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Subsurface Biospheres

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Executive Summary

A better understanding of the Earth's deep biosphere is essential because it can serve as a model for life on other planets and it is a critical component of the Earth's biogeochemical cycles. This proposal is aimed at gaining a fundamental understanding of the life in deeply buried marine sediments. We propose to conduct an interdisciplinary set of projects that takes advantage of our considerable expertise in marine sedimentary microbiology, sedimentary biogeochemistry and deep ocean drilling. Our objectives are to understand the subsurface microbial ecosystems of marine sediments, their role in Earth's biogeochemical cycles, and their relevance to the search for life on other planets. We will focus on three major projects.

The first project will explore the taxonomic composition, metabolic activity and geochemical consequences of buried microbial ecosystems in marine sediments with widely different physical and chemical characteristics. Environments will include hot, deeply buried anoxic sediments where life may exist independently of the photosynthesis-based ecosystem at Earth's surface, and old, deeply buried sediments where life may be limited by the availability of electron donors or key nutrients. The ecosystems of such subsurface habitats are potentially representative of the ecosystems that may exist on other planets.

The second project will document the nature, extent and perturbation-sensitivity of microbial activity in marine sediments and the effect of that activity on Earth's biogeochemical cycles of various chemical species, particularly sulfate and methane. Sulfate, the second most abundant anion in seawater, is the dominant terminal electron acceptor in marine sediments. Methane is climatically active and marine methane deposits comprise the largest untapped hydrocarbon reservoir on Earth. The biogeochemical cycles of these and other chemical species are potentially sensitive to centennial to millenial changes in Earth's surface temperatures, nutrient fluxes to the ocean, and the structure of marine ecosystems.

The third project will focus on identifying signatures of present and past microbial processes in Earth's subsurface as a guide to predicting such signatures in extraterrestrial subsurface environments. The molecular-isotopic based data that will be generated as part of this project, will complement the objectives of the first two projects. For example, the study of biomarkers produced by deeply buried methanotrophic microbes will give us an indication of both past and present activity of this specific taxonomic group.

We have already obtained extensive sample sets from deep Pacific drill-holes for analysis. We developed procedures for identifying contamination of samples, spearheaded the drive to instrument a microbiology laboratory on the Ocean Drilling Program (ODP) drill-ship *JOIDES Resolution*, and compiled relevant global geochemical and physical databases. We proposed and will participate in the first ODP Leg (Leg 201) dedicated to Subsurface Biosphere research scheduled for 2002. These and other developments provide the framework for collecting, analyzing and interpreting microbiological, biogeochemical and physical data from a wide range of deep subsurface samples. This framework leaves us poised to greatly advance Subsurface Biosphere objectives.

Our approach is fundamentally interdisciplinary and we will use advanced techniques from various fields, including microbiology, molecular biology, organic and inorganic biogeochemistry, isotope geochemistry, large data set integration and computational modeling. The work will be field-, laboratory-, and model-based. In order to effectively accomplish the program's goals, we will build teams of investigators, post-doctoral researchers, graduate students and select undergraduate students with diverse skills.

We are fully committed to implementing the collaborative and networking concepts of the NASA Astrobiology Institute.

Integrated Research Plan

The research plan of this proposal is divided into three sections. Each section is focused on one of the proposal's three major projects. The first project will explore the taxonomic composition, metabolic activity and geochemical consequences of deeply buried microbial ecosystems in marine sediments with widely different physical and chemical characteristics. The primary goal of this project will be to identify the nature of individual taxa, microbial communities and limits to subsurface life under such conditions as high burial temperatures, and extremely low availability of electron donors or nutrients. The second project will document the nature and extent of marine-sedimentary microbial control of Earth's cycles of sulfur, nitrogen, and carbon (particularly methane). The primary goal of this project will be to understand how the dominant microbial processes in deep-sea sediments control (1) the flux of sulfate (the second most abundant anion in seawater) from the ocean, and (2) the creation and destruction of marine sedimentary methane deposits (the world's largest untapped fossil fuel reservoir and an extremely large greenhouse-gas source in close proximity to the Earth's ocean and atmosphere). The third project will focus on identifying organic and inorganic signatures of present and past microbial processes in Earth's subsurface. The primary goal of this project will be to help inform the search for microbial signatures in extraterrestrial subsurface environments and to add complementary molecular-isotopic information to resolution of objectives stated in projects one and two.

The first and third projects "address the general issue of the nature and/or search for habitable worlds outside the solar system, as well as methods for detecting the presence of life thereon" [CAN preference (a)}. The second project "explores the role of ecosystem processes (e.g., large-scale episodic disturbance, nutrient dynamics and trace gas production, biogeographic change) in the global carbon cycle and other biogeochemical cycling processes on centennial to millennial time scales with a goal of contributing understanding that could advance our ability to predict future conditions" [CAN preference (b)].

Background.

Advances in microbial monitoring and identification, and increasing knowledge about the metabolic capacities of microorganisms provide a solid basis for addressing fundamentally important questions about deep marine subsurface microbiota. Significant progress has already been made in extending our knowledge about the diversity of microbially inhabitable subsurface environments and microbial types. Cruise-of-opportunity studies have documented the existence of a widespread deep biosphere in marine sediments (*e.g.*, Parkes *et al.*, 1994). Recent studies have pushed the maximum burial depth of that deep biosphere to more than 807 meters below seafloor (Ocean Drilling Program Leg 180 Preliminary Report).

Recent microbiological investigations of sediments in the deepest Mariana Trench, close to 11000 m depth, have made clear that depth and hydrostatic pressure *per se* do not preclude vigorous and highly diverse microbial life (Tamegai et al., 1997; Takami et al., 1997). The finding of microbial "burrowing" structures in volcanic glass (Fisk et al., 1998), and the isolation of a new strain of the thermophilic bacterial genus *Thermus* from 3.5 km deep, geothermally heated ore of a South African gold mine (Kieft et al. 1998), indicate that both deep sediments and oceanic crust, including their geothermally heated portions, provide potential habitats for microbial life.

Meanwhile, novel forms of bacterial metabolism with subsurface potential are being discovered. As an example, systematic studies of sulfate- and sulfur-reducing bacteria and archaea have shown that many representatives of these organisms, among them an astonishing set of phylogenetically very deep lineages (Vargas et al. 1998), share an unexpected capacity for Fe-III reduction (Lonergan et al. 1996). Respiration of metal oxides could allow bacteria and archaea a respiratory

mode of life even after other electron acceptors, including oxidized sulfur species, become extinguished with increasing distance from the oxidized biosphere. Given such novel metabolisms, it is likely that temperature, not lack of respiration substrates or carbon, will pose the ultimate limits to life in the deep subsurface. The record growth temperature for a microbial culture in the laboratory is currently 113°C, for the microaerophilic and denitrifying, autotrophic Crenarchaeon *Pyrolobus fumarius* (Blöchl et al. 1997). The success of this culture indicates that temperature regimes between 110 and 120°C are still compatible with microbial life.

The study of the deep microbial biosphere is becoming an essential and interdisciplinary research focus of the geological and biological sciences. The important questions are as varied as the viewpoints. Geochemistry: What are the relationships between microbial activity, pore-water chemistry, and post-depositional alteration of marine sediments? What can be learned from the isotopic record of microbially mediated processes in deep sediments? What are the organic-biomarker and isotopic signatures of deeply buried microbial activity? Microbial physiology: What are the physiological adaptations of microorganisms under the various extreme conditions in the subsurface? Genetics and Evolution: Are there new and possibly ancient types of bacteria to be found? And from the most far-reaching perspective, Astrobiology: If some extremophilic bacteria are known to survive in deep sediments, in hot or cold crust, could similar life forms also survive or thrive on other planets? Can we recognize unprededented biomarkers in the earth's crust in order to be prepared for elusive evidence of life in non-terrestrial samples?

First project: Taxonomic composition, metabolic activity and geochemical consequences of microbial ecosystems in marine sediments with widely different physical and chemical characteristics.

Sediment chemistry, microbial activity and community composition.

The primary goal of our research on sediment chemistry and microbial communities will be to document the limits to life imposed by the availability of electron donors and electron acceptors. The availability of electron donors (i.e., H_2, CH_4, organic matter) may ultimately control the presence or absence of life in some deeply buried sediments. In contrast, the presence or absence of specific electron acceptors (such as Fe(III)-oxides, Mn(IV)-oxides, NO_3^-, SO_4^{2-}) may control taxonomic composition and the nature of microbial activity, but is not likely to ultimately determine whether life is present or absent, because the electron acceptor CO_2 is always present in marine sedimentary porewaters.

Chemical and sedimentary data from Ocean Drilling Program (ODP) and Deep Sea Drilling Project (DSDP) drill holes can be used to identify regions and burial depths where this goal can be most readily met (Fig. 1). For example, geographic regions and stratigraphic (burial depth) intervals of extremely low microbial activity can be predicted from the vertical profiles of dissolved sulfate and methane concentrations in sedimentary porewaters of ODP and DSDP drill holes.

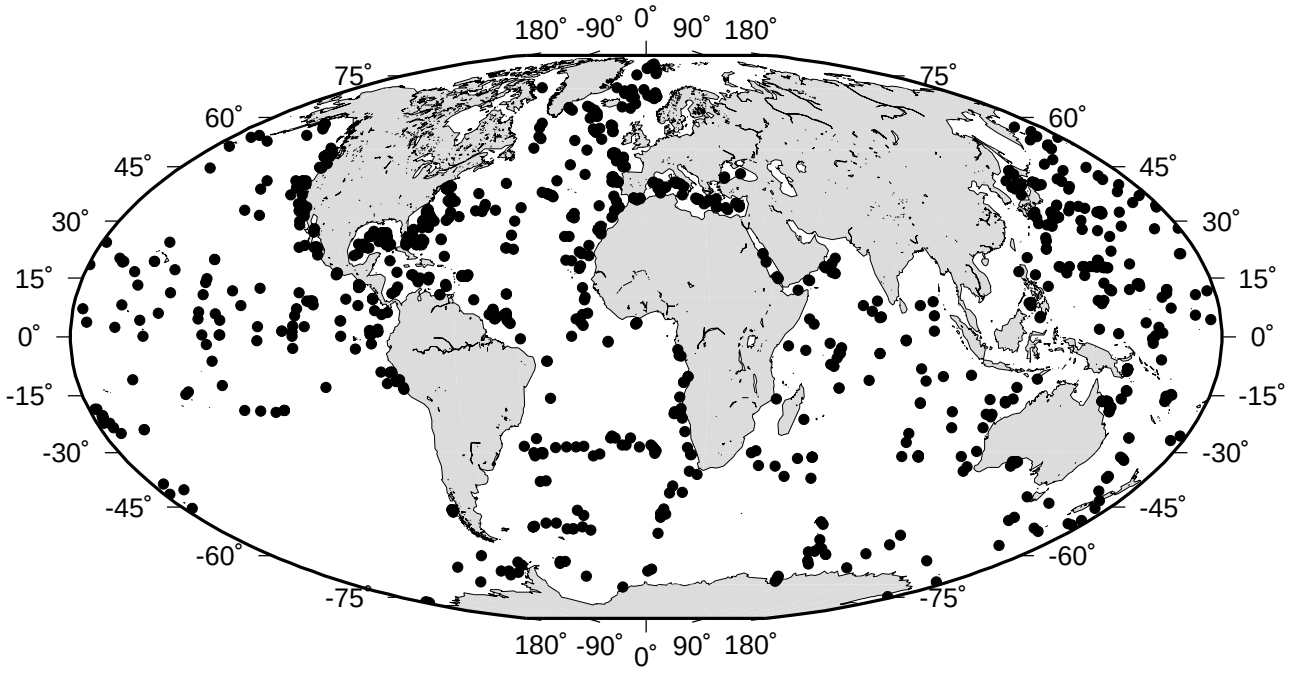

Figure 1. Global map indicating all Ocean Drilling Program (ODP) and Deep Sea Drilling Program (DSDP) sites.

We can infer that sulfate reduction and methanogenesis are the dominant microbial processes in deeply buried marine sediments, because (1) in deep-sea porewaters, initial concentrations of dissolved sulfate are nearly 50 times as great as concentrations of all other electron acceptors combined, (2) depending on the flux of organic carbon to the sediments, the other external electron acceptors typically disappear within the first few centimeters to tens of meters of burial depth (Froelich et al., 1979), and (3) when all external electron acceptors have been reduced, fermentation and methanogenesis are the only known avenues of microbial activity that remain.

Typical downhole profiles of dissolved sulfate and methane are presented in figure 2 a & b. The concentration of dissolved sulfate in sediments results from the balance between (1) diffusion of sulfate from the overlying ocean and (2) reduction of sulfate by microbial activity in the sediments. Because the sulfate diffuses down from the overlying ocean, peak sulfate concentrations typically occur at the sediment-water interface. In contrast, methane is produced by buried microbes and typically diffuses up toward more shallowly buried sediments, where it is consumed by sulfate-reducing microbial consortia (e.g., Hinrichs et al., 1999; D'Hondt et al., 2000). Consequently, peak methane concentrations usually occur at some depth below the seafloor.

