation).

The marine deep biosphere and continental subsurfaces represent the largest deep biomes on Earth. Both present significant but different technical challenges for obtaining samples. Currently the marine deep biosphere is sampled nearly entirely via ocean drilling program. To date, all methods developed to detect microbial life in the marine deep biosphere involve *ex situ* analysis of recovered materials from boreholes, mainly sediments. Sediment samples are analyzed for microbial biomass by extracting cells or cellular components, or by application of dyes to samples and performing cell counts. The latter methodology is standard in the industry, but represents a very time and labor-intensive effort of visually counting of fluorescent cells under a microscope. Scientists working on the continental deep biosphere have been able to work on both core samples as well as water samples, but opportunities for acquiring these samples rely entirely upon piggy backing on commercial drilling or mining operations. This is a cost effective approach, but has the distinct disadvantage of reducing the quality of the material, removing the choice of targets, making the timing of sampling somewhat unpredictable and eliminating the ability of recovering more samples from the same site at a later time.

Surface-bound bacteria predominate in all environments on Earth and pose particular challenges for researchers; dyes that bind to DNA or proteins often sorb non-specifically to organic and inorganic materials; mineral surfaces on which the microbes attach are often highly fluorescent. These problems make microbes difficult (sometimes impossible) to clearly resolve microscopically. Because of the problems associated with fluorescence of minerals and staining to detect microbial cells, some researchers resort to physically removing cells from surfaces to stain and count them separately from their matrix (e.g., Kallmeyer et al., 2009). This is a laborious process that involves some degree of cell loss and loss of information about the mineralogical context of the microbial ecology. However, this approach also has advantages: it allows enumeration of very low-density populations and it provides appropriate samples for single-cell molecular studies.

The challenges associated with detecting and quantifying microbial life in an environmental matrix have motivated the development of optical and spectroscopic methods that enable (i) detection and imaging of bacterial cells on natural and opaque surfaces and (ii) assessment of microbial density and distribution on spatial scales ranging from centimeters to microns. A method that has been in technological development for several years (specifically aimed at life detection on other planets using rovers) utilizes deep UV (<250 nm) laser-induced native fluorescence (DUV). DUV imaging targets organic components intrinsic to the cell or spore while avoiding autofluorescence interference from the substrate (Bhatia et al., 2010; "Label-free bacterial imaging with deep UV Laser induced native fluorescence" in review). DUV laser-induced native fluorescence can detect bacteria on opaque surfaces at spatial scales ranging from tens of centimeters to micrometers; hence it can detect both communities of microbes and single cells. Given exposure times of 100 μs and low excitation intensities, this technique enables rapid imaging of bacterial communities and cells without irreversible sample alteration or destruction. Importantly, DUV-induced native fluorescence is a strong signal, and hence it is possible to spatially scale this approach over three orders of magnitude while maintaining single-cell sensitivity. The microscope configuration enables a resolution of 300 nm over a 700 μm^2 area and a large area raster scanning configuration enables a resolution of 200 μm where the area is limited by 1 m x 0.5 m raster tracks. As a consequence of DUV laser developments, it is possible that a tool could be developed for wire-line deployment and mapping of the distribution and abundance of cells in a marine subsurface drill hole (bore hole) environment. A related instrument could be modified for terrestrial subsurface work.

Hand-held and robotic variations of the DUV tool have been deployed in the Arctic, Antarctica and in the deep ocean at hydrothermal vents. The lasers have been shock-and vibration-tested in accordance to launch specification for missions to Mars, and temperature-tested to below -80°C while the instruments have been operated at less then -25°C and greater than 50°C, under both low- and high-humidity conditions. An Antarctic ice-hole instrument design provides the basis for this DUV tool to fit within the borehole, provide sufficient structural support for pressures relevant to the deep biosphere, and analyze borehole walls. The current version of DUV instruments developed for NASA, Army and Navy programs will be used as the basis for the software to be used for near-real-time analysis of the biomass and low-error logging of the fluorescence data. The DUV

tool will be nearly fully autonomous, will have redundancy on single point failures (electronically switchable backup laser sources), video logging, the capability to communicate via fiber optics to the ship, and on board-archival data storage. A particular challenge unique to the borehole environment is that of varied surface topography effecting the intensity measurements and as such a focusing optic for the DUV tool will be needed.