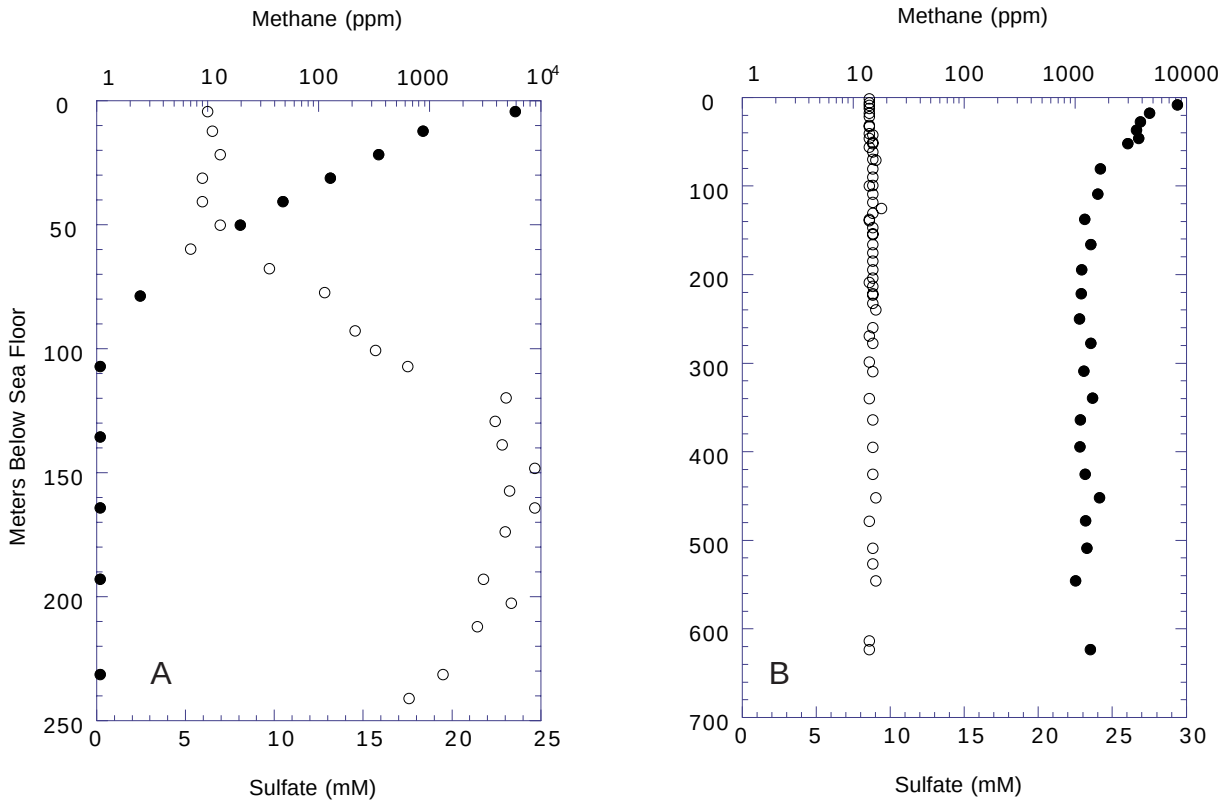

Figure 2a. Profiles of downhole methane and sulfate concentrations at a) a typical coastal site and b). a typical open ocean site.

In open-ocean regions, dissolved sulfate profiles (and where present, methane profiles) typically reach a stable non-zero value within the first few meters to few tens of meters below the seafloor (D'Hondt et al., 2000) (Fig. 3 a & b). Below this burial depth, any effect of microbial activity on dissolved sulfate profiles is indistinguishable from analytical uncertainty in the sulfate measurements. Most deep-sea sediments that are characterized by such stable downhole sulfate profiles contain very low concentrations of organic carbon and are relatively cool. Hence, life in these sediments is likely to be limited by electron donor availability. The nature of microbial assemblages in these intervals of stable sulfate concentrations remains largely unknown. Are they unique communities of slow-living microbes with extraordinarily low nutrition requirements? Or do they merely represent a dead or relatively inactive ecosystem whose microbial components exhibit greater levels of activity in organic-rich sediments at shallower sediment depths and in other regions of the world ocean? If slow-living, dead and inactive microbial assemblages all occur in deep-sea sediments, under what physical and chemical conditions does each type of assemblage occur?

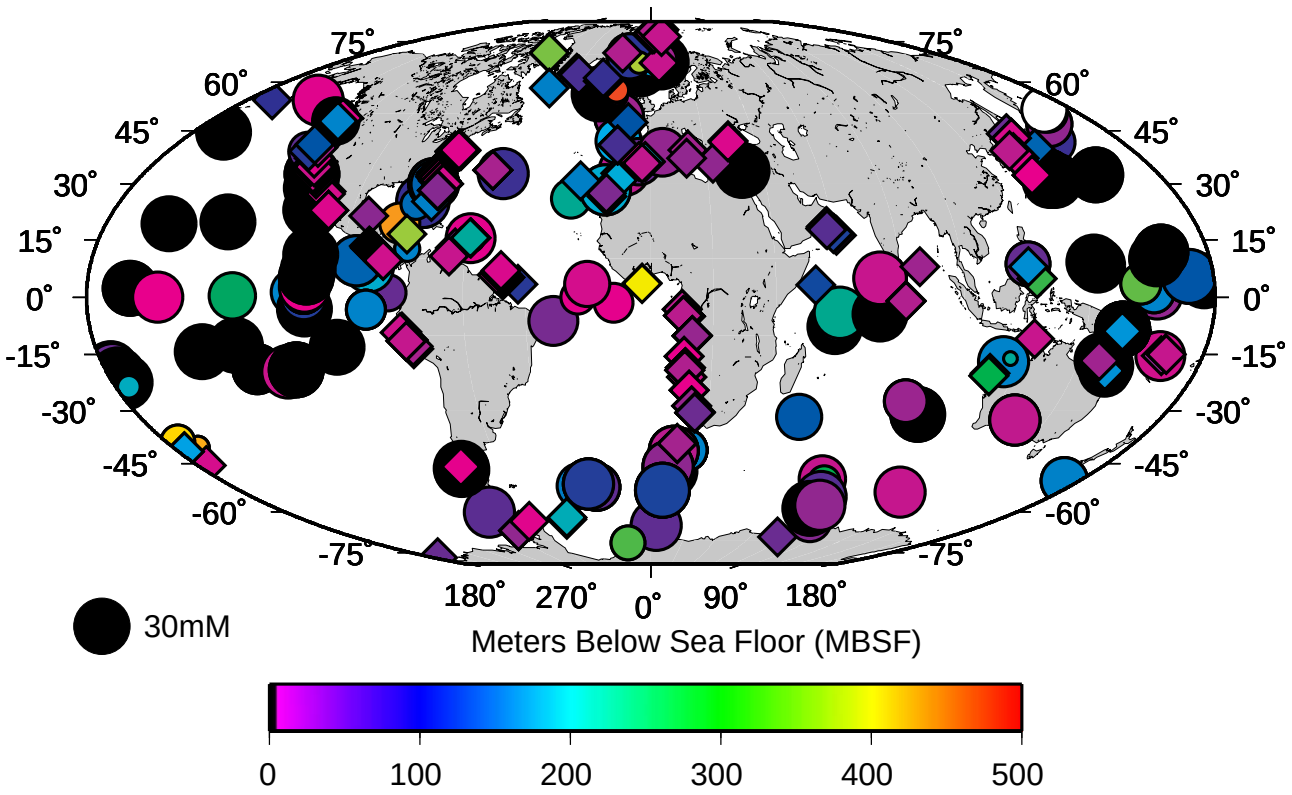

Figure 3a. Global map of dissolved sulfate concentrations in deep sea sediments. Color of symbol indicates the sediment depth at which dissolved sulfate concentrations reach a stable value. Size of dot indicates sulfate concentration at the depth of stabilization. Diamonds mark sites where dissolved sulfate disappears at the depth of stabilization.

Characterization of these subsurface microbial communities and the sedimentary environments in which they occur will have a host of benefits for our understanding of subsurface life on Earth and other planetary bodies. Most obviously, it will provide us with models for estimating (1) the lowest levels of electron donor concentrations that can sustain life, (2) how long microbial communities can remain active and still be reactivated, and (3) what kinds of metabolic and other cellular adaptations, if any, are required to sustain life at extremely low concentrations of electron donor or nutrients.

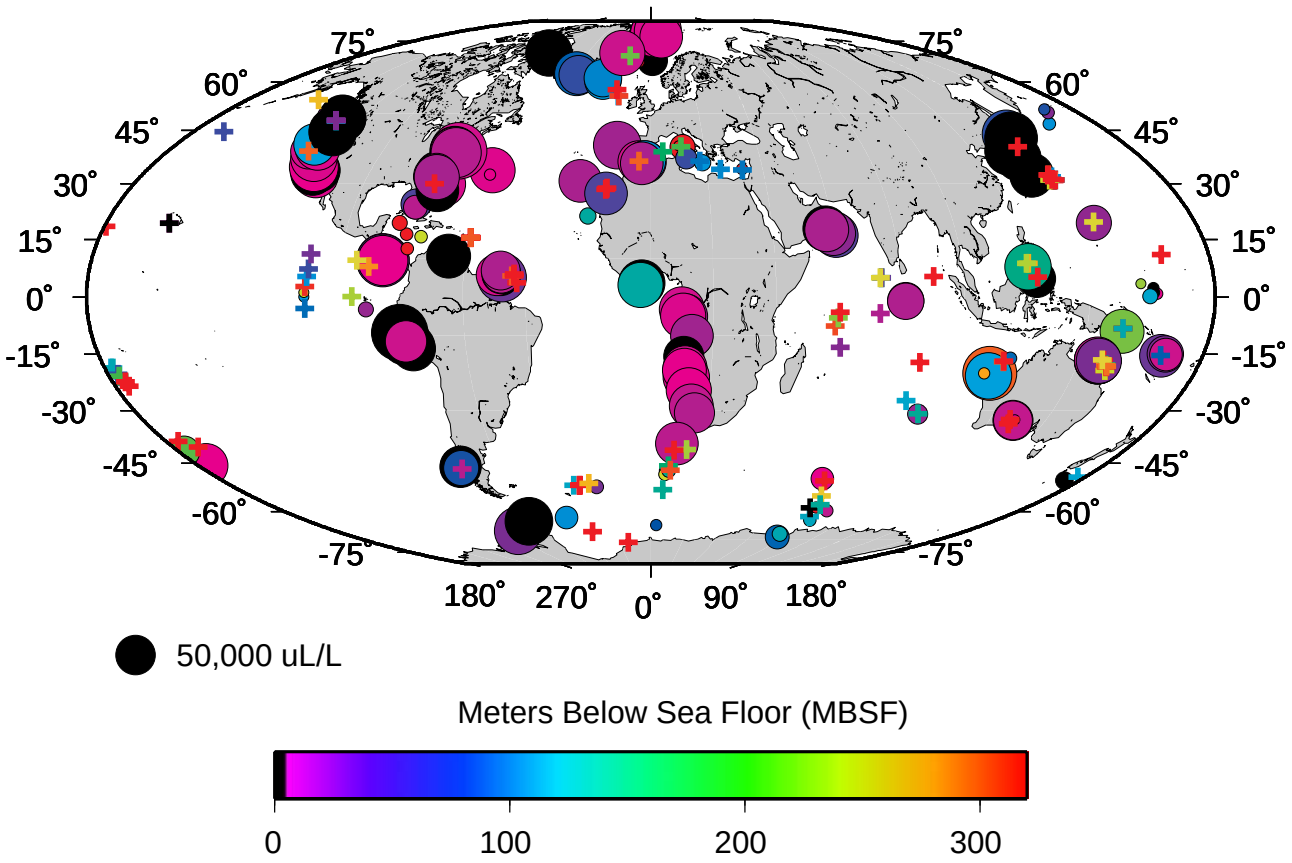

Figure 3b. Global map of methane concentrations in deep sea sediments. Color of symbol indicates the minimum sediment depth at which methane rises above the detection limit. Size of dot indicates the peak subsurface methane concentration at each site. Crosses mark sites with no subsurface methane.

Dissolved nitrogen species in subsurface ecosystems.—

In addition to sulfate and methane a well-developed understanding of the ecology of the deep biosphere requires quantifying the role of nitrogen. Traditionally, the study of sedimentary microbial activity has focussed on net dissimilatory activity, in effect the unidirectional decomposition of detrital organic compounds supplied from the overlying water column. However, there are a number of observations that point to the significance of the uptake, assimilation and recycling of material through the buried microbial biomass. The significance is twofold; it is directly related to the controls on the activity and structure of sedimentary ecosystems and the global geochemical cycle of nitrogen.

The existence of an active microbial nitrogen cycle is pointed to by two observations, the existence of deeply buried bacteria and sedimentary pore fluid ammonium profiles. Clearly, if bacteria are active they require reduced nitrogen or must actively fix N_2. In shallowly buried anaerobic sediments there is abundant dissolved ammonium and microbial activity is associated with a net conversion of organic nitrogen to dissolved ammonium. However, in deeply buried sediments, microbial activity appears to be a net sink of dissolved ammonium. At many ODP sites (e.g. Site 1173, fig. 4) there is a mid-core maximum in dissolved ammonium.

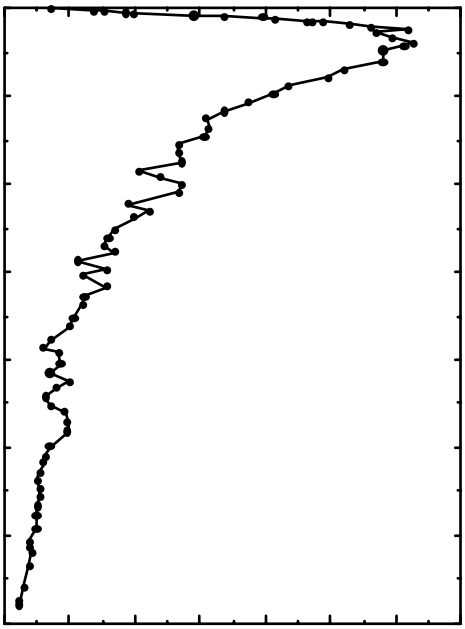

Figure 4. Concentration of dissolved ammonium as a function of depth, ODP Site 1173, Nankai Trough.

This decrease with depth has often been attributed to inorganic ion exchange reactions because deep microbial activity was not previously recognized and ammonium is not involved in significant authigenic mineral formation. We, however, argue that net uptake via ion exchange is unlikely as it is not consistent with the profiles of the major ions. Our alternative hypothesis is that there is active microbial uptake of ammonium in the depth interval of the ammonium decrease and that the decrease is effectively due to the sequestering of nitrogen into refractory compounds synthesized by microbes. For example, Tanoue et al. (1995) showed the ubiquity of a trans-outer-membrane protein (porin P) in vertical profiles in the water column. This protein, produced by Gram negative bacteria, is well know to be resistant to degradation by protease (Lugtenberg and Alphen, 1983). This sequestration of nitrogen appears to be effective enough to drive deep pore fluid ammonium to nearly zero concentrations. This depletion may lead to the nitrogen limitation of deeper microbial activity and require deeper metabolic activity to be dependent on nitrogen fixation. In this regard, it is interesting to note the universal presence of nitrogen fixing enzymes in sulfate reducing bacteria has been considered a "mystery" as the possibility of microbial activity in deeply buried ammonium depleted sediments was not recognized (Fenchel et al., 1998).

A complete understanding of the dynamics of nitrogen cycling in these deep food webs will require quantifying gross rates of 1) ammonium production from detrital organic matter, 2) assimilation of ammonium into both labile and refractory organic nitrogen compounds, 3) recycling of microbially produced organic nitrogen and 4) nitrogen fixation. These effectively are the same issues being addressed by scientist studying the role of microbes in the oceanic water column and soil food webs. To quantify these rates, nitrogen isotopes ($^{15}N/^{14}N$) have been used in two ways. Tracer enrichment studies have been conducted and natural abundance variations are measured and modeled (e.g., Tietema and Vandam, 1995; Sigman et al., 1999). Our calculations indicate that the turnover time of ammonium is too long to allow for tracer studies using stable isotopes in deeply buried sediments. However, a combination of net fluxes derived from diffusion modeling of pore fluid profiles and measured variations in the nitrogen isotopic composition of ammonia should allow for the determination of the individual fluxes that are components of the deep biosphere nitrogen cycle.

The presence or absence of subsurface life on Earth and other planetary bodies is commonly recognized to depend on the presence or absence of water, electron donors and electron acceptors. The potential role of nutrients in limiting subsurface life—and in shaping the evolutionary history of subsurface cellular machinery—is more rarely considered. Studies of the nitrogen cycle in deeply buried marine sediments and their microbial communities will document the effect of nutrient availability on subsurface life help to shape predictive models for the nature of subsurface life on other planetary bodies.

Dissolved hydrogen in subsurface ecosystems.—

Dissolved hydrogen plays a critical and unique role in the biology of deeply buried sediments. It is involved in almost every transformation catalyzed by anaerobic bacteria. Hydrogen is an end product of fermentation and it is well documented that hydrogen consumers are necessary for the survival of hydrogen producers via interspecies hydrogen transfer. Bacteria that perform the complete oxidation and bioremineralization of organic compounds to CO_2, in shorthand, terminal oxidizers, consume hydrogen as electron donor for their energy metabolism. In this way, dissolved hydrogen concentrations represent a balance between microbial production and consumption of hydrogen, with very fast hydrogen turnover times and low steady-state concentrations (Jørgensen, 1989). Recent work on shallow near-shore sediments supports the hypothesis that terminal oxidizers that use a specific electron acceptor regulate hydrogen concentrations to gain a competitive advantage over bacteria that utilize other electron acceptors (Hoehler et al., 1998). For example, sulfate reducers regulate hydrogen to a concentration that is too low for it to be of use to methanogens.

In addition to this role in influencing community structure via competition, hydrogen has also been suggested as a substrate that may support chemolithotrophic communities, a process that has been identified as clearly relevant to the possibility of life on other planets. However, despite its clear biogeochemical importance, previous published studies of sedimentary hydrogen have been limited to shallow, near-shore sediments; there are no published data on the distribution and dynamics of hydrogen in deeply buried sediments. From an astrobiology perspective, this lack of information must be remedied as soon as possible, because the most deeply buried sediments and rocky crust are the farthest removed from the photosynthetic world—and the likeliest model for life-bearing subsurface environments on other planetary bodies.

During ODP Leg 190 we established and tested procedures for the measurement of hydrogen in deep-sea sediments recovered on the ODP drillship *JOIDES Resolution* (Spivack et al., 2000). In addition, we analyzed a limited number of sediment samples covering a depth range of a few meters below the seafloor (mbsf) to 1,100 mbsf and spanning an estimated temperature range of 3 to 140°C. Hydrogen concentrations in the upper sections of the sediment columns were similar to those in shallow near shore sediments suggesting similar microbial control as argued for in near shore sediments. Concentrations generally increased with *in situ* temperature as predicted by thermodynamic calculations. Preliminary results from sterilization experiments indicate the possibility of significant abiological hydrogen production at higher temperatures. However fundamental questions remain concerning the relationship between hydrogen, pore fluid composition (e.g., total dissolved organic carbon, specific organic compounds such as acetate and formate, and electron acceptors), sediment composition (e.g., organic carbon content and iron abundance), temperature and microbial community structure.

Effect of sedimentary temperature on microbial community composition and activity.

The primary goal of our research on sedimentary temperature and microbial communities will be to (1) document the nature of subsurface life at a wide range of ambient temperatures, and (2) identify sedimentary microbial communities that are fully independent of photosynthesis. The latter communities may serve as a reasonable model for the subsurface life that may exist on other planetary bodies.

In approaching these goals, we expect to address a number of closely related questions. What is the effect of temperature on the phylogenetic composition of the deep-sea sedimentary biosphere? Where might the upper thermal bound to life be encountered in deep-sea sediments? What is the

importance of temperature for independence from photosynthesis of the deep-sea sedimentary biosphere? For example, is sustenance by abiological H_2 production an effective growth strategy in or near hot (deeply buried) anaerobic sediments? Are these hot sedimentary environments reasonable analogues for environments on extraterrestrial bodies—or for environments inhabited by Earth's early biosphere?

Except in hydrothermal vents, seafloor temperature approximates that of the overlying deep sea (about 1°C in most of the ocean). The diffusive thermal balance between the cold seafloor and geothermal heat below causes sedimentary temperatures to increase with burial depth. This can be expected to affect microbial activity and community composition in two ways. First, temperature sets a physical boundary to the kinds of microbes that can survive at increasing burial depths. The ultimate high-temperature limit to buried microbial communities is the temperature above which no microbes can survive.

Microbial studies of the surface Earth demonstrate that normal marine bacteria (mesophiles) grow best at ca. 20 - 25°C and do not survive prolonged exposure to 40°C or higher (ZoBell, 1946). Normal thermophiles, which include numerous gram-positive spore-forming anaerobes, fermenters, and sulfate- and sulfur reducers, exhibit a temperature range of ca. 50 to 70°C (examples in Hippe et al., 1992; Widdel, 1992). Most known hyperthermophilic Archaea have an optimal temperature range of 85 to 95°C (Blöchl et al., 1995). At present, the upper thermal limit to life appears likely to be in the range of 110 to 120°C; the most heat-tolerant microbes that are presently known were isolated from hydrothermal vent chimneys and can grow and divide at 113°C (Blöchl et al., 1997).

These generalizations provide a basis for predicting the composition of subsurface microbial communities at different sedimentary temperatures. For example, most sediment in the world's ocean basins is cooler than 30°C (Fig. 5) (Rutherford and D'Hondt, unpublished). Consequently, it is likely, but largely untested, that the microbes in most deep-sea sediments are not particularly adapted for thermal extremes. The relative coolness of most deep-sea sediments largely stems from their relative thinness; throughout most of the ocean, the 30°C isotherm is actually in the oceanic crust that underlies the sediments. However, much higher temperatures occur within sediments in regions of high geothermal heat flux, such as active plate tectonic margins, and relatively thick sediment cover, such as near continental margins. For example, the 120°C isotherm occurs in the first kilometer of burial depth in many regions of the world ocean (Fig. 6) (Rutherford and D'Hondt, unpublished). Although the taxonomic composition and metabolic activity of the highest-temperature deep-subsurface microbial communities are presently unknown, such communities are likely to occur close to the burial depth of the 120°C isotherm in deep-sea sediments.

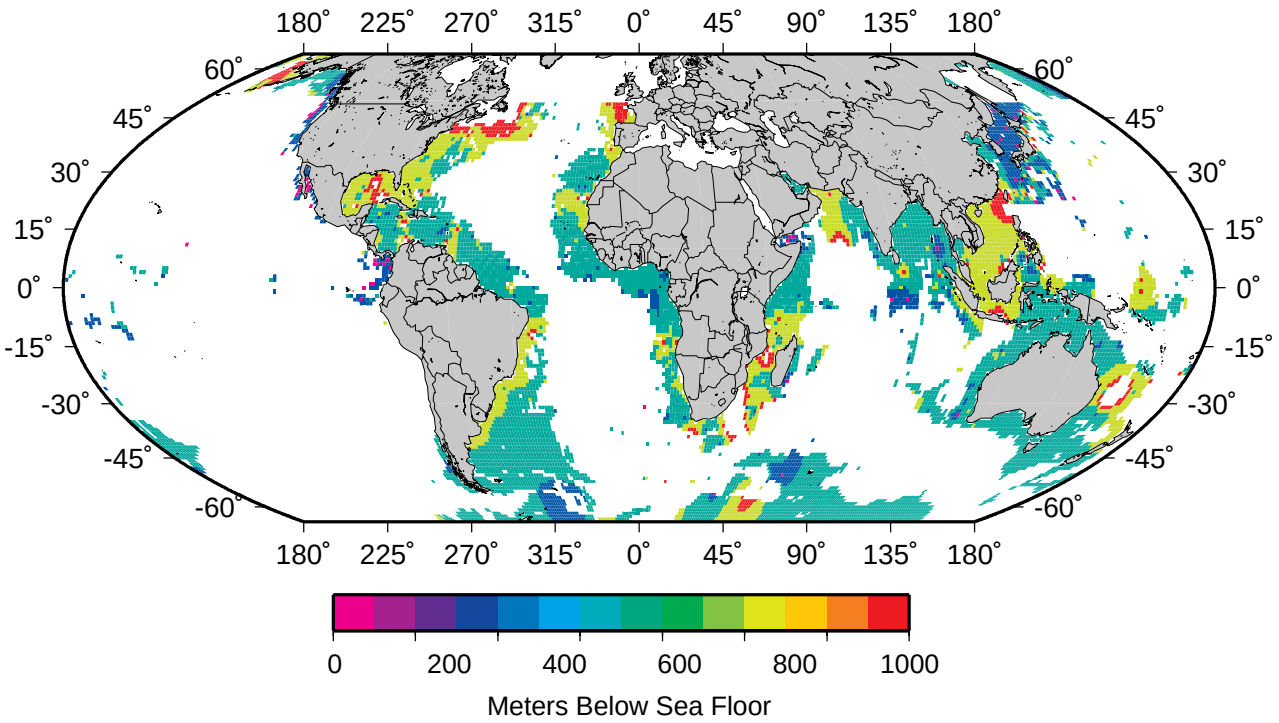

Figure 5. Global map indicating the depth (meters below seafloor) of the 30 °C isotherm in ocean sediments. In white regions, the isotherm typically occurs within the underlying crust (rather than the sediments).

The second way that burial temperature can affect microbial communities is by changing the availability of electron donors. Increased temperature can increase electron donor availability by enhancing degradation of buried organic matter (Wellsbury et al., 1997) and by enhancing the reaction rates and equilibrium concentrations of inorganic electron donors, such as H_2. These two processes have different implications for the nature of subsurface microbial communities. The first process, enhanced degradation of refractory organic carbon, may not be truly independent of photosynthesis, since most of the organic carbon in deep-sea sediments was originally synthesized in the overlying surface ocean. However, the second process, enhancement of the abiological release of electron donors, may support subsurface microbial communities that are fully independent of photosynthesis. In at least one respect, these hot subsurface communities are even farther removed from the photosynthetic world than hydrothermal vent communities, which have access to highly oxidizing electron acceptors, such as dissolved oxygen, nitrate and sulfate (these electron acceptors are relatively abundant in seawater because high oxygen concentrations are maintained in Earth's atmosphere by photosynthesis). Consequently, the anaerobic chemolithotrophic communities of hot deeply buried sediments may provide a reasonable terrestrial model for (1) Earth's early life, and (2) the subsurface life that may exist on other planets and planetoids.