Another tool that is used in the context of planetary exploration that is ripe for being developed into a subsurface tool is in Raman laser spectroscopy. Raman spectroscopy generally uses a monochromic source – usually in the form of a laser – for the detection of chemical and structural information of materials in solid, liquid, or gaseous states. Similar to the DUV system, the analysis is done in the native state, without disturbing the system. Tools could be developed that encompass DUV, Raman, and optical (microscopic) imaging for exploration of the deep subsurface biosphere. Furthermore, telemetry systems and software that permit immediate integration with real-time data transmission and analysis can be integrated with these tools. These advances would result in the ability to use the data immediately in guiding, for example, observatory creation, sampling strategies, and other factors that help scientists develop the very best strategies for complex subsurface exploration programs.

Molecular analyses will play a key role in defining microbial population structures and in exploring evolution of deep subsurface microbial communities. Metabolomics, lipidomics and proteomics will extend beyond genomics and enable examination of biological processes under low- to no-growth conditions, which require maintenance energy for survival. The "omic" technologies must seek improvements in extraction of biomolecules (broadly defined, including nucleic acids, proteins, lipids etc) from recalcitrant sample materials, especially rock-hosted systems, but also low-biomass sediment. Closely related challenges include the need for improved detection and monitoring of microbial processes through metagenomic surveys. Advances in high-throughput DNA sequencing have ushered in a new age of shotgun metagenomics where catalogues of randomly collected DNA sequences describe the metabolic potential of a complex microbial community. However, deep sequencing surveys of microbial population structures reveal that diversity is so great that contemporary shotgun metagenomic surveys only capture information about the most abundant taxa and fail to detect DNA and RNA sequences from low abundance organisms that constitute the "rare biosphere". Exploring the spatial and temporal distribution of deep subsurface microbes and their metabolic properties will require improvements in deep sequencing technology or the application of relevant array technology (e.g. GeoChip or digital qPCR) to collect increased numbers and longer DNA sequences.

It will also be important to operate laboratory experiments that emphasize cultivation and physiological aspects of microbial ecology that cannot presently be investigated *in situ*. We must take advantage of emerging microbiological approaches, especially cultivation/enrichment techniques for pure cultures and consortia, with a long time horizon (1-10 years) that takes the slow growth of oligotrophs into account. Our experiments will make use of novel laboratory cultivation devices, flow-through devices,

single-cell sorting and high throughput cultivation approaches, *in situ* incubators, and advanced imaging technologies that can concurrently differentiate between multiple phylotypes and link phylotypes to functional properties through stable isotope probing. These investigations into physiological processes will dramatically facilitate annotation and inferences from the omic analyses. Examples of laboratory experiments would include the (i) cultivation of enrichments for investigation of consortia, individual microbial strains or single cell isolation, and (ii) growth or maintenance experiments using retentostats.

Finally we must account for spatial and temporal heterogeneity and gradient structure of the deep biosphere in microbial surveys. For meta-analysis of global deep biosphere surveys, it will be necessary to establish dedicated and well-curated databases that can receive, organize, and integrate molecular results with contextual information from globally and locally distributed samples.

Other relevant tools include but are not limited to new dyes to better monitor surface-life contamination of deep subsurface samples, single cell isolation by laser micro-dissection, magnetic capture of cells, nano-scale visualization of stable isotope incorporation, 3D imaging of sub-cellular architecture using electron tomography, next-generation sequencing technologies, spectroscopic analyses of metal biotransformations (i.e., Mössbauer and energy dispersive X-ray absorption spectroscopy), high-pressure and high-temperature diamond-anvil cell spectroscopic analyses of cell metabolism, and high-throughput retentostat cell cultivation.

VI. Technological outputs

Enzyme function in solvents

The extreme physical conditions that exist in deep biosphere environments could select for metabolic activities that tolerate conditions close to those in supercritical and ionic fluids. These enzymes could be useful for ethanol, butanol or fatty acid fermentations for transportation and jet fuels or even photovoltaic catalytic processes.