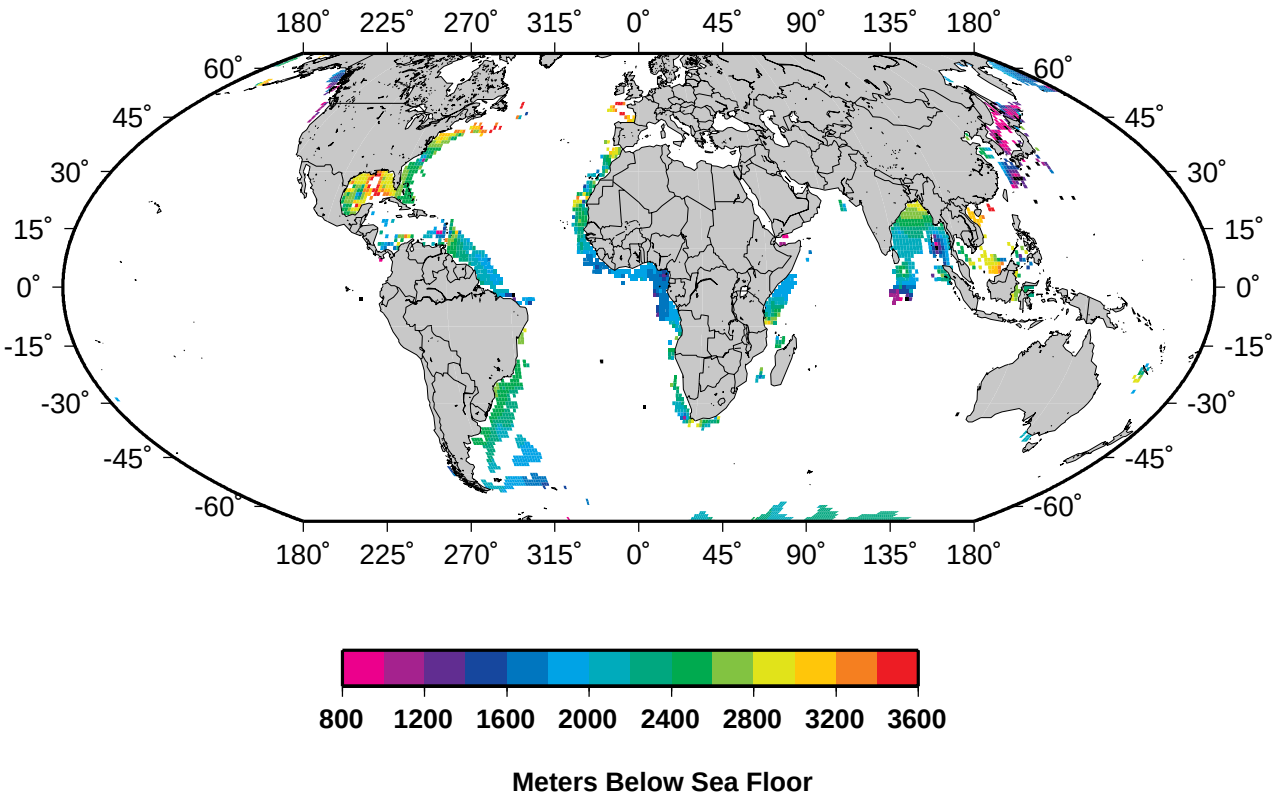

Figure 6. Global map indicating the depth (meters below seafloor) of the 120 °C isotherm in ocean sediments. In white regions, the isotherm typically occurs within the underlying crust (rather than the sediments).

Second project: Nature and extent of marine-sedimentary microbial control of Earth's cycles of sulfur and carbon (particularly methane).

In addressing this theme, we propose to focus primarily on the microbial communities responsible for sulfate reduction, methanogenesis, anaerobic methanotrophy, and the sequestration of nitrogen and phosphorus in deep-sea sediments. Numerous studies suggest that these sedimentary microbial processes are respectively responsible for (1) the dominant flux of sulfate (the second most abundant anion in seawater) from the ocean, (2) the creation of the world's largest methane deposits (which incidentally constitute the world's largest untapped fossil fuel reservoir), (3) the ongoing natural destruction of those methane deposits by microbial methanotrophy, and (4) the dominant fluxes of bioavailable nitrogen and phosphorus from the oceans. Consequently, focusing on these processes will allow us to better understand the dominant microbial processes in deep-sea sediments, the ways in which those processes affect the surface earth, and the sensitivity of those processes to changes in Earth's surface conditions. Conditions to which those processes may be sensitive include: marine ecosystem structure, sea-surface temperature, deep-ocean temperature and fluxes of dissolved nutrients to the surface ocean.

Nature of the relevant (sulfate-reducing, methanogenic and methanotrophic) microbial consortia.

The processes of sulfate reduction, methanogenesis, and methane oxidation in marine sediments are tied to each other in several ways; together, they perform the complete bioremineralization of organic compounds in the seabed. Organic compounds are oxidized by sulfate reducers as long as sulfate is available. In deep layers of marine sediments where sulfate is depleted, the terminal steps of carbon remineralization are taken over by methanogens, which can utilize a variety of carbon sources; besides CO_2 and H_2 also acetate, ethanol, formate, methanol and methylamines are metabolized. Methyl compounds such as methanol or methylamines are preferentially utilized by genera within the Methanosarcinales (*Methanosarcina*, *Methanosaeta*, *Methanococcoides*, *Methanomethylovorans*, and *Methanolobus*) which possess methyl-disproportionating pathways for concomitant oxidation of methyl carbon to CO_2 and reduction to methane (Whitman et al., 1992). These methanogens may be viewed as preadapted to anaerobic methane oxidation to CO_2, since they lack only a methane-activating enzyme system to bridge the gap between methane and methyl group oxidation (Hoehler and Alperin, 1996). While the responsible microorganisms have not yet been cultured, the process of anaerobic methane oxidation dominates sulfate reduction / methanogenesis transition layers in marine sediments on a global scale. Here, sulfate reducing bacteria occur in association with methane-oxidizing organisms and oxidize methane stoichiometrically, most likely by remineralization and removal of acetate and hydrogen, the presumed intermediates of methane oxidation (Valentine and Reeburgh, 2000). Understanding the composition of sulfate-reducing, methanogenic, and methane-oxidizing microbial communities in marine sediments and in the subsurface is essential to grasp the microbial physiology and therefore the effective environmental controls behind these pervasive processes that shape the anaerobic portion of Earth's carbon cycle. Here we give a summary of the relevant microbial communities in marine sediments and in the subsurface.

Sulfate-reducing bacteria extend deeply into marine sediments. Bacterial sulfate reduction is the dominant terminal anaerobic oxidation process in marine sediments, and in coastal sediments this process can be responsible for more than 50% of the organic matter degradation (Jørgensen, 1982). The importance of sulfate reduction decreases in deeper-water sediments, where the diagenetic zones are greatly extended (Froelich et al., 1979; Jørgensen, 1983) and sulfate can be present to a depth of several hundred meters in some pelagic sediments (Canfield, 1991; D'Hondt et al., 2000) (Figs. 2, 3). Although the peak activities of sulfate reduction were always found within the uppermost meters of the sediment column, the process was still measurable at depths of ca. 100 m in diverse organic-rich marine sediments (Cragg et al., 1990, 1995). Microbial sulfate reduction has been recorded at sediment depths of at least 425 m (Parkes et al., 1994).

At present, we do not have a working inventory of sulfate-reducing bacterial diversity in cold deep-sea sediments, the geothermally heated subsurface, or generally subsurface habitats; few species have been described in any detail. The barophile *Desulfovibrio profundus* from 500 m depth in sediments of the Sea of Japan remains so far the only described species from deep marine sediments (Bale et al., 1997). Its unusually wide growth temperature range (15 – 65°C) has no counterpart in any other sulfate-reducing bacterium and is a unique physiological feature of this subsurface organism. The new species *Desulfomicrobium hypogeium* was isolated from deep sandstone in Mew Mexico (Krumholz et al., 1999), and the thermophile *Desulfotomaculum putei* from 2.7 km deep terrestrial sandstone (Liu et al., 1997). Sulfate-reducing microorganisms exist that can cope with several of the environmental extremes of the subsurface. For example, the sulfate-reducing Archean *Archaeoglobus* grows at temperatures of 85 - 90ºC, and has been found in geothermally heated, deep oil reservoirs (Stetter et al., 1993). In thermal gradients of hydrothermal vent sediments, different suites of sulfate reducers perform sulfate reduction over a continuous temperature spectrum of 10 - 102ºC (Elsgaard et al., 1994). Members of the thermophilic sulfate-

reducing genera *Thermodesulfobacterium* and *Thermodesulfovibrio* are possible players (Henry et al. 1994, Zeikus et al. 1983).

Methanogenesis is a pervasive process in the Earth's subsurface. In deep marine sediments, porewater methane may accumulate over depth gradients of several hundred meters (Parkes et al., 1994), and is subject to in-situ reoxidation processes within the sediment (Cragg et al., 1995). Methanogenesis in the subsurface was originally regarded as the most likely chemolithoautotrophic basis for a deep microbial biosphere that is fuelled by hydrogen and CO_2 (Gold, 1992). An unexpected dimension was added by the discovery that methanogenesis in deep marine sediments can utilize acetate - and probably other low-molecular weight carbon compounds - that is released from recalcitrant organic matter during burial and reheating (Wellsbury et al., 1997). In concert with other microbial populations, even extremely recalcitrant carbon compounds such as alkanes can be degraded and finally fuel methanogenesis (Zengler et al., 1999).

Although this large body of evidence emphasizes the importance of methanogenesis for the subsurface biosphere, the record of methanogenic isolates from the subsurface is surprisingly spare. Most-Probable-Number enumerations of methanogens in deep marine sediments have yielded cultured methanogens in much smaller numbers than sulfate-reducing bacteria (in the Peru margin; Cragg et al., 1990) or not at all (in the Japan Sea; Cragg et al., 1992). These surveys have, to our knowledge, not lead to the description of new methanogen species from the marine subsurface. This may, in part, be a problem of slow growth of methanogens, or their extreme sensitivity to elevated redox potential during shipboard handling (Rothe and Thomm, 2000). Candidates for subsurface methanogens are versatile acetate- and methyl compound-utilizing methanogens (Methanosarcinales), that were found down to 112 m below sea level, and chemolithoautotrophic methanogens from 45 to 446 m below sea level, both from cold granitic groundwater (Kostelnikova and Pedersen, 1998). A different group of deep-subsurface methanogens is associated with hydrothermal vents. Isolations of extremely thermophilic, chemolithoautotrophic *Methanococcus* spp. from moderately warm, mixed vent fluids which are too cold to permit their growth suggest that these thermophiles thrive in the hydrothermal vent subsurface (Holden et al., 1998); their actual occurrence depth remains unknown. Chemolithoautotrophic methanogens with subsurface potential also include the hydrothermal vent genus *Methanopyrus*, the current most thermophilic methanogen with a growth temperature of up to 110 °C (Kurr et al., 1991).

Sulfate reduction and methanogenesis interact with each other in the process of anaerobic methane oxidation which takes place in a distinct sulfate-methane interface in the sediment. Methane produced in deep sediment layers diffuses upward and is oxidized in a microbial process where sulfate acts as the terminal oxidant; sulfate reduction deep in the sediment can be driven by methane oxidation (Niewohner et al., 1998). Although most studies of anaerobic methane oxidation have focused on near-shore shallow sediments, the process is also documented in deep marine sediments, including methane hydrate sites (Cragg et al., 1995, 1996). The working hypothesis that a microbial consortium of methane-oxidizing Archaea and sulfate-reducing bacteria performs anaerobic methane oxidation (Hoehler et al., 1994) is generally consistent with the available geochemical evidence. The intermediates of methane oxidation, either hydrogen alone (Hoehler et al., 1994), or - with improved energy yield - hydrogen and acetate together, can be utilized and removed by syntrophic sulfate-reducing bacteria (Valentine and Reeburgh, 2000).

These anaerobic methane-oxidizing microbial populations have not been cultured, but small subunit RNA surveys have identified a number of candidates among methanogen-related Archaea. Whenever possible, sequence profiles of anaerobic methane-oxidizing communities are corroborated with diagnostic Archaeal lipids. By their unusually light stable carbon isotopic composition, these lipids reveal their origin from biogenic methane that has been assimilated, oxidized to the level of a coenzyme-bound acetyl group, and channeled into lipid biosynthesis. Several examples exist here: 1) A combined study of 16S rRNA and lipid biomarkers at the Eel River methane seeps offshore northern California identified a novel phylogenetic cluster among the

methanogens (ANME-1) that is not specifically related to any cultured methanogen. The sequence signature was complemented by isotopically light archaeal signature lipids, which included archaeol, the general lipid of nonthermophilic methanogens and their relatives, and (2-sn-hydroxyarchaeol) that is diagnostic for members of the Methanosarcinales (Hinrichs et al., 1999). 2) Sediment cores from the Guaymas Basin hydrothermal vent sites, an unusual, sediment-covered vent site with ammonia- and methane-enriched vent fluids, yielded sequences of the ANME-1 cluster, a new side branch of the ANME-1 cluster, and members of the Methanosarcinales (Teske et al., 2000). The sequence profile was consistent with the parallel data set of isotopically light archaeol and *sn*-2-hydroxyarchaeol signatures. 4) 16S rRNA sequence signatures of a methane clathrate from the Gulf of Mexico, a methanogenic sediment from the sea floor near the Cascadia Margin, and borehole fluid from ODP site 891 offshore Oregon at the Cascadia margin, yielded predominantly sequences related to members of the *Methanosarcinales* (Lanoil et al., 2000). A fluorescent-in situ hybridization study of methane-rich Cascadia margin sediments visualized tight clusters of methanosarcinales and sulfate-reducing bacteria, the first direct look at methane-oxidizing consortia (Boetius et al., 2000). While the involvement of members of the methanosarcinales in anaerobic methane oxidation is increasingly well supported, the role of the novel ANME-1 cluster that is detected frequently in anaerobic methane oxidation sites remains open; it could be an active participant in anaerobic methane oxidation, or have an altogether different function.

Quantifying rates of sulfate reduction, methanotrophy, methanogenesis—and the total activity of the deeply buried biosphere.

Sulfate reduction by subsurface microbes is a major sink of sulfate from the world ocean. Methanogenesis by buried microbes is the principal source of methane over the 70% of Earth's surface that is covered by oceans. Anaerobic methanotrophy by more shallowly buried microbes appears to be the primary mechanism of methane destruction over that same 70% of Earth's surface (e.g. Reeburgh, 1996; D'Hondt et al., 2000). Despite their importance to the global cycles of methane and sulfate, the rates of sulfate reduction, methanogenesis and methanotrophy in deep-sea sediments are poorly known. Furthermore, although the total biomass of microbes in deeply buried marine sediments and crust has been estimated to approximate the biomass of Earth's surface, the total activity of those deeply buried microbes remains poorly constrained.

Rates and kinds of microbial activity in deep-sea sediments are primarily controlled by the availability of electron donors (usually organic matter) and electron acceptors. The most concentrated microbial activity occurs in the shallowest sediments, where organic matter and the most energy-yielding electron acceptors [O_2, Mn(IV)-oxides, NO_3^-, Fe(III)-oxides) are most abundant. Rates of sulfate reduction are highest in deeper sediments, where electron acceptors with higher energy yields are absent. Rates of methanogenesis are highest in deeper organic-rich sediments that have already been stripped of dissolved sulfate by microbial activity. Because sulfate diffuses down into the sediment from the overlying seawater, the rate of sulfate reduction increases where the distance to the sulfate-reducing zone decreases and concentrations of organic matter in that zone increase. Similarly, the rate of methanogenesis is higher where the concentration of organic matter in the methanogenic zone is greater.

In relatively cool sediments, to a first approximation, total microbial activity varies directly with the flux of organic matter to the sediment surface from the photosynthetic zone of the overlying surface ocean; when the organic flux to the sediment is high, sedimentary microbial activity in the sediment is also high. The flux of organic matter to the sediment surface varies as a function of (1) the distance from the upper water column (the photosynthetic zone) to the sediment surface (Berger et al., 1989), (2) biomass production in the photosynthetic zone (Eppley and Peterson, 1979), and (3) the trophic structure of the marine ecosystem in the upper water column (Michaels and Silver,

1988). The second and third variables are closely linked. Biomass production and ecosystem structure are highly correlated and appear to be affected by dissolved nutrient availability. A recent modeling study suggests that, at least in the modern ocean, these biological variables are united by sea-surface temperature (Laws et al., 1999); in that study, 86% of the variance in the proportion of total production that sinks from the surface ocean is correlated to temperature.

At least some of these properties can change on human timescales and due to human activities. For example, the long-term warming of Earth's atmosphere over the last few decades has been paralleled by ocean warming (Levitus et al., 2000) and may eventually warm large areas of the ocean surface. Warming of the ocean surface may in turn cause widespread changes in marine trophic structure and biomass production. Even on short timescales, in relatively large key areas, such as large river deltas and other nearshore environments, the organic flux to deep-sea sediments may be changed by human agricultural activity. Such changes are due to both the increased organic load of agriculturally influenced rivers and the increased flux of dissolved nutrients to the sea. For example, the annual flux of dissolved phosphate to the ocean has doubled since the advent of widespread fertilization (Pilson, 1998).

The total subsurface activity that relies on any single microbial metabolic pathway, such as sulfate reduction or methanogenesis, depends on (1) how much organic matter survives O_2-fueled microbial activity at the sediment surface and gets buried, and (2) how much sulfate diffuses into the sediment to be reduced. The first parameter depends on both the dissolved oxygen concentration of the deep sea and the rate of sediment accumulation. The second parameter depends on the diffusivity of the sediment and the distance over which the ion must diffuse. The diffusional distance depends on sedimentation rate, diffusivity depends on the porosity and tortuousity of the sediment, and the oxygen content of the deep sea varies with both the temperature of the deep sea and the amount of oxygen that has already been stripped from the deep water by microbial activity.

At any given site, depth-integrated rates of sulfate reduction can be estimated from porewater geochemical profiles and core-based estimates of effective diffusivity. After local rates have been determined for large numbers of sites, a predictive model can be developed by correlating those rates to relevant site-variable properties (such as water depth, sediment type, productivity of the overlying water column, seasurface temperature, etc.) (e.g., Canfield, 1991). Such models could be used for estimating global or regional rates by using the fit of rate estimates to geographically continuous variables (i.e., water depth, sediment type, productivity of the overlying water column, seasurface temperature) to extrapolate over the area of the entire deep seafloor. These models could also be used to predict the sensitivity of subsurface sulfate reduction to widespread (global or regional) changes in such oceanographic properties as seasurface temperature, deep-sea temperature, or ecosystem structure or nutrient availability in the upper water column.

In principle, similar techniques could also be used to estimate local, regional and global rates of methanogenesis and anaerobic methanotrophy in deep-sea sediments. Unfortunately, measured methane concentrations in recovered drill cores often under-represent in situ concentrations because methane is outgassed as the cores are brought up through the water column and ambient pressure decreases (Dickens et al., 1997). Fortunately, the recent development and ODP deployment of a downhole high-pressure sampling apparatus now allows this problem to be circumvented (albeit at a moderately high cost in drilling time and effort) by recovering cores at *in situ* pressures (Dickens et al., 1997). Over the next few years, utilization of this apparatus on selected ODP cruises should allow measurements of methane concentrations that are accurate enough to directly estimate rates of methanogenesis and methanotrophy for key regions of the world ocean. Furthermore, two recent intellectual developments collectively indicate that it should be possible to indirectly estimate rates of anaerobic methanotrophy from downhole profiles of dissolved sulfate. The first of these developments is the recent recognition that almost all of the methane diffusing up through deep-sea sediments is destroyed by sulfate-reducing microbial consortia at the buried interface between

methane-rich sediments and overlying sulfate-rich sediments (e.g., Hinrichs et al., 1999; D'Hondt et al., 2000). The second development is the recognition that sulfate concentrations linearly decline from the sediment-water interface to the depth of anaerobic methanotrophy in regions of abundant subsurface methane (such as methane clathrates) (Borowski et al., 1999). This relationship implies that virtually all of the sulfate entering the sediment diffuses to the sulfate/methane interface, where it is reduced by anaerobic methanotrophy.

The census of DSDP/ODP data by Borowski et al. (1999) and our more recent census of nearly 600 ODP sites suggest that this linear relationship of downhole sulfate and methane profiles is characteristic of most geochemical records in all the major methanogenic regions that have been drilled by ODP (Fig. 3). The constancy of this relationship effectively indicates that local, regional and global rates of anaerobic methanotrophy may be estimated from rates of subsurface sulfate reduction in methane-rich regions.

Almost all microbial activity in deeply buried marine sediments is supported by either sulfate reduction or methanogenesis [because dissolved sulfate is nearly 50 times as abundant as all other external electron acceptors combined in deep-sea sediments (data from Froelich et al., 1979 and Pilson, 1998) and fermentation and methanogenesis are the only avenues of microbial activity that remain after all external electron acceptors have been reduced]. Consequently, to a first approximation, total rates of microbial activity in the buried biosphere of deep-sea sediments can be estimated from subsurface rates of sulfate reduction and anaerobic methanotrophy, if subsurface methanotrophy is assumed to be in balance with methanogenesis in the underlying sediments.

The geographic density of Deep Sea Drilling Project (DSDP) and ODP pore-water data appears sufficient to estimate profiles of sulfate and methane concentrations in deep-sea sediments throughout most of the world's ocean basins. Our preliminary census of DSDP Leg 1 through ODP Leg 170 indicates that pore-water sulfate data are available from well over 600 DSDP and ODP Sites. Furthermore, because the methane concentrations of recovered sediments are routinely measured for safety reasons on all ODP drilling legs, methane concentration data (measured as headspace ppm) are available for nearly all ODP Sites (there are nearly 600 ODP Sites). Methane concentrations in deep-sea sediments range from 0 ppm in shallow sediments and at many mid-ocean sites to values approaching 1,000,000 ppm in sub-surface sediments of some near-shore basins (at least 30 ODP Sites have peak headspace methane values of 900,000 ppm or higher).

Pore-water concentrations of inorganic electron acceptors other than sulfate are more poorly documented in the DSDP and ODP databases (see fig. 3 for a subset of the ODP data coverage). Our preliminary census of those databases shows that they include sedimentary manganese data for approximately 100 sites and sedimentary iron data for a few tens of sites. The sedimentary records of only seven ODP Sites are represented by nitrate data in the DSDP and ODP geochemical databases. It should be possible to find in the peer-reviewed literature more data on the concentrations of these electron acceptors in pore waters from DSDP and ODP Sites. However, it will probably be necessary to measure downhole profiles of dissolved NO_3^-, Mn(IV)-oxides, and Fe(III)-oxides at many more sites before robust global estimates of subsurface nitrate, manganese and iron reduction can be developed.

Third Project: Organic signatures of present and past microbial processes in Earth's subsurface.

Chemical approaches will complement traditional isolation and characterization of microbes inhabiting the deep marine subsurface and their characterization by small subunit 16S rRNA sequence techniques. In principle, two analytical avenues with distinct target compounds – (1) geologically relatively stable biomarkers and (2) their mostly unstable biological

precursors - promise to provide independent insights on microbial ecology, on patterns of carbon flow, and on populations of living microorganisms. Chemical techniques for the study of environmental microbiology provide information on a large variety of microorganisms whereas traditional isolation techniques are limited to the small percentage of cultivable organisms.

The first group of compounds comprises extractable, volatile lipids that are derived by slight chemical transformation from their polar and, under environmental conditions, highly labile biological precursor molecules produced by archaea and bacteria. The volatile lipids are commonly analyzed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). Many of these compounds serve as chemotaxonomic markers for the detection of certain microbes in complex communities. Their mostly conveniently determinable carbon isotopic composition (e.g. Hayes et al., 1989) provides information on their carbon fixation pathway and on the carbon source utilized (Hayes, 1993). For example, archaea and a few deeply-branching bacteria synthesize distinct glycerol diether lipids with isoprenoid and non-isoprenoid alkyl moieties, respectively (Langworthy et al., 1982; 1983). Bacteria synthesize a large variety of fatty acids as part of their phospholipid membranes. Their structural features can often provide important insights on the structure of the microbial community, for example on environmental populations of sulfate-reducers (e.g. Taylor and Parkes, 1983). An additional group of exclusive prokaryotic products is the hopanoids, again structural elements of cell membranes from many bacteria. Although this group of compounds has to date exclusively been isolated from aerobic bacteria (Rohmer et al., 1984; Farrimond et al., 1998), their occurrence is probably not categorically limited to aerobes. In addition, recent developments of new analytical protocols (Hopmans et al., 2000) now allow determination of intact archaeal tetraethers by liquid-chromatography-mass spectrometry (LC-MS). This technique has revealed a previously unknown diversity and abundance of these potentially powerful biomarkers for the study of the deeply buried biosphere (Schouten et al., in press).

Lipid-biomarker approaches to the understanding of microbial processes and ecology have been particularly fruitful in near-surface environments such as Hot Springs (e.g. Zeng et al., 1992a,b), hydrothermal vents (Teske et al., 2000), microbial mats (Ward et al., 1989) and methane seeps (e.g. Hinrichs et al., 1999; 2000; Pancost et al., 2000). Especially in methane-rich environments, the structural features of archaeal and bacterial biomarkers in combination with their carbon isotopic compositions provided a previously unobtainable view of complex consortia of anaerobic methane-oxidizers (Hinrichs et al., 1999; 2000) which have escaped cultivation in spite of multiple attempts. These findings set the stage for a new era in the study of these biogeochemically significant microbes. Particularly the combination of molecular-isotopic lines of evidence with modern, culture-independent microbiological techniques has proven to be fruitful in the elucidation of the anaerobic oxidation of methane (AOM) and the participating microorganisms (Hinrichs et al., 1999; Boetius et al., 2000).