Polymer breakdown

Aromatic biopolymers such as lignin and sporopollenin from plants and trees and aliphatic biopolymers such as algaenan from certain algae as well as geopolymers generated during diagenesis through oxygen and sulfur crosslinking of low-molecular-weight lipids make up the bulk of resistant organic matter in the geosphere. Upon catagenesis these substances will to some extent generate low-molecular-weight aromatic and aliphatic hydrocarbons including methane and CO_2 with residues becoming even more resistant. The deep biosphere may provide access to unusual enzymes, syntrophic microbial assemblages or unusual metabolic pathways leading to depolymerization of such partly altered bio- and geopolymers important for biomass conversion to fuels and detoxification of contaminated soils and sediments. Similarly, unusual lipases or proteases from extremophilic microorganisms may continue to improve industrial or consumer products such as environmentally friendly degreasers and detergents.

Sequestration of the green house gases CO_2 and CH_4

Subsurface microbes in both marine and terrestrial settings produce and consume both of these carbon compounds. Improved understanding of the controls on these biogeochemical processes is needed in order to assess the risks and promise associated with subsurface carbon sequestration (whether hydrate, liquid, or gas) as an approach for climate change remediation.

In situ conversion of hydrocarbon reservoirs

Approximately 30% of hydrocarbons in reservoirs cannot be physically removed. Stimulation of indigenous microbial communities could induce conversion of these hydrocarbons to CH4 that could then be easily extracted.

Toxic and beneficial metals

Microbe-mineral interactions are major determinants of metal toxicity. Manipulating microbe-mineral interactions are crucial for immobilizing metallic wastes such as radionuclides or toxic heavy metals such as cadmium, chromium or mercury. In addition, some microbes synthesize magnetic nanoparticles of specific dimensions that could be used in nanotechnology, including photovoltaic production.

VII. Science Coordination

Given the scope of the Deep Carbon Observatory and the DLD initiative, coordination and prioritization are particularly important. This coordination must be done among marine, continental and terrestrial environments, across disciplines (geology, biology, chemistry, and engineering). It must include experimental, observational, and theoretical approaches. Coordination is also needed to develop resources infrastructure and to respond effectively to funding opportunities. For these reasons and to establish synergy and avoid duplication, it is important to establish a science coordination framework for the Deep Life Directorate. This framework could be modeled after the very successful Sloan-sponsored International Census of Marine Microbes initiative, or NASA's mission Scientific Definition Team or some other model of a successful international multidisciplinary scientific program. A key element of the framework will be the multidisciplinary network of researchers. The network will conduct a variety of functions, including operating community web resources, and organizing specialized workshop and annual meetings. One immediate need for coordination is in the area of site selection as outline above. Another function could be funding of relatively short-term proof-of-concept projects as listed in section VII below.

VIII. Potential pilot studies that could be accomplished in one to two years

1. Improved taxonomic characterization of deep life

Using available deep subsurface samples (i.e., from past or near term International Continental Drilling Program and Integrated Ocean Drilling Program operations), the extent of taxonomic novelty among microbes (Bacteria, Archaea, Eukarya) present in the deep biosphere will be better constrained by obtaining ribosomal RNA gene sequence

information and IPL analyses. One model that could be adopted in selecting samples for analysis is that adopted by the International Census of Marine Microbes.

2. Single-cell isolation and preliminary characterization

Deep subsurface life exists mostly on mineral surfaces and techniques are needed for cell removal and characterization. Single-cell isolation techniques such as laser microdissection and fluorescently activated cell sorting will be used to isolate single cells. Subsequent to single cell isolation, single-cell genome sequencing will be optimized and the extent of genome coverage and its functional significance will be assessed.

3. Cultivation under deep biosphere conditions

High-throughput cultivation techniques such as microdroplet encapsulation and incubation in high temperature, high-pressure incubators will be used to attempt to culture important representatives of the deep biosphere.

4. Additional Pilot Projects

The scientific community will be encouraged to submit mini-proposals to a DLD Review Committee for funding a select number of additional small-scale short term projects.

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