In deep-subsurface sediments, however, such an integrated approach faces potentially significant challenges that are caused by the decline of microbial activity with burial depth. Such a decline with depth should lead to an increasingly disadvantageous signal-to-noise ratio for the study of microbial products produced by a presently active *in situ* community. This signal-to-noise issue is exacerbated by the accumulated remnants of microbial biomass from earlier (nearer-surface) microbial activity. One means of overcoming these problems is to search for molecular biomarker signals related to active microbial communities at sediment horizons with distinct chemical features. In such microenvironments, the presence certain types of microbial activity can be predicted with some confidence. One example for such a focused strategy is the successful search for products of anaerobic methanotrophic microbes at the transition zone at ~ 2 m depth in anoxic sediments at Kattegat (Bian et al., submitted) – the sediment horizon defining the boundary between sulfate-reduction zone and methanogenesis where anaerobic oxidation is commonly observed (e.g. Iversen and Jørgensen, 1985). Methane-oxidation rates in that horizon are several orders of magnitudes lower than at methane-hydrate-related seep sites, where near-surface sediments contain significant populations of active methanotrophic communities which facilitate the study of their products.

Nevertheless, in those sediments with presumably less-active communities of lower density, the combination of geochemical search criteria and the very distinct isotopic signature allowed the characterization of methanotrophic products such as crocetane with a carbon isotopic composition of ~-100‰. This lipid has never been isolated from a cultured microorganism, but is presumably of archaeal origin and has been found to date only in environments with active AOM.

The understanding of the role of deeply buried anaerobic methanotrophic communities and their role for their importance in the deep biosphere is one major objective in our research plan. In the case of methanotrophy the "isotopic labeling" caused by consumption of strongly ^{13}C-depleted methane (e.g. Kvenvolden, 1995) at sediment horizons in which the chemical environment favors AOM, e.g. the deep, sulfate-rich brine incursion intertwining into methane-laden sediments (targeted during Leg 201; D'Hondt et al., 1999), will guarantee the distinction of methanotrophic products from other microbial biomarker products that are unrelated to *in situ* activity.

In contrast, the group of relatively stable biomarkers may be only of limited use in the study of microbial processes that are not linked to methane-consumption and therefore do not possess a distinct isotopic signal. In fact, the commonly praised stability of biomarkers impedes the differentiation between a signal related to an active community and one that was active possibly a million years ago in a much shallower sediment or even in the water column. Therefore, we envision an approach that combines the analysis of established, stable biomarker as well as more labile microbial products that, to a first approximation, will represent the living stock of prokaryotes thriving in deeply buried sediments.

One particularly labile group of abundant building blocks for bacterial cell membranes – phospholipids – is widely used in environmental, microbiological and biogeochemical studies to assess the amounts of living bacterial biomass (e.g. White et al., 1979, Balkwill et al., 1988) and to characterize microbial communities based on chemotaxonomic information embedded in these compounds. These compounds are associated with living microbes because the phospholipid-head groups are labile to hydrolysis soon after cell death (White et al., 1979). In addition to information on the amount of living biomass, the distribution and structural features of phospholipids carry important information on the structure of a microbial community. Traditional approaches to the analysis of bacterial phospholipid isolate and quantify the phospholipids to provide estimates of living biomass (e.g. White et al., 1997). The ester-linked fatty acids become GC-amenable as fatty acid methyl esters (FAMEs) following mild alkaline trans-methylation (Fang and Findlay, 1996). Their structural features, such as double-bond configuration, methyl-branching patterns, and chain lengths of FAMEs provide chemotaxonomic information on participants in microbial consortia (e.g., Boschker et al., 1998). An analogous approach is often taken for archaeal phospholipids such as glycerol di- and tetraethers, the corresponding membrane elements in archaea, which possess a diverse group of ether-linked, often genera-specific, isopranyl moieties (e.g. Langworthy et al., 1982; Kates, 1993). In environmental studies in marine and sedimentary habitats, however, only the ether-linked isopranyl moieties are commonly analyzed upon chemical isolation and degradation procedures, with both the phospholipid head group and the glycerol being removed (e.g. DeLong et al, 1998, Hoefs et al., 1997).

However, these procedures do not permit coupled analysis of the distinct structural features of the associated phospholipid head groups of bacterial and archaeal products, which themselves bear an additional wealth of chemotaxonomic information (White et al, 1979; Kates, 1993). The bacterial fatty acids and archaeal ispopranyl moieties determined as such are a mixture of compounds derived from an assemblage of distinct intact phospholipids from different microbial populations. As a result, information on the microbial population is lost, and the specificity of the phospholipid-bound microbial marker determination to estimate biomass and determine community structure is lost.

Recently, a different path was chosen for the analysis of intact bacterial phospholipids by direct coupling of liquid chromatography to mass spectrometry (LC-MS; e.g. Kim et al., 1994; Karllsson et al., 1996; Fang and Barcelona, 1998; Fang et al., 2000). This approach allows analysis of the intact phospholipids and therefore simultaneous determination of the ester-bound fatty acids and the phosphorus-containing head group, an approach which, due to its increased specificity, is superior to traditional phospholipid analysis protocols as outlined below (Fang et al., 2000). The phospholipid head group is a phosphate linked to the *sn*-3-glycerol position (*sn*-1 in case of archaeal phospholipids) by an ester bond and, by a second ester bond, to a variety of additional substituents such as amino acids (phosphatidylserine, PS), amines (phosphatidylethanolamine, PE; phosphatidylcholine, PC), sugars (phosphatidylinositol, PI), and glycerol (phosphatidylglycerol, PG). Analogous rapid approaches using LC-MS for the analysis intact archaeal membrane phospholipids have not yet been attempted, although the chemical and chromatographic features of these compounds predict successful analysis by very similar analytical protocols. For example, Hopmans et al. (2000) have successfully used LC-MS techniques to analyze intact archaeal tetraethers from which the polar head groups had been removed. More recently, this techniques has been successfully applied to the study of archaeal and bacterial diversity in marine and lacustrine sedimentary environments (Schouten et al., in press). Notably, in a single study, this advancement revealed previously unknown details about the structural diversity of intact archaeal ether lipids.

The analysis of intact bacterial phospholipids has been pioneered by H. Rütters (University of Oldenburg, Ph.D. thesis in progress, pers. communication, 2000), who demonstrated how their characterization in anoxic, near-surface sediments allows a chemotaxonomic description of a complex community of sulfate-reducing bacteria. The extent of information about the composition of a complex natural community that can be derived in this way is directly linked to the extent of available information about the distribution of phospholipids in isolates and to an appropriate numerical treatment of complex data (Fang et al., 2000). Despite the present paucity of data derived from cultured organisms (the diversity of intact phospholipid distributions may resemble that found in assemblage patterns of DNA), other features of phospholipids confirm their importance as analytical targets. Specifically, the phospholipids provide a chemical indicator for living biomass and provide access to isotopic information about that material. Such points are of great importance in the thorough exploration of an unknown microbial habitat. Integrated in a program with other molecular-isotopic and culture-independent microbiological tools discussed earlier, the analysis of intact bacterial and archaeal phospholipids has great potential to open new dimensions for the study of environmental microbial habitats. For example, interesting information can be expected from comparisons of the sedimentary inventories of archaeal glycerolethers and of bacterial acyl glycerides with the respective head group-containing phospholipid precursors. Since the head group is readily lost after an organism's decay (White et al., 1979), these relative amounts may bear important details on the turnover rate of certain microbes and/or microbial communities and thereby on their overall activity.

The unique analytical facilities being assembled at WHOI will allow the study of complex lipids such as intact phospholipids and their head-group-liberated analogs. The recently acquired moving-wire interface coupled to an isotope-ratio mass spectrometer will allow on-line and off-line analysis of these microbial biomarkers (n. b., the moving-wire interface is performing well in the analyses of intact nucleic acids; the successful analysis of sub-nanomole-C quantities of phospholipids can be predicted with confidence). The moving-wire interface in Woods Hole (Brand and Dobberstein, 1996) is a prototype and is the only such instrument available in institutional laboratories, world-wide. The analytical capability to study simultaneously the structural and isotopic compositions of classes of compounds that were previously largely ignored or analytically inaccessible in studies of environmental microbiology and biogeochemistry opens new doors to the study of microbial habitats, not only in deeply buried sediments, and is of major relevance for astrobiological research. No other single analytical approach offers a comparable amount of information in the study of unknown microbial habitats in terms of microbial diversity, amount and possibly activity of living biomass, and possible substrates and metabolic pathways.

In principle, each of the targeted biomarkers can serve as an indicator for extraterrestrial subsurface life. In the case of a hypothetic living extraterrestrial biosphere, tests for life using biomarkers such as those described in this section would likely be trivial. This task would be more challenging for past extraterrestrial biospheres that may have become extinct billions of years ago. Here, examples of recoveries of chemotaxonomically diagnostic biomarkers from 2.7 billion years old, highly metamorphic rocks on earth (Summons et al., 1999; Brocks et al., 1999) suggest that such information may be preserved on other planets as well.

Microbiological approaches to these research projects.

Contamination tests.—

Contamination tests by ODP Leg 185 shipboard scientists now provide robust shipboard protocols for assessing whether or not the microbial assemblages recovered from cored deep-sea sediments are indigenous to those sediments or are potential contaminants. These protocols use perflorocarbons as contamination tracers and are based on continental drilling protocols (Harvey *et al.*, 1989; Senum and Dietz, 1991) introduced to the ODP by its Deep Biosphere Program Planning Group. Two proponents of this Astrobiology Institute proposal, David Smith and Arthur Spivack, successfully pioneered these tests on Leg 185 (Smith *et al.*, 2000 a & b) and again successfully applied them on Leg 190. Their studies demonstrated that core interiors are typically uncontaminated. With their shipboard colleagues, they also demonstrated the feasibility and power of paired microbiological and geochemical studies on-board the *JOIDES Resolution.*

Sample retrieval.—

Fresh ODP cores will be sampled within one or two hours after arrival on the ship for cultivable microorganisms. Cultivations and molecular analyses require fresh samples, the main reason why a microbiology lab is indispensable directly on the ship. Within a few hours of core recovery, microbial communities undergo drastic alteration in composition (Rochelle et al., 1992), and do not allow inferences about their original microbial populations.

Subcores from sediments will be placed in an anaerobic chamber filled with an oxygen-free N_2/CO_2 gas mixture and subsampled with sterile metal or plastic sampling cores. The inner portions of the subcores which do not show the presence of contaminant tracers (i.e. fluorescent dye, perfluorocarbon or microscopic beads) will be selected for cultivations and molecular and organic biomarker analyses. -Solid rock and highly compacted sediments will be sampled with the use of a rock-splitter. The rock splitter will be placed in the anaerobic chamber and subsamples from the interior of the rock will be used in further analyses.

Cultivations.—

Cultivations are our principal handle to demonstrate microbial diversity, not only under a taxonomic viewpoint (different isolates with different 16S rRNA sequences), but actually as a proof that bacteria of specific metabolic types, according to the specificity of the media, exist in deep sediment and rock samples. 16S rRNA surveys are not always convincing in that respect, especially if the connection between sequence type and metabolism is ambiguous. Cultivations will focus on anaerobic prokaryotes, since oxygen disappears a few centimeters under the sediment-water interface. Inoculations and transfer of oxygen-sensitive anaerobes into anaerobic medium must proceed without delay from freshly retrieved cores, unaffected by prolonged oxygen exposure. For example, mesophilic sulfate-reducing bacteria begin to show oxygen damage after a few hours of air exposure (Cypionka et al. 1985, Fukui and Takii, 1990).

Quantification of cultivable microbial populations will be done with the Most Probable Number (MPN) technique (American Public Health Association, 1989). In short, homogenized sample materials will be serially diluted, and the pattern of positive samples in three or more parallel dilution series will be used to infer the most likely population density of bacteria in the original sample. The cultures in the highest positive dilutions will represent bacteria which occur in relatively high densities in the sampled habitat. Sometimes these dominant populations are novel and unexpected (Teske et al., 1998). We will use Hungate techniques (Breznak and Costilow, 1994) and anaerobic media under N_2/CO_2 gas mixtures for cultivations of sulfate reducers (carbonate-buffered as described by Widdel and Bak, 1991), iron reducers (specifications in Vargas et al., 1998), and methanogens (Balch et al., 1979).

If surplus deep sediments are available for this purpose, sterilized sediment portions can be added to MPN culture tubes on the shipboard laboratory. Such additions increase the sensitivity of MPN counts by factors of 100 to 1000 and thus push MPN counts close to the actual *in situ* bacterial densities (Vester and Ingvorsen, 1998). The reason for this increased efficiency of natural media is not only the presence of a substrate spectrum that matches the needs of the *in situ* populations, but also the availability of particles which facilitate growth of many "difficult", surface-associated or clump-forming bacteria which do not grow in homogeneous suspension (Widdel et al., 1983).

Temperature is a decisive factor in cultivations. The heat sensitivity of most mesophilic marine bacteria was recognized early (ZoBell, 1941). Generally, incubation temperatures over 20-25°C have to be avoided, except in cultivations which aim explicitly at thermophiles and hyperthermophiles. Cold can be tolerated much more easily. Thermophilic anaerobes cease to grow during cold spells and also become less sensitive to oxygen exposure; they disperse widely through cold water and colonize new habitats (Stetter et al., 1993), or they survive as spores (Isaksen et al., 1994).

Pressure is an important factor in cultivations from deep-sea cores. Obligately barophilic bacteria, so far isolated from depths greater than 6000 m, require cultivation under elevated pressure (Yayanos et al., 1981), and the ability to reproduce at high pressure is considered a hallmark characteristic of barophilic bacteria (Yayanos et al., 1982). Facultatively barophilic bacteria also grow at atmospheric pressure, but have their growth optimum at elevated pressure—frequently the *in situ* pressure of the depth they were isolated from (see compilation in DeLong et al., 1997). Acquisition of high-pressure culture vessels will allow for the cultivation of microbes from samples collected from extreme depths. Samples collected at more moderate depths may not require the use of such vessels.

Molecular approaches.—

In order to detect microbial populations which do not grow under the culture conditions employed, cultivation surveys have to be complemented with molecular, DNA- and rRNA-based approaches. Samples for molecular work have to be taken within a short time after core retrieval, in parallel with samples for cultivations. After just a few hours, cored sediments undergo drastic alteration of their microbial community composition (Rochelle et al., 1992), and do not allow inferences about their original microbial populations. Core samples for nucleic acid isolation can be kept deep-frozen at - 80°C or in liquid nitrogen for months until arrival in the home laboratory and further processing.

The potentially large number of ODP core samples will require high-throughput techniques for nucleic acid based microbial community analysis. We plan to use Denaturing Gradient Gel Electrophoresis (DGGE) as a means to monitor changes in the microbial community structure over the extended spatial gradients of ODP cores. DGGE separates homologous double stranded DNA

fragments according to their melting domain stability and primary sequence. DGGE is most commonly used to separate mixtures of double-stranded PCR products from PCR-amplification of environmental DNA mixtures (Muyzer et al. 1993). The PCR products migrate into an acrylamide gel of increasing denaturing concentration, melt at different points in the gradient, stop their migration, and sort themselves into distinct bands of one sequence type each, thus avoiding the cloning bias inherent in shotgun cloning separation of mixed PCR products (Muyzer et al. 1993, 1997). Side by side comparisons of DGGE patterns from different samples over the length of a core make it possible to track and identify individual microbial populations in a spatially changing pattern of microbial community composition; as demonstrated with millimeter layers of a microbial mat core (Teske et al. 1998). Individual DGGE bands can be eluted from the gel, reamplified, and sequenced for phylogenetic identification, or DGGE patterns can be transferred to a nitrocellulose carrier membrane for hybridization with genus- or group-specific probes (reviewed in Muyzer and Smalla 1998).

A variety of other nucleic-acid based survey techniques can be applied to nucleic acids extracted from core samples. These techniques include terminal restriction fragment length polymorphism (T-RFLP; Liu et al., 1997). This method is suitable for side-by-side comparisons and identification of metabolic key genes such as dissimililatory sulfite reductase, the key gene of sulfate reduction (Wagner et al., 1998) which remains inaccessible to DGGE due to insufficient sequence conservation (D. Stahl, pers. comm.).

These molecular analyses do not have to be performed in the shipboard laboratory. For practical reasons, core samples for nucleic acid isolation can be kept deep-frozen for months until arrival and further processing in the home laboratory. Here, the specialized, expensive, and partially fragile equipment will be available, along with more time than the sample retrieval schedule of the JOIDES Resolution can accommodate. However, good -80°C and liquid nitrogen storage facilities will be necessary on board to preserve samples for nucleic acid extraction and molecular analyses.

Microscopic observations.—

Direct microscopic observation is an indispensable source of information about microbial community composition. It requires a well-equipped microscope on the ship. Generally, direct observation is one of the first steps (never the only step) in identifying microbial community members, using clues from morphology or chemotactic behavior for a tentative identification. Some microbial groups are recognizable by autofluorescence. For example methanogens show a distinct blue-green autofluorescence. Different fluorescent stains have to be used to detect non-fluorescent and inconspicuous microbial cells. General DNA-stains such as Acridine orange allow total community cell counts (Hobbie et al. 1979). When microbial activities and intracellular rRNA levels are high, fluorescent in-situ hybridization (FISH) of individual cells with rRNA-targeted fluorescent oligonucleotide probes (Amann et al. 1995) allows to analyze the phylogenetic community composition of sediment samples (Llobet-Brossa et al. 1998). The caveat has to be kept in mind that many cells in natural microbial assemblages show significantly diminished FISH fluorescence, due to low activity and rRNA content (Ramsing et al. 1996); this could apply especially to deep sediments with low nutrient content.

Microbial activity determinations.—

The use of radioisotopes in microbial ecology has led to both qualitative and quantitative discoveries which were possible only due to the extreme sensitivity of these detection methods. Radioisotopes can be used to determine which individual cells are active by the use of microautoradiography [a technique that Brock (1966) introduced into the field of microbial ecology]. In addition to cell-specific assays, radiolabeled compounds can be used to determine the rates of bulk processes such as methanogenesis and sulfate reduction. ^{3}H-thymidine has been widely used to determine DNA synthesis rates of a variety of microorganisms. While there is evidence that

many anaerobes do not incorporate thymidine there are cases where it has been successfully employed (Winding 1992, Wellsbury et al. 1993). Other radiolabeled precursors have also been used to measure microbial growth (e.g., ^{3}H -leucine for protein synthesis rates).

Biogeochemical approaches to these research projects.

Quantitative and isotopic analysis of microbial molecular biomarkers.—

The molecular biogeochemical component of the proposed research plan will focus on the extractable organic fraction of microbial origin and will be combined of analyses of relatively stable biomarkers and on intact phospholipids and their head-group liberated derivatives. Always, these analyses will be performed on samples for which complementary traditional and culture-independent microbiological data will be generated in parallel.

For the analysis of stable biomarkers, we will employ established protocols developed in our laboratories (e.g. Hinrichs et al., 1999, 2000). These compounds are largely GC-amenable. Presumably, this group of compounds will consist of archaea-specific ether lipids such as archaeol and hydroxyarchaeol (e.g., Hinrichs et al., 1999), irregular isoprenoid hydrocarbons (Elvert et al., 1999; Thiel *et al.*, 1999; Hinrichs et al., 2000; Bian et al., submitted), and products of sulfate-reducing bacteria such as mono- and dialkylglycerolethers (Hinrichs et al., 2000), and free fatty acids (e.g., Taylor and Parkes, 1983; Dowling *et al.*, 1986). In addition to environments with active anaerobic methanotrophy, bacterial ether lipids are expected to be particularly abundant in geothermally heated subsurface environments, where phylogenetically deeply-branching relatives of known producers may be abundant (e.g. L'Haridon et al., 1995). The compound-specific isotopic compositions (Hayes et al., 1989) of these groups can indicate mechanistic interactions within microbial communities. For the study of deeply buried methanotrophic communities – one major objective on the forthcoming Peru margin expedition ODP Leg 201 - the isotopic composition will unambiguously identify products from methane-consuming microbes. In such a case, the structural features of ^{13}C-depleted compounds in combination with phylogenetic analyses will allow the culture-independent characterization of deeply buried anaerobic methanotrophs and their comparison to near-surface communities.

The isotopic compositions can also be used, in combination with those of total organic carbon and carbon-containing solutes in pore waters, respectively, to identify carbon flows within the microbial community. As analyses accumulate from a substantial number of samples and environments, abundances of biomarkers (incl. ntact phosholipids) will be tested for correlation with abundances of organisms represented by specific 16S rRNA sequences, and MPN-dilutions. These tests should allow identification of relationships between biomarkers and phylogenetic types. In addition, products of cultivation experiments of deep subsurface microbes will be continuously monitored for biomarkers incl. phospholipids in order to allow both chemotaxonomic assignment of biomarker distributions observed in environmental samples to certain microorganisms.

All necessary equipment is available for these analyses. Non-phospholipid biomarkers will be extracted from aliquots of the freeze-dried and homogenized sediment by accelerated solvent extraction (ASE, Dionex) with a solvent mixture of DCM/MeOH (9:1). The solvent will be evaporated under a stream of N_2, using a Zymark –turbo evaporator™. When needed, extracts for analysis will be separated into fractions (hydrocarbons, ketones and esters, alcohols, multifunctional compounds, and fatty acids) using commercial glass cartridges (Supelclean™ LC-NH2) eluted with solvent mixtures of increasing polarity according to a protocol developed in our group (Hinrichs et al., 2000). Prior to gas-chromatographic analyses, fractions containing alcohols and fatty acids will be derivatized to produce TMS-ethers and TMS- or methyl esters, respectively. Analytes will be quantified relative to internal standards added to samples prior to extraction.

Analyses of non-phospholipid biomarkers cultivated biomass will employ protocols developed in our group and successfully tested with sub-mg amounts of biomass from cultured archaea and bacteria. Briefly, they involve cell lysis by sonication of cell material with combusted, analysis-grade sand in MeOH (2.5% HCl) and subsequent heating of the mixture to 70°C for 16h to remove the phospholipid-head groups, with subsequent extraction by sonication in DCM/MeOH (9:1, four times). Novel and previously uncharacterized compounds (in particular those related to glycerol-ether lipids) will be identified by comparison to reference standards and to materials from pure cultures of archaea and deeply-branching bacteria.

Starting in year 2002, distribution and abundance of intact phospholipids of archaeal and bacterial origin will be monitored on a regular basis from samples retrieved from different boreholes. Their abundance will provide independent information on the density of deeply buried microbial communities. Complementary monitoring of the abundance and distribution of related analogs that lost their head group already in the sediment (representative for decayed biomass) will establish a data base that may contain valuable information on the activity of a microbial community. For example, in an extremely slow growing community under low-energy conditions, the ratio of phospholipids to their analogs without head group is expected to be large relative to the ratio in a fast-growing community**.** Statistical approaches will be employed to relate their distribution to those of relevant cultured organisms (Fang et al., 2000; H. Rütters, pers. commun., 2000) and to other relevant data indicative for the microbial ecology, which will be generated from comparable samples. Stable carbon isotopic compositions of phospholipids and glycerolether and –acyl derivatives will be determined on-line by transferring the LC effluent to the moving-wire interface (Brand and Dobberstein, 1996) coupled to a Finnigan MAT 252 isotope ratio mass spectrometer, and off-line when poor LC compound resolution requires additional clean-up steps by solid phase extraction or thin layer chromatography.

Since no published material exists on studies of microbial polar lipids such as intact phospholipids in marine sediments, in year 1 a particular focus will be given to development of analytical protocols under use of standards, cultured microorganisms, and marine sediments with abundant microbial biomass. The specification of the Thermoquest Deca XP liquid chromatograph coupled to an ion trap mass spectrometer allows two ionization modes, electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). Phospholipids have been successfully studied using ESI (Kim et al., 1994; Karlsson et al., 1996; Fang and Barcelona, 1998), whereas for bacteriohopanepolyols (Fox et al., 1998) and archaeal tetraethers from which the head groups had been removed (Hopmans et al., 2000) good experience has been made using APCI, in the latter case even in sediment samples (Schouten et al., in press). Additional development is planned for the optimization of extraction procedures of intact phospholipids. To keep up with high numbers of samples we intend to establish focused protocols for rapid analysis like those applied for non-phospholipid biomarkers. For that purpose we will compare established extraction procedures for the analysis of intact phospholipids (modified Bligh and Dyer: e.g., White et al., 1979a) with the automated extraction system in our laboratory, which allows variation of temperature, pressure, and solvent mixtures. Different LC stationary phases will be tested for the optimization of analytical conditions for different analytes.

Hydrogen analyses and experiments.—

There are two interrelated components to the proposed work involving hydrogen. The first component includes the quantification of hydrogen in deeply buried sediments. In order to understand the dynamics of hydrogen and its relationship to environmental conditions, a number of other chemical constituents must be determined in addition to the hydrogen. Therefore, we intend to measure pore fluid acetate, formate, methane, alkalinity, sulfate, sulfide, and calculate pH (based on in situ T, P, alkalinity and Ca^{+2}) as part of our planned ODP drilling leg. We will also determine the isotopic composition (D/H) of the pore fluid hydrogen in order to quantify the relative roles of

biological and abiological hydrogen production. While the existing protocols allow for the reliable determination of dissolved sedimentary hydrogen, further refinements of the methods in a shore-based laboratory are warranted. These methodological studies will focus on the development of vessels for the quantitative storage of hydrogen gas produced during ship-board incubations and the development of low-level hydrogen gas standards for both ship-board and shore-based analysis.

The second component involves experimental manipulations of sediment samples to determine the factors critical in controlling hydrogen concentrations in sediments. The experiments have two principle goals, 1) quantifying the role of hydrogen production via fermentation, inorganic reactions, and abiological diagenesis of organic matter, and 2) testing the theoretical relationships between hydrogen concentration, pore fluid composition and temperature. Specifically we intend to quantify the relationship between abiological hydrogen production, temperature and sediment composition. We will employ two general approaches to this problem.

The first approach involves inhibiting biological production (sterilizing samples by autoclaving) followed by purging of all the pre-existing hydrogen. The samples will then be heated at in-situ temperatures and hydrogen production will be quantified. We conducted preliminary experiments of this type during ODP Leg 190 and, as stated above, observed the production of hydrogen at higher temperatures (Spivack et al. 2000). We intend to refine and expand these experiments based on the results of the method development. In these experiments we also intend to determine the hydrogen isotope systematics that are associated with abiological production. This information will be used in the interpretation of the pore fluid hydrogen isotope data to differentiate biogenic (produced via fermentation) and abiogenic hydrogen (as separately produced from both inorganic and organic substrates).

In the second group of experiments we intend to apply techniques that have been successfully employed in near shore shallowly buried sediments (Hoehler et al., 1998). Specifically, we will conduct incubation enrichment experiments to test the dependence of pore fluid hydrogen on the concentration of specific substrates (sulfate, sulfide, acetate, carbon dioxide and methane), pH and temperature.

Analyses of nitrogen species and isotopes.—

The long-term goal of the proposed work on nitrogen is to quantify rates of nitrogen transformations and net fluxes in the deeply buried sediments and to relate these rates to bacterial abundance and community structure. Our approach will be fourfold involving the measurement of dissolved ammonium, ammonium nitrogen isotope ratios, and dissolved dinitrogen (N_2). In addition we will look for evidence of active biological nitrogen fixation utilizing molecular techniques. Estimates of net fluxes and reaction rates will be based on the diffusion modeling of measured ammonium profiles (Berner, 1980). Since ammonium behaves as a strong ion exchanger, this modeling will require the determination of ammonium adsorption coefficients along with porosity and tortuosity. Tortuosity will be inferred from sediment electrical resistivity measurements. Resistivity, ammonium concentrations and porosity are routinely determined on the *JOIDES Resolution*. Adsorption coefficients will be determined in shore-based experiments.

As described above, nitrogen isotopes may enable the quantification of gross rates. Our first goal with regards to the development of nitrogen isotopes for this purpose will be the determination of a pore fluid ammonium nitrogen isotope profile. This will allow us to evaluate the sensitivity of this approach for partitioning net transformations into its components (degradation of detrital nitrogen, uptake of ammonia, and microbial production of refractory nitrogen containing compounds). We will use the results of this initial work as a basis for laboratory studies aimed at determining the isotopic fractionations associated with specific transformations, such as the bacterial uptake of ammonium. Nitrogen concentrations will be determined by gas chromatography with thermal conductivity detection and by mass spectrometry (N_2/Ar ratios). The GC analyses can

be done on the *Resolution* during core recovery and mass spectrometric analyses will be conducted shore-based.

Where nitrogen fixation is occurring we expect dissolved dinitrogen concentrations to be depleted relative to atmospheric equilibrium concentrations. Based on measured profiles, uptake rates can be modeled. This method should be sensitive to low levels of nitrogen fixation as the equilibrium concentration of dissolved dinitrogen is only approximately 600 micromolar. While we expect nitrogen fixation to occur in intervals where ammonium is depleted we will examine regions of high ammonium as well for comparative purposes. Nitrogen concentrations will be determined by gas chromatography with thermal conductivity detection. These analyses can be done on the *Resolution* during core recovery.

As noted above, nitrogen fixation genes are found in sulfate reducing bacteria, thus the presence of these genes in the sediments is expected whether there is active nitrogen fixation or not. Therefore, we will look for the evidence for the transcription of these genes by using reverse transcriptase polymerase chain reaction (RT-PCR) analysis to detect the presence of mRNA specific to nitrogen. The presence of this mRNA would indicate active nitrogen fixation activity.

Other sedimentary and porewater chemical analyses.—

A variety of measurements on interstitial waters and solid phases are necessary to characterize the chemical environments of buried microbial communities. Many of these chemical measurements are already routinely undertaken on ODP cruises. These include measurements of: pH and alkalinity (via titration); ammonium (by spectrophotometry); sulfate (by ion chromatography); total organic carbon (TOC) and total inorganic carbon (TIC) (by coulometry); headspace methane and other evolved gases; total Fe and Mn (by atomic absorption spectrometry); dissolved silica and phosphate (both by spectrophotometry); Na, Ca, and Mg by ion chromatography; and Sr, Li, Rb, and other metals by atomic absorption.

More complete characterization of microbial environments would require additional measurements to be made. These include measurements of nitrate (by spectrophotometry), total CO_2 (by coulometry), total H_2S (by spectrophotometry), H_2 (by gas chromatography), and dissolved organic carbon (DOC) (by Shimadzu analyzer). In general, these measurements can be done in the *Resolution's* shipboard chemistry laboratory during relevant cruises. Some of these analyses, such as the H_2S measurements, can be done on preserved samples.

Computational approaches.—

The computational analyses required for this Subsurface Biospheres Program fall into two distinct categories: 1) the integration and analysis of existing data to help target investigations to particular geographic regions and subsurface depths, and 2) the modeling of geochemical profiles and calculations of the global impact of microbiological processes in deep sea sediments. An example of the first category is provided by our use of over 24,000 existing heat-flow measurements (Pollack et al., 1991) to estimate the depth of microbially important isotherms in marine sediments (Figs. 4 and 5). During ODP Leg 190 the results of these isothermal mapping exercises served as a guide for targeting microbiological sampling. Through analysis of existing sulfate, methane, ammonium, and organic carbon measurements in deep sea drill holes, we can similarly expand and refine our ability to target specific geochemical regimes conducive to or indicative of different microbiological processes (as described in the section on the First Project of our Integrated Research Plan).

As described in the subsection on our Second Project, one-dimensional modeling of down-hole geochemical profiles can provide local estimates of microbial influence on cycles of sulfur and methane. These models must at least incorporate the open-ocean and coastal-ocean profile types

shown in Figure 2, and in reality must be flexible enough to embrace a wider range of profile types, including those influenced by anhydrite ($CaSO_4$) precipitation and subsurface brine flow. With sufficient geographic coverage, we can combine the results of these one-dimensional models with global fields of primary production at the sea surface, water depth, and distance from shoreline, for example, to link the subsurface microbial biosphere with surface processes. These quantitative links can provide the basis for estimating the global impact of microbial processes (e.g. sulfate reduction) occurring in deep sea sediments.

Initial data compilation and quality assessment will be performed with a combination of the Unix tools Sed and Awk in addition to FORTRAN programs. For global and regional color contour maps we will use The Generic Mapping Tools (GMT) (as in Fig. 3). GMT will also be used to create downhole profiles. One of the main advantages of GMT over other mapping/plotting utilities is its ability to work within Unix shells or Perl programs to automate mapping and profile plotting. These tools are also applicable to World Wide Web based applications suitable for outreach and communication with other researchers.

Geochemical modeling may be best accomplished with Matlab and its associated optimization and statistics toolboxes. Matlab also provides basic environment for solving inverse problems that can encompass multiple spatial fields and is flexible enough to be adapted for almost any computational requirement.

Sample acquisition through deep-sea drilling.

The microbiological and geochemical samples that are required to directly study the deeply buried biosphere of marine sediments can only be recovered by deep-sea drilling. Such samples are recovered by the Ocean Drilling Program (ODP) on a near-daily basis, using the ocean drillship *JOIDES Resolution*. The *Resolution* spends approximately 340 days at sea every year, drilling and recovering sediments and crust from every part of the world ocean. At water depths ranging from 100 meters to several kilometers, the ship routinely recovers deep-sea sediments and oceanic crust from burial depths in excess of one kilometer. All that has been needed for routine global collection of microbes from those sediments and crust is (1) a well-equipped microbiology laboratory on the *Resolution*, and (2) the presence of microbiologists on the shipboard scientific crew. Since the microbial populations of samples change irreversibly and beyond recognition within a few hours, it is mandatory that microbiological work be carried out onboard immediately after sample retrieval.

A well-equipped microbiology laboratory has recently become part of the permanent scientific facilities on the *Resolution*. Basic instrumentation for that lab was purchased by ODP for western Pacific Leg 185 (April – June, 2000). In order to further advance studies of the deep biosphere in oceanic sediments and crust, a wide range of additional microbiological and geochemical instrumentation was purchased by a grant from the U.S. National Science Foundation (NSF) Life in Extreme Environments (LExEn) Program to the proponents of this Astrobiology Institute proposal (Teske *et al.*, 1999). The shake-down cruise for this fully equipped laboratory was ODP Leg 190 (May – July, 2000).

We have already acquired many of the deep-biosphere samples necessary for our proposed Research Plan through the participation of co-proponents David Smith and Arthur Spivack on western Pacific ODP legs 185 and 190. We propose to acquire future deep-biosphere samples in the same manner. Because the scientific participants on each cruise must undergo a competitive ODP application and selection process, we cannot guarantee that we will be allowed to participate on any individual future ODP cruise. Nonetheless, we do not anticipate any serious trouble participating in future cruises on a fairly regular basis. The Ocean Drilling Program has just scheduled the first-ever scientific ocean-drilling cruise to focus primarily on deep-biosphere objectives. That cruise

(ODP Leg 201, scheduled for Feb – April, 2002) is based on a drilling proposal by the proponents of this Astrobiology Institute proposal (D'Hondt et al., 1999/2000). Furthermore, microbiologists and geochemists can apply to participate as shipboard scientific staff on any ODP cruise, regardless of its primary scientific objective. This open application process effectively renders every ODP cruise a cruise of opportunity for scientists interested in deep-biosphere studies. Cruises scheduled for the next two years will collectively sample every deep-sea sedimentary environment mentioned by this proposal, including: deep hot anoxic sediments; organic-poor open-ocean sediments where life is likely to be limited by electron-donor availability; and organic-rich sediments with very active sulfate-reducing, methanogenic and methanotrophic microbial communities.

The Ocean Drilling Program does not provide significant support for large shore-based scientific efforts (such as the Astrobiology Institute Subsurface Biospheres Program that we propose here). However, ODP does pay all of the operational costs associated directly with the ocean-drilling cruises of the *JOIDES Resolution*. These operational costs include the shipboard salaries of participating scientists. Furthermore, shipboard scientists and technicians on every ODP cruise routinely measure a host of geochemical, geophysical and sedimentary properties for each recovered core and drilled hole. These routine measurements facilitate close integration of microbiological, geochemical and geophysical studies. This close integration will allow scientists to routinely document the environmental limits and biogeochemical effects of the deep biosphere, at no real cost to the proposed Astrobiology Institute Deep-Biosphere Program.

Roles of individual participants

As described in the relevant sections of this proposal, all of the Investigators will be expected to participate in all aspects of the proposed program, including Research, Training, and Education / Public Outreach.

The PI, Steven D'Hondt, will bear the ultimate responsibility for guiding and coordinating all aspects of the proposed Subsurface Biospheres Astrobiology program, including its Research, Education and Outreach activities. The WHOI Institutional PI, Andreas Teske, will bear the primary responsibility for guiding and coordinating the research, education and outreach activities of the Subsurface Biospheres program at Woods Hole. We anticipate that both D'Hondt and Teske will spend at least one month per year on these activities.

The major components of the Integrated Science Plan can be grouped into three broad categories: microbiological studies, porewater and inorganic biogeochemical studies, and organic-biomarker biogeochemical studies. Research in each of these categories will be undertaken by teams of Investigators, post-doctoral scholars and graduate students. For short periods of time, summer undergraduate research fellows and visiting (guest) undergraduates and (at WHOI) select high-school students will also be members in those research teams.

Andreas Teske and GSO Co-investigator David Smith will coordinate and oversee all of the microbiological studies. In particular, Andreas Teske will tutor the microbiology postdocs and coworkers on anaerobic cultivation, ribosomal RNA sequence analysis and microbial systematics and phylogeny. David Smith and Andreas Teske will take care of and supervise shipboard cultivation of bacteria and Archaea from fresh deep subsurface samples Co-Investigator Arthur Spivack and Steven D'Hondt will coordinate and oversee the porewater and inorganic biogeochemical studies. Spivack will bear primary responsibility for the analytical aspects of those biogeochemical studies. D'Hondt will bear primary responsibility for the computational aspects of those and other studies (such as the mapping of sedimentary isotherms). Both Spivack and D'Hondt will actively participate in the modelling and interpretation of those

data. Co-investigator Kai-Uwe Hinrichs will coordinate, oversee, and bear primary responsibility for guiding and interpreting all of the organic-biomarker biogeochemical studies.

Spivack and D'Hondt will work closely with Smith to keep the microbiological studies closely integrated with the porewater and inorganic biogeochemical studies. Hinrichs will work closely with Teske to maintain integration of the microbiological and organic biogeochemical studies. As discussed in the Management Plan, all five Investigators will meet regularly to coordinate the overall program and all program participants will meet regularly to share their results and advance their collective understanding of (and enthusiasm for) Astrobiology and Subsurface Biospheres.

If this proposal is funded, we anticipate that all of the Investigators will spend at least three months per of their NASA-funded time on the Research activities of this Astrobiology Program. Most of that time will be spent on personal research efforts. However, much of that time will also be spent guiding the post-doctoral scholars, graduate students and summer undergraduate research fellows in their respective efforts.

